Importância da dopamina e 5-hidroxitriptamina no transporte de electrólitos e água a nível do epitélio jejunal

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Doutor Manuel José Bragança Tender

Doutor Manuel Teixeira Amarante Junior

Doutor Mário José Cerqueira Gomes Braga

Doutor Walter Friedrich Alfred Osswald

Introdução e objectivos **8**

Capítulo 1 **18**

Formação e metabolismo da dopamina: linha celular intestinal vs células isoladas do epitélio intestinal.

a) Vieira-Coelho MA, Lucas-Teixeira V, Guimarães JT, Serrão MP & Soares-da-Silva P.1999. Caco-2 cells in culture synthesize and degrade dopamine and 5-hydroxytryptamine: a comparison with rat jejunal epithelial cells. *Life Sci.* **64**: 69-81

Capítulo 2 **32**

Factores que modulam a resposta da dopamina sobre o transporte epitelial.

b) M Augusta Vieira-Coelho, Vera A Lucas Teixeira, Yigael Finkel, Patrício Soares-da-Silva, and Alejandro M Bertorello. 1998. Dopamine-dependent inhibition of jejunal Na^+-K^+-ATPase during high-salt diet in young but not in adult rats. *Am. J. Physiol.* **275 (6)**: G1317-G1323

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Capítulo 3 **56**

Efeito da activação de receptores α- adrenérgicos sobre o transporte epitelial: factores que modulam essa resposta.

a) Lucas-Teixeira V, MA Vieira-Coelho, MP Serrão & Soares-da-Silva P. 2000. Food deprivation increases α_2-adrenoceptor-mediated modulation of jejunal epithelial transport in young and adult rats. *J. Nutr.* "in press"

Capítulo 4 **64**

Factores que modulam a resposta da 5-HT sobre o transporte epitelial.

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Capítulo 5 **72**

Funcionamento do sistema dopaminérgico intestinal em situações patológicas

a) Lucas-Teixeira V, MA Vieira-Coelho, MP Serrão, M Pestana & Soares-da-Silva P. 2000. Salt intake and sensitivity of intestinal and renal Na^+,K^+-ATPase on inhibition by dopamine in Spontaneous Hypertensive and Wistar-Kyoto rats. *Clin. Exp. Hypert.*. **22(5)**:455-69

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Discussão e conclusão **102**

Bibliografia **114**

Resumo **122**

Summary **124**

O aparelho digestivo fornece ao organismo toda a quantidade de água, electrólitos e nutrientes necessários à vida. Para que isto seja possível o tracto gastrointestinal possui uma série de funções que tornam possível a realização dessa tarefa, nomeadamente uma grande variedade de mecanismos de transporte intestinal que se encontram sujeitos a vários tipos de regulação.

O aparelho digestivo é constituído por partes distintas cada uma adaptada à função que desempenha. O jejuno é uma das parte que forma o intestino delgado e é responsável pela maior parte da absorção de água, electrólitos e nutrientes no intestino. Todas estas funções ocorrem ao nível da mucosa jejunal. A mucosa é a camada mais interna da parede do intestino. É composta por três estruturas: a muscular da mucosa, a lâmina própria e o epitélio intestinal. Esta última camada é responsável pela formação das vilosidades e das criptas intestinais (Madara & Trier, 1994). As criptas são pequenas estruturas cilíndricas situadas na base das vilosidades onde se encontram as células embrionárias indiferenciadas, que dão origem a todas as outras células. As vilosidades intestinais são estruturas alongadas viradas para o lúmen que têm como função um aumento da área de contacto com o conteúdo intestinal. As mais longas localizam-se no jejuno e vão diminuindo progressivamente em direcção ao cólon. As células mais comuns no epitélio intestinal são os enterócitos. Estes são células altamente especializadas nos processos absorptivos e secretivos e têm a particularidade de possuírem inúmeras microvilosidades na zona apical, o que permite deste modo um aumento adicional da superfície de contacto com o lúmen intestinal.

Princípios gerais do transporte epitelial

Considerando que a função primordial do intestino é o transporte de água, electrólitos (Na^+, K^+, HCO_3^-, Cl^-) e nutrientes, o estudo da regulação dos mecanismos básicos responsáveis pelo transporte transepitelial é de extrema importância. Os vários sistemas de transporte presentes nas células são, assim, importantes para a manutenção do volume, da água e concentração iónica intracelular (Eveloff & Warnock, 1987; Spring, 1985).

O transporte intestinal pode ocorrer quer a nível paracelular quer a nível transcelular. O transporte paracelular é um transporte passivo dependendo, por isso, exclusivamente de gradientes electroquímicos transepiteliais e corresponde à passagem através das junções apertadas de água e electrólitos. O transporte transcelular, pelo contrário, envolve mecanismos activos e passivos, e é possível devido à distribuição polarizada de canais, transportadores, bombas e trocadores iónicos entre as membranas apical e basolateral. É aliás esta polaridade funcional resultante da distribuição diferencial, entre as membranas apical e basolateral, de uma grande variedade de enzimas digestivas e proteínas envolvidas no transporte transmembranar de iões e nutrientes uma das característica principais das células epiteliais que permite o transporte vectorial ou transcelular de electrólitos.

Existem 4 tipos de transporte transmembranar: (1) transporte activo primário, (2) transporte activo secundário, (3) transporte activo terciário e (4) transporte passivo. É a acção conjunta destes sistemas de transporte que mantém a electronegatividade típica das células epiteliais. Esta electronegatividade é consequência de vários factores: (1) da actividade electrogénica da bomba de sódio

na membrana basolateral; (2) uma maior permeabilidade da membrana das células epiteliais para o K^+ do que para o Na^+, o que facilita uma maior difusão para fora da célula de K^+ do que de Na^+; (3) da presença no interior das células de uma elevada concentração de proteínas com carga negativa. Estas duas características, baixa concentração intracelular de Na^+ e electronegatividade, favorece a existência de um gradiente electroquímico fundamental para todos os sistemas de transporte que permitem a absorção e secreção e que irão ser descritos nos próximos capítulos. A análise de todos estes mecanismos de transporte revela que na base deles existe um elemento comum. Este é a ATPase-Na^+,K^+ que devido à sua localização basolateral (DiBona and Mills, 1979; Stirling et al, 1972) é o transporte activo primário mais utilizado em células epiteliais. Esta bomba iónica na presença de Mg^{2+} catalisa o efluxo de 3 iões Na^+ e o influxo de 2 iões K^+ utilizando a energia resultante da hidrólise de 1 molécula de ATP (Kaplan, 1983; Kirk et al, 1980; Carafoli & Zurini, 1982; Kaplan, 1983) gerando, assim, o gradiente de sódio necessário à ocorrência de todos os outros tipos de transporte.

É a acção conjunta e em simultâneo de todos estes mecanismos de transporte que culmina nas duas principais funções do epitélio intestinal, a absorção e a secreção. A primeira depende essencialmente de um ião, o sódio, enquanto que a secreção depende, de acordo com a região do tracto gastrointestinal principalmente de dois iões, o cloro e o bicarbonato (HCO_3^-) (Armstrong, 1987; Binder & Sandle, 1987; Donowitz & Welsh, 1987; Powel, 1987). O sódio pode ser absorvido por dois mecanismos distintos, um mecanismo electroneutro em que o resultado do transporte das diferentes cargas eléctricas é neutro, e um mecanismo electrogénico que gera uma corrente eléctrica. A absorção electroneutra de sódio depende da actividade simultânea da ATPase-Na^+,K^+ e dos trocadores Na^+/H^+ e Cl^-/HCO_3^-, ambos de localização apical (Foster et al, 1990; Knickelbein et al, 1983; May et al, 1993; Knickelbein et al, 1983; Liedtke & Hopfer, 1982b; Lubeke et al, 1986; Binder et al, 1987; Foster et al, 1986). É a regulação do pH intracelular que faz a ligação entre estes dois trocadores (Liedtke & Hopfer, 1982; Lowe & Lambert, 1983; May et al, 1993). Um aumento da concentração intracelular de sódio conduz a uma alcalinização do citoplasma que tem como consequência o aumento da actividade do trocador Cl^-/HCO_3^- que elimina o excesso de HCO_3^- produzido por troca com Cl^-. Este mecanismo permite a entrada de Na^+ e H^+ por troca com Cl^- e HCO_3^-, que são produzidos no interior das células pela acção da anídrase carbónica a partir do CO_2. O Na^+ é depois bombeado para fora da célula através da ATPase-Na^+,K^+ localizada na membrana basolateral. A água segue os iões absorvidos e sai através das junções apertadas. A acção conjugada destes dois trocadores resulta no influxo de NaCl e na manutenção do pH intracelular.

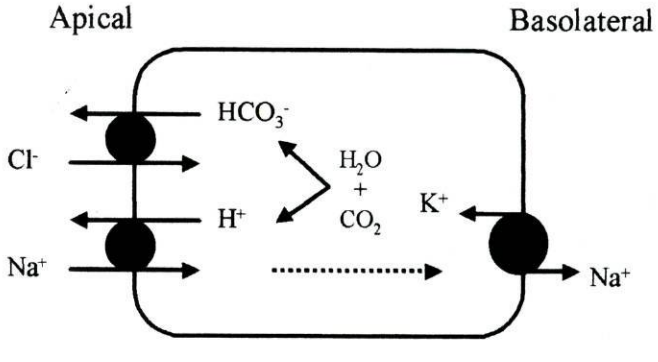


Figura 1. Representação esquemática da absorção electroneutra de NaCl.

A absorção electrogénica reflecte-se na formação de uma corrente eléctrica transepitelial. Está também dependente da ATPase-Na^+,K^+ e adicionalmente de canais de sódio localizados na membrana apical dos enterócitos e ocorre predominantemente no cólon (Lubeke et al, 1986).

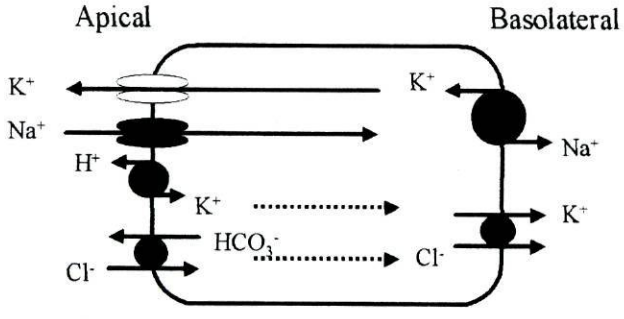


Figura 2. Representação esquemática da absorção electrogénica de NaCl.

Ao nível do intestino delgado é a acção conjunta da absorção de nutrientes acoplada ao Na^+, como por exemplo o co-transporte de Na^+/glicose, e do transporte electroneutro de Na^+ e Cl^-, o responsável pela maior parte da absorção de água e electrólitos.

Tal como o transporte de sódio fornece à célula o gradiente necessário à absorção de fluídos, o cloro é fundamental para gerar o gradiente necessário à secreção desses mesmo fluídos. O epitélio intestinal mantém um nível basal de secreção de cloro, que pode ser modulado por uma variedade de hormonas e mediadores parácrinos, neuroniais, luminais e inflamatórios. A secreção de cloro também é um mecanismo electrogénico, uma vez que não ocorre em simultâneo nem com o transporte activo de um catião nem por troca com um outro anião. Durante este processo o cloro é transferido para o interior da célula a partir da corrente sanguínea via o co-transportador- Na^+,K^+, 2 Cl^- (NKCC) de localização basal (Dhamasathaphorn et al, 1985; Weymer et al, 1985), sendo a energia para a actividade deste transportador fornecida pela ATPase-Na^+,K^+ (Weymer et al, 1985; Thorens, 1993). O cloro é depois secretado pelo bordo apical através de canais específicos para este anião. Globalmente o processo secretor resulta da passagem transcelular do Cl^- acompanhada por água que atravessa o epitélio de um modo paracelular através das junções apertadas.

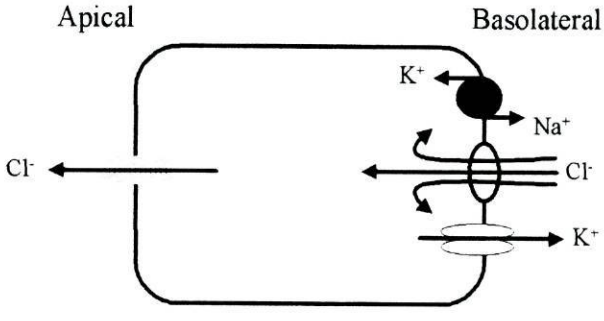


Figura 3. Representação esquemática da secreção electrogénica de Cl^-.

Relativamente ao bicarbonato este é primordialmente secretado no duodeno onde desempenha um importante papel protector (Quigley & Turnberg, 1987; Flemstrom, 1987; Flemstrom et al, 1986), apesar de também estar presente no íleo e cólon, onde é importante na conservação do cloro através da actividade do trocador Cl^-/HCO_3^- (Davis et al, 1983). Vários mecanismos têm sido propostos para a secreção de bicarbonato (Yao, 1993; Sullivan & Smith, 1986). Um dos mecanismo depende da actividade do trocador Cl^-/HCO_3^- sendo, por isso, electroneutro. Segundo este modelo, a secreção electrogénica de cloro fornece a quantidade deste ião necessária à sua troca,

através do trocador Cl^-/HCO_3^-, pelo bicarbonato. O outro mecanismo é electrogénico e resulta da extrusão de iões HCO_3^- através de canais localizados na membrana apical (Yao, 1993). O bicarbonato secretado pode ser produzido no citoplasma por acção da anídrase carbónica ou pode ser translocado da corrente sanguínea para o interior das células pelo trocador Na^+/HCO_3^-, cuja actividade é mantida pela ATPase-Na^+,K^+. Tal como ocorre para a secreção de cloro, a secreção de bicarbonato induz a secreção de vários catiões bem como de água por uma via paracelular.

Além da secreção de cloro, outros iões existem que desempenham um importante papel na secreção intestinal. Um destes iões é o potássio (K^+). No intestino delgado a maior parte da secreção de K^+ é passiva ocorrendo secundariamente como resultado da diferença de potencial negativa existente através do epitélio. A secreção deste ião é semelhante ao mecanismo proposto para a secreção de cloro requerendo a presença da ATPase-Na^+,K^+ e do co-transportador Na^+,K^+,$2Cl^-$ na membrana basolateral. A única diferença reside na presença na membrana apical de um canal específico para o K^+ que é sensível ao bário e ao tetraetilamónio. Este é um processo electrogénico produzindo uma corrente contrária à secreção de Cl^- e à absorção de Na^+.

Tal como foi referido relativamente aos mecanismos absorptivos e secretivos quase todos, se não todos, requerem a actividade da ATPase-Na^+,K^+. Foi verificado em inúmeros estudos que a presença de um inibidor desta enzima, como por exemplo a ubaína, impedem a activação de todos os mecanismos activos de transporte que se conhecem.

Quer a nível intestinal quer a nível renal a actividade desta bomba é essencial na manutenção da homeostasia hidroelectrolítica e é modulada por diversos factores.

Mecanismos e factores que regulam o transporte hidroelectrolítico

Inúmeros factores regulam o transporte epitelial. De uma forma muito simplista, podemos dividir esses factores em imunológicos, neurócrinos, endócrinos e parácrinos/ autócrinos. No entanto, apesar desta separação não existe uma fronteira rígida, uma vez, que todos eles interagem entre si, na modulação desse mesmo transporte.

A regulação parácrina/autócrina baseia-se na capacidade que as células epiteliais têm para sintetizar e libertar moléculas, que actuam não apenas sobre as células vizinhas (regulação parácrina), mas também sobre as próprias células que as sintetizam e libertam (regulação autócrina).

Sistemas monoaminérgicos não neuroniais

Um dos sistemas mais amplamente estudados e que exerce uma regulação parácrina/autócrina é o sistema monoaminérgico periférico em particular o sistema dopaminérgico renal. Tal como acontece para o epitélio renal, existem evidências que sugerem a presença de um sistema semelhante responsável pela regulação do transporte de água e electrólitos ao nível do aparelho digestivo (Vieira-Coelho et al, 1997). A existência deste sistema monoaminérgico periférico, em particular dopaminérgico e 5-hidroxitriptaminérgico a nível do epitélio jejunal é possível, uma vez que, também na mucosa jejunal, estão presentes todos os

elementos essenciais à sua existência, que incluem todos os mecanismos de captação intracelular dos percursores, L-DOPA e L-5-hidroxitriptofano (L-5-HTP), bem como de toda a maquinaria enzimática necessária à síntese e metabolização da dopamina (DA) e 5-hidroxitriptamina (5-HT) (Vieira-Coelho e Soares-da-Silva, 1993; Vieira-Coelho et al, 1997). Igualmente semelhantes são a presença de receptores ao nível da membrana plasmática, das vias de transdução de sinal e dos efectores alvo para estas aminas. A enzima descarboxílase dos aminoácidos L-aromáticos (AADC) é a enzima que permite a síntese de DA e 5-HT a partir de L-DOPA e L-5-HTP circulantes, respectivamente (Landsberg and Taubin, 1973; Soares-da-Silva, 1993) e está presente ao longo do aparelho gastrointestinal sendo a sua actividade, no entanto, mais elevada no duodeno, jejuno e íleo.

Sabe-se que a nível renal o sistema monoaminérgico é fundamental na manutenção da homeostasia hidroelectrolítica, porque se por um lado a dopamina produz um efeito natriurético por activação de receptores de tipo D_1 de localização basolateral com consequente inibição da actividade da ATPase-Na^+,K^+ (Aperia et al., 1994; Baines et al., 1992; Bertorello & Aperia, 1990; Jose et al, 1992; Lokhandwala and Amenta, 1991) e de receptores D_1 no bordo apical com inibição do trocador Na^+/H^+ (Felder et al., 1990). Por outro, a 5-HT tem um efeito anti-natriurético, uma vez que estimula a bomba de sódio (Soares-da-Silva et al, 1996b; Soares-da-Silva e Pinto-do-Ó, 1996; Soares-da-Silva e Vieira-Coelho, 1998; Soares-da-Silva et al, 1996a). Um equilíbrio entre estes dois efeitos é fundamental, por exemplo, na regulação da pressão arterial (Vieira-Coelho, et al, 1997). Além do papel que desempenham a nível renal a dopamina e a 5-HT também são importantes na regulação da actividade e transporte hidroelectrolítico a nível do epitélio jejunal (Binder, 1993).

A dopamina e a 5-HT no intestino

No sistema digestivo a dopamina exerce inúmeras funções, nomeadamente ao nível da modulação da motilidade intestinal (Bueno et al, 1984; Van Nueten and Schuurkes, 1984; Marzio et al, 1986; Bonaz et al; 1991; Bueno et al, 1992), da protecção do intestino através da secreção de fluídos (Knutson et al, 1993; Flemstrom et al, 1993; Flemstrom et al, 1994; Mezey et al; 1996), da regulação da circulação sanguínea (Sjovall et al, 1984; Aliabadi-Wahle et al, 1999) e do transporte de água e electrólitos (Donowitz et al, 1982; Donowitz, 1983; Donowitz et al; 1983). De modo semelhante ao epitélio renal, existem evidências que sugerem que a dopamina através da activação de receptores específicos, pode estar implicada na regulação de água e electrólitos no sistema digestivo (Donowitz et al, 1982), através da alteração das actividades da ATPase Na^+, K^+ e do trocador Na^+/H^+ (José et al, 1992, Soares-da-Silva et al, 1996). A dopamina pode ainda activar no jejuno outro tipo de receptores, em particular α_2-adrenérgicos, com um consequente efeito anti-secretivo e pro-absorptivo (Wahawisan et al, 1997; Vieira-Coelho and Soares-da-Silva, 1998).

Relativamente à 5-HT o intestino é a maior fonte de todo o organismo desta amina (Thompson, 1971), e apesar de as células epiteliais (enterócitos) possuírem toda a maquinaria enzimática necessária à sua síntese, ela encontra-se principalmente nas células enterocromafins (Erspamer, 1954), podendo ser libertada por estímulos mecânicos ou químicos (Burks and Long, 1966). Outra fonte de 5-HT, encontra-se no plexo mientérico que enerva a parede

gastrointestinal (Gershon et al, 1965). A 5-HT libertada dos terminais nervosos desempenha um papel muito importante na regulação quer da motilidade (Sirek and Sirek, 1970) quer da circulação sanguínea (Biber et al, 1973) a nível intestinal. A associação entre situações de diarreia e elevadas concentrações de 5-HT (Oates and Sjoerdsma; 1962, Kowlessar, 1989; Challacombe et al, 1977) vieram apoiar a ideia de que esta amina é importante na regulação do transporte intestinal e consequentemente na secreção de água e electrólitos pelo intestino (Donowitz and Binder, 1975), uma vez que, o maior efeito no intestino deste transmissor é, assim, uma potente estimulação da secreção de fluídos e electrólitos. A secreção intestinal induzida por 5-HT deve-se à secreção de cloro pelas células das criptas jejunais em conjunto com uma inibição do mecanismo electroneutro de absorção de sódio (Hardcastle et al, 1981, Zimmerman and Binder, 1984).

O facto de a DA e a 5-HT poderem ser localmente sintetizadas e libertadas (Soares-da-Silva e Pinto-do-Ó, 1996; Sole et al, 1986; Stier et al, 1984) associado à possibilidade de estas aminas actuarem através de receptores específicos nas próprias células do epitélio, poderá constituir a base para a presença do sistema dopaminérgico e 5-hidroxitriptaminérgico autócrino e parácrino intestinal.

Factores que modulam o transporte do epitélio intestinal

Como foi mencionado anteriormente a actividade da bomba de sódio, e outros transportadores como o NKCC, encontram-se na base de todos os mecanismos de transporte de cuja a actividade dependem todos os processos absorptivos e secretivos. O efeito do sistema monoaminérgico periférico sobre a actividade intestinal e consequentemente sobre as estruturas responsáveis pelo transporte de variados iões, nutrientes e água depende de inúmeros factores que se relacionam com características inerentes ao próprio organismo e com factores e estímulos externos. Alguns destes factores que incluem a presença ou ausência de alimentos no intestino, o conteúdo em sal e proteínas da dieta, idade e estádio de desenvolvimento (Binder, 1983) têm um grande impacto e na manutenção da homeostasia electrolítica e metabolismo hídrico ao longo do desenvolvimento do organismo em condições fisiológicas normais, mas também em determinadas patologias (Herbst e Suskind, 1969; Younoszai et al, 1978).

A idade e o conteúdo em sal são dois dos factores que determinam a resposta da mucosa jejunal ao sistema monoaminérgico intestinal. Finkel e col. (1994) demonstraram que o jejuno de ratos jovens além de um maior conteúdo de dopamina possui uma absorção de sódio mais elevada do que animais adultos da mesma estirpe (Sprague-Dawley). Adicionalmente foi observado que apenas os primeiros respondem com uma diminuição da absorção de sódio e um aumento dos níveis de catecolaminas a uma dieta hiperssalina. A ausência de resposta nos animais adultos ao aumento do aporte de sódio coincidiu com o período em que o rim atingiu a maturidade (Robillard et al, 1992a; Robillard et al, 1992b). Estes dados permitiram assim concluir que a resposta à dopamina está dependente do factor idade e do conteúdo de sal da dieta, além de sugerir ainda uma possível complementaridade funcional entre o rim e o intestino, uma vez que, ao inibir a absorção de sódio durante a ingestão de uma dieta rica em sódio, o intestino está a contribuir para a homeostasia hidroelectrolítica.

Para além da idade e do teor em sódio da dieta, a função da mucosa intestinal assim como a sua estrutura podem também ser reguladas por factores sistémicos associados com a alimentação e com o contacto da própria mucosa com os alimentos. Não apenas a presença, mas também a ausência de alimentos parece ter um papel preponderante na regulação da mucosa intestinal. Vários estudos demonstraram que durante períodos de jejum quer a função quer a estrutura da mucosa são alteradas. Por exemplo, a mucosa intestinal de esquilos sujeitos a um período de 3 dias de jejum apresenta um aumento de peso assim como uma alteração significativa da razão das dimensões vilosidades/criptas quando comparadas com animais seguindo um regime alimentar normal (Carey, 1992). Foi também verificada uma redução da taxa de proliferação e migração dos enterócitos (Carey e Cooke, 1991) que resulta num aumento do número de enterócitos mais desenvolvidos, ou seja, com maiores capacidades absorptivas (Thompson e Debnam, 1986) resultante de um aumento do número de transportadores presentes membrana apical (Robinson et al, 1980), de um aumento da extensão das microvilosidades (Smith et al, 1984). Todas estas alterações têm como consequência uma alteração nas características das membranas epiteliais, nomeadamente ao nível da permeabilidade (Meddings, 1990) e da conductância do epitélio. Young e Levin (1990a, 1990b) observaram especificamente um aumento da função secretora no jejuno de rato após que curtos períodos de jejum. Por outro lado, um aumento da absorção também foi demonstrada após um "bypass" intestinal, jejum ou deficiente nutrição (Butzner et al, 1985; Carey e Cooke, 1991; Marciani, et al 1987; Robinson et al, 1980; Thompson e Debnam, 1986). Apesar de todas estas observações, não se conhece ainda o mecanismo que se encontra na base de todas estas alterações funcionais e estruturais.

Por tudo o que foi mencionado anteriormente é possível concluir que o correcto funcionamento dos sistemas monoaminérgicos intestinal e renal, e em particular o dopaminérgico (que comparativamente ao 5-hidroxitriptaminérgico se encontra melhor estudado) são essenciais para o normal funcionamento de todo o organismo. Esta conclusão levanta, no entanto, uma questão interessante relacionada com o que sucede quando surge uma anomalia num destes sistemas. A hipertensão é uma patologia que muitos estudos indicam possa ser causada por esta situação. Sabe-se que a nível renal o sistema dopaminérgico perdeu a capacidade de regular a excreção de sódio, ocorrendo deste modo uma retenção de sal e consequente aumento da tensão arterial. Estas anomalias podem estar relacionadas com inúmeros factores, nomeadamente com uma diminuição da capacidade de síntese da amina pelas células epiteliais (Kuchel and Shigetomi, 1992; Soares-da-Silva et al, 1995; Yoshimura et al, 1987), com um deficiente acoplamento entre os receptores dopaminérgicos e as vias de transdução de sinal (Felder et al, 1993; Hussain, and Lokhandwala, 1997; Kansra et al, 1995) ou ainda com a falta de receptores dopaminérgicos específicos (Albrecht et al, 1996; Asico et al, 1998). Além deste modelo de hipertensão, outros existem onde também se verificam alterações no funcionamento do sistema dopaminérgico renal. É o que sucede no modelo animal de obesidade em ratos da estirpe Zucker. Estes animais têm sido extensivamente utilizados no estudo da relação entre a obesidade e a hipertensão (Boese et al, 1985; Kurtz et al, 1989). Tal como acontece para o modelo animal de

hipertensão, verificou-se que a dopamina não exerce qualquer efeito inibitório sobre a bomba de sódio renal. Se considerarmos a existência de uma complementaridade funcional entre o intestino e o rim, uma das questões que estes dois modelos animais levantam está relacionadas sobre qual será o estado de activação do sistema dopaminérgico intestinal, numa situação onde declaradamente o sistema renal se encontra com uma capacidade de manter o equilíbrio iónico e hídrico diminuída.

Pelo exposto anteriormente existem evidências que apoiam a hipótese de que a resposta do sistema monoaminérgico periférico sobre a actividade do epitélio intestinal pode ser modulada, e que um dos alvos dessa modulação é a bomba de sódio, uma vez que se encontra na base de todos os mecanismos de transporte de electrólitos. Adicionalmente também é possível verificar que os sistemas renal e intestinal funcionam com um elevado grau de complementaridade, sendo este último um sistema altamente dinâmico cuja regulação depende de inúmeros factores intrínsecos e extrínsecos ao próprio organismo cuja natureza e modo de acção não são totalmente conhecidos.

Sendo assim, o objectivo deste trabalho foi a determinação da importância das aminas, dopamina e 5-hidroxitriptamina, sobre a actividade da ATPase-Na^+,K^+ e outros transportadores responsáveis pela absorção e secreção de água e electrólitos (Na^+, K^+, Cl^-) pela mucosa jejunal em diferentes condições experimentais: dietas hipo, normo e hiperssalinas, jejum e em situações de ingestão após um período de jejum, bem como em modelos experimentais onde se verifica uma diminuição na capacidade do rim para manter a homeostasia hidroelectrolítica, no sentido de determinar se o intestino é capaz de executar parte das funções normalmente atribuídas ao rim e de que modo é que o sistema monoaminérgico intestinal é influenciado por todos os factores mencionados anteriormente (Figura 4).

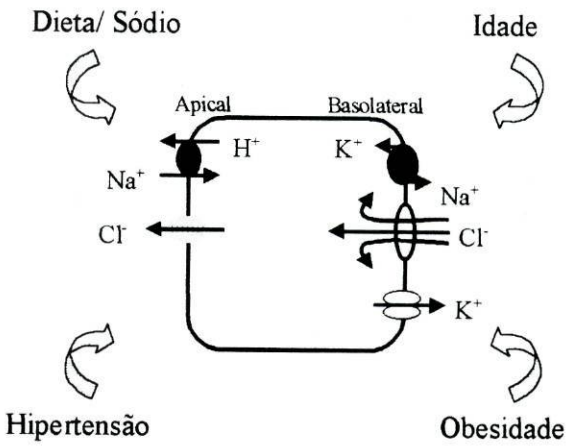


Figura 4: Esquema representativo dos factores que modulam o transporte epitelial a nível jejunal e que são objecto de análise neste projecto.

De seguida estão divididas por capítulos as questões mais relevantes levantadas no decurso deste projecto.

Capítulo 1. Formação e metabolismo da dopamina: linha celular intestinal vs células isoladas do epitélio intestinal.

- Possui a mucosa jejunal toda a maquinaria molecular necessária à existência de um sistema monoaminérgico periférico?

a) Vieira-Coelho MA, Lucas-Teixeira V, Guimarães JT, Serrão MP & Soares-da-Silva P.1999. Caco-2 cells in culture synthesize and degrade dopamine and 5-hydroxytryptamine: a comparison with

rat jejunal epithelial cells. *Life Sci.* **64**: 69-81

Capítulo 2. Factores que modulam a resposta da dopamina sobre o transporte epitelial.

- Quais o mecanismos celulares que estão na base da inibição da absorção de sódio pela dopamina?
- O efeito da dopamina sobre o transporte epitelial está exclusivamente dependente da fase do desenvolvimento ontogénico dos animais?
- Podem factores extrínsecos ao organismo, tais como uma dieta com diferentes concentrações de sódio, modular o papel do sistema dopaminérgico ao nível intestinal?
- Pode o tempo de exposição a uma dieta com diferentes concentrações de sódio modular a actividade do sistema dopaminérgico intestinal em animais adultos?
- Além da concentração salina pode o tipo de regime alimentar alterar o efeito da dopamina sobre os mecanismos celulares envolvidos no transporte de electrólitos?

a) M Augusta Vieira-Coelho, Vera A Lucas Teixeira, Yigael Finkel, Patrício Soares-da-Silva, and Alejandro M Bertorello. 1998. Dopamine-dependent inhibition of jejunal Na^+,K^+-ATPase during high-salt diet in young but not in adult rats. *Am. J. Physiol.* **275 (6)**: G1317-G1323

b) V Lucas-Teixeira, MP Serrão & P Soares-da-Silva. 2000. Effect of salt intake on jejunal dopamine, Na^+,K^+-ATPase activity and electrolyte transport. *Acta Physiol. Scand.* **168**: 225-231

c) Lucas-Teixeira V, Vieira-Coelho MA, Soares-da-Silva P. 2000. Food intake abolishes the response of rat jejunal Na^+,K^+-ATPase to dopamine. *J Nutr.* **130(4)**: 877-881

Capítulo 3. Efeito da activação de receptores α-adrenérgicos sobre o transporte epitelial: factores que modulam essa resposta.

- Que tipo de receptores, além dos receptores dopaminérgicos, poderão estar envolvidos na resposta da dopamina sobre o transporte epitelial?
- Qual o efeito da activação de receptores α-adrenérgicos sobre o transporte epitelial?
- Quais os mecanismos celulares subjacentes ao efeito da activação de receptores α-adrenérgicos sobre o transporte de electrólitos?

a) Lucas-Teixeira V, MA Vieira-Coelho, MP Serrão & Soares-da-Silva P. 2000. Food deprivation increases α_2-adrenoceptor-mediated modulation of jejunal epithelial transport in young and adult rats. *J. Nutr.* "in press"

Capítulo 4. Factores que modulam a resposta da 5-HT sobre o transporte epitelial.

- Quais o mecanismos celulares que estão na base da inibição da absorção de sódio dependente da 5-HT?
- Pode o desenvolvimento ontogénico ser um factor importante no efeito da 5-HT sobre os mecanismos celulares subjacentes ao transporte hidroelectrolítico?
- Além da idade podem outros factores como o regime alimentar modular o efeito da 5-HT?

a) Lucas-Teixeira V, MP Serrão & Soares-da-Silva P. 2000. Response of jejunal ATPase-Na^+,K^+ to 5-hydroxytryptamine in young and adult rats: effect of fasting and refeeding . *Acta Physiol. Scand.* **168**: 167-172

Capítulo 5. Funcionamento do sistema dopaminérgico intestinal em situações patológicas.

- Qual o estado de activação do sistema dopaminérgico intestinal em modelos animais que apresentam uma anomalia ao nível do sistema dopaminérgico renal?

a) Lucas-Teixeira V, MA Vieira-Coelho, MP Serrão, M Pestana & Soares-da-Silva P. 2000. Salt intake and sensitivity of intestinal and renal Na^+,K^+-ATPase on inhibition by dopamine in Spontaneous Hypertensive and Wistar-Kyoto rats. *Clin. Exp. Hypert.*. "in press"

b) V.A. Lucas-Teixeira, T. Hussain, P. Serrão, M Lokhandwala & P. Soares-da-Silva. Intestinal dopaminergic activity in obese and lean Zucker rats: response to high salt intake. *(artigo submetido para publicação)*

Formação e metabolismo da dopamina: linha celular intestinal vs células isoladas do epitélio intestinal.

a) Caco-2 cells in culture synthesize and degrade dopamine and 5-hydroxytryptamine: a comparison with rat jejunal epithelial cells.

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CACO-2 CELLS IN CULTURE SYNTHESIZE AND DEGRADE DOPAMINE AND 5-HYDROXYTRYPTAMINE: A COMPARISON WITH RAT JEJUNAL EPITHELIAL CELLS

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Summary

To explore the usefulness of *Caco-2* cells in the study of intestinal dopaminergic and 5-hydroxytryptaminergic physiology, we have undertaken the study of aromatic L-amino acid decarboxylase (AADC), catechol-O-methyltransferase (COMT) and type A and B monoamine oxidase (MAO-A and MAO-B) activities in these cells using specific substrates. The activity of these enzymes was also evaluated in isolated rat jejunal epithelial cells. The results showed that V_{max} values (in nmol mg protein^{-1} h^{-1}) for AADC, using L-DOPA as the substrate, in rat jejunal epithelial cells (127.3±11.4) were found to be 6-fold higher than in Caco-2 cells (22.5 ± 2.6). However, K_m values in Caco-2 cells (1.24±0.37 mM) were similar to those observed in rat jejuanl epithelial cells (1.30±0.29 mM). Similar results were obtained when AADC activity was evaluated using L-5HTP as substrate; in rat jejunal epithelial cells V_{max} values (in nmol mg prot^{-1} h^{-1}) were found to be 5-fold that in Caco-2 cells (16.3±1.0 and 3.0±0.2, respectively), and K_m values in Caco-2 cells (0.23±0.08 mM) were again similar to those observed in rat intestinal epithelial cells (0.09±0.03 mM). Caco-2 cells were not able to O-methylate dopamine, in contrast to rat jejunal epithelial cells (V_{max} = 8.6 ± 0.4 nmol mg protein^{-1} h^{-1}; K_m = 516±57 µM). V_{max} values (in nmol mg protein^{-1} h^{-1}) for type A and B MAO in Caco-2 cells (19.0±0.6 and 5.4±0.6, respectively) were found to be significantly lower ($P<0.05$) than those in rat jejunal epithelial cells (46.9±3.1 and 9.6±1.2, respectively); however, no significant differences in the K_m values were observed between Caco-2 and rat jejunal epithelial cells for both type A and B MAO. In conclusion, Caco-2 cells in culture are endowed with the synthetic and metabolic machinery needed to form and degrade DA and 5-HT, though, no COMT activity could be detected in these cells.

Key Words: Caco-2 cells, rat jejunal epithelial cells, dopamine, metabolism, 5-HT synthesis

Dopamine (DA) and 5-hydroxytryptamine (5-HT) are believed to exert opposite autocrine effects upon renal epithelial transport of electrolytes (1,2). Both amines can be synthesized locally and

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the activation of specific receptors results in changes in Na^+,K^+-ATPase activity, with inhibition for DA and activation for 5-HT (2,3). When the prevalent effects are those of DA the net effect is an increase in urinary excretion of sodium; by contrast, antinatriuresis occurs when the prevalent effects are those of 5-HT (1,4,5). Epithelial cells of the intestinal mucosa are rich in aromatic L-amino acid decarboxylase (AADC) activity (6), and, therefore, have the capacity to decarboxylate circulating or luminal L-3,4-dixydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan (L-5-HTP) to DA and 5-HT, respectively. Similarly to that occurring at the kidney level, the presence of an intestinal autocrine monoaminergic systems responsible for the fine regulation of intestinal electrolytes transports has been also hypothesized (7). In agreement with this view are the following findings. Endogenous DA reduces jejunal sodium transport in young rats submitted to a high salt diet (8). Under *in vitro* conditions DA inhibits, in a concentration-dependent manner, Na^+-K^+ ATPase activity in isolated jejunal epithelial cells from 20 day-old rats, and this can be prevented by pre-treatment with 5-HT (9). The study of an intestinal autocrine monoaminergic system, similar to that describe in the kidney, may be further complicated by the presence of a heterogeneous population of cells in the intestinal mucosa, namely enterochromaffin cells, which are known to be an important source for 5-HT (7). On the other hand, the amount of the amine that is available for the activation of specific receptors may depend not only on the delivery of the corresponding precursor and on the activity of AADC, but also on the magnitude of the metabolism to which the amine is submitted. In fact, the intestinal mucosa is endowed with one of the largest monoamine oxidase (MAO) and catecol-*O*-methyltransferase (COMT) activities in the body (10).

Several intestinal cell lines are often used as physiological model systems of intestinal absorptive and secretive function, namely because, in most cases, their utilisation enables the evaluation of a given process in a single population of cells. Caco-2 cells are an established epithelial cell line derived from a human colon adenocarcinoma that undergoes enterocyte differentiation in culture (11). This cell line has been also suggested to possess attributes that make it a suitable *in vitro* model system for the investigation of transport across the small intestinal epithelium (12,13). However, in contrast to the process described in the intestinal mucosa of several species (human, dog, cat and rat), to our knowledge there is no information available in the literature on the presence of the enzymes involved in the synthesis and degradation of monoamines in Caco-2 cells. To explore further the usefulness of Caco-2 cells for the study of intestinal monoaminergic epithelial systems, we have undertaken this study to evaluate the ability of Caco-2 to synthesize and degrade DA and 5-HT. Since most of the information on the intestinal monoaminergic system has been obtained using the rat intestine or intestinal epithelial cells, we decided also to use this preparation for the sake of comparison.

Materials and methods

Cell culture

The Caco-2 cells (ATCC 37-HTB) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a humidified atmosphere of 5% CO_2-95% air at 37°C. Caco-2 cells (passages 23-30) were grown in Minimal Essential Medium (Sigma Chemical Company, St. Louis, Mo, USA) supplemented with 100 U/ml penicillin G, 0.25 µg/ml amphotericin B, 100 µg/ml streptomycin (Sigma), 20% foetal bovine serum (Sigma) and 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanosulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.05% trypsin-EDTA, split 1:3 and subcultured in Costar flasks with 75- or 162-cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). Cells used in measurements of transepithelial resistance were cultured in 1-cm^2 Snapwell filters (Costar 3407). The cell medium was changed every 2 days, and the cells reached confluence after 5-7 days of initial seeding. For

24 hours prior to each experiment, the cell medium was free of foetal bovine serum. Experiments were generally performed 2-3 days after cells reached confluence and 7-10 days after the initial seeding and each cm^2 contained about 100 µg of cell protein.

Cell isolation

The preparation of jejunal epithelial cells was based on the techniques previously described (14,15), with minor modifications. In brief, animals (male Wistar rats 60 day old) were killed by decapitation under anaesthesia and a jejunal segment approximately 10 cm in length removed through a midline abdominal incision. The jejunal segment was placed on an ice cold glass plate and subsequently cut to segments of approximately 1.5 cm in length and rinsed free from blood and intestinal contents with saline (0.9% NaCl). The fragments were everted with fine forceps and incubated for 45 minutes in 5 ml warm (37°C) and gassed (95% O_2 and 5% CO_2) Hanks' solution with 0.06% collagenase type I (Sigma Chemical Co, St Louis, MO). At the end of the incubation period the preparation was gently vortexed to allow the epithelial cells to detach. The fragments were then removed from the solution and the medium containing the detached cells centrifuged (200 g, 4 min, 4°C). The pellet was resuspended in Hanks' medium. Cell viability was estimated by the Trypan blue (0.2%; 2 min) exclusion method, and the percentage of viable cells was > 90% (excluding the dye), determined by hemocytometer counting.

AAAD preparation and decarboxylation studies

Caco-2 cells and rat jejunal epithelial cells were homogenised in 0.5 M phosphate buffer (pH=7.0) with a Thomas teflon homogeniser kept continuously on ice. Aliquots of 250 µl of cell homogenate plus 250 µl incubation medium were placed in glass test tubes and preincubated for 15 min. Thereafter, L-DOPA (50 to 5,000 µM) or L-5HTP (50 to 5,000 µM) were added to the medium for a further 15 min; the final reaction volume was 1 ml. The composition of the incubation medium was as follow (in mM): NaH_2PO_4 0.35, Na_2HPO_4 0.15, sodium borate 0.11 and pyridoxal phosphate 0.12, pH=7.2; tolcapone (1 µM) and pargyline (100 µM) were also added to the Hanks' medium in order to inhibit the enzymes COMT and MAO, respectively. The pH of the reaction medium was kept constant at an optimal pH=7.0 (16). Assay of DA or 5-HT was performed by HPLC with electrochemical detection.

COMT preparation and O-methylation studies

COMT activity was evaluated by the ability of cell homogenates to methylate dopamine to 3-methoxytyramine, as previously described (17). Aliquots of 125 µl of the homogenate were preincubated for 20 min with 125 µl of phosphate buffer (0.5 mM); thereafter, the reaction mixture was incubated for 30 min with increasing concentrations of dopamine (1 to 2000 µM; 50 µl) in the presence of a saturating concentration of the methyl donor (S-adenosyl-L-methionine, 100 µM); (18) the incubation medium contained also pargyline (100 µM), $MgCl_2$ (100 µM) and EGTA (1 mM). The assay of 3-methoxityramine was performed by HPLC with electrochemical detection.

MAO preparation and deamination studies

MAO activity was determined in cell homogenates, as previously described (19). Caco-2 cells and rat jejunal epithelial cells were homogenised in 67 mM phosphate buffer, pH=7.2, at 4°C with a Thomas teflon homogeniser kept continuously on ice. MAO activity was determined with 5-hidroxytryptamine (5-HT) as a preferential substrate for MAO-A and [^{14}C]-β-phenylethylamine ([^{14}C]-β-PEA) as a preferential substrate for MAO-B. After 20 min of incubation at 37°C with

oxygenation and continuous shaking, the tubes were transferred to an ice water bath and the reaction was stopped by the addition of 150 µl of 2M perchloric acid or 10 µl of 3 M HCl for MAO-A and MAO-B respectively. The deaminated product of [^{14}C]-β-PEA was extracted with ethyl acetate (500 µl) and measured by liquid scintillation counting. 5-hydroxyindolacetic acid (5-HIAA), the deaminated metabolite of 5-HT, was measured by HPLC with electrochemical detection.

Assay of monoamines

The assays for DA, 5-HT, 3-methoxytyramine and 5-HIAA were performed by means of high-pressure liquid chromatography, as previously described (3,5). The detection was carried out electrochemically with a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141); the detector cell was operated at 0.75 V. The current produced was monitored using the Gilson 712 HPLC software. The lower limit for detection of DA, 5-HT, 3-methoxytyramine and 5-HIAA ranged between 350 to 500 fmol.

Transepithelial resistance

Rat jejunum epithelial sheets (exposed area of 0.28 cm^2) or Snapwell filters were mounted in Ussing chambers equipped with water-jacketed gas lifts bathed on both sides with 10 ml of Krebs-Hensleit solution, gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. D-Glucose (10 mM) was added to the serosal-side reservoir and an equimolar amount of mannitol was added to the mucosal-side reservoir. The Krebs-Hensleit solution contained (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2; the pH was adjusted to 7.4 after gassing with 5% CO_2 and 95% O_2. The tissues were continuously voltage clamped to zero potential differences by application of external current, with compensation for fluid resistance, by means of an automatic voltage current clamp (DVC 1000, World Precision Instruments, Sarasota, Florida, USA). Transepithelial resistance (Ω .cm^2) was measured by altering the membrane potential stepwise (± 5 mV) and applying the Ohmic relationship. The voltage/current clamp unit was connected to a PC via a BIOPAC MP1000 data acquisition system (BIOPAC Systems, Inc., Goleta, CA, USA). The data analysis were analysed using Acq*Knowledge* 2.0 software (BIOPAC Systems, Inc., Goleta, CA, USA).

Na^+,K^+-ATPase assay

Na^+,K^+-ATPase activity was measured by the method of Quigley and Gotterer with minor modifications (20). Briefly, Caco-2 cells and isolated rat jejunal epithelial cells were pre-incubated for 15 min at 37°C. After the pre-incubation period the cells were permeabilized by rapid freezing in dry ice-acetone and thawing. The reaction mixture, in a final volume of 1.025 ml, contained (in mM) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium EDTA, 5 $MgCl_2$, NaN_3, 75 tris(hydroxymethyl)aminomethane(tris) hydrochloride and 100 µl cell suspension (100 µg protein). The reaction was initiated by the addition of 4 mM ATP. For determination of ouabain-sensitive ATPase, NaCl and KCl were omitted, and Tris-HCl (150 mM) and ouabain (1 mM) were added to the assay. After incubation at 37°C for 15 min, the reaction was terminated by the addition of 50 µl of ice-cold trichloroacetic acid. Samples were centrifuged (3,000 rpm), and liberated P_i in supernatant was measured by spectrophotometry at 740 nm. Na^+,K^+-ATPase activity is expressed as nanomoles P_i per milligram protein per minute and determined as the difference between total and ouabain-sensitive ATPase.

Protein assay

The protein content in cell homogenates (approximately 2 mg ml^{-1}), as determined by the method of Bradford (21) with human serum albumin as a standard, was similar in all samples.

Cell viability

Caco-2 cells and jejunal epithelial cells were preincubated for 15 min at 37°C and then incubated in the absence or the presence of L-DOPA, L-5HTP, 5-HT and [^{14}C]-β-PEA or DA for further 15 min. Subsequently the cells were incubated at 37°C for 2 min with trypan blue (0.2% w/v) in phosphate buffer and examined using a Leica microscope. Under these conditions, more than 90% of the cells excluded the dye.

Data analysis

V_{max} and K_m values for the decarboxylation of L-DOPA, L-5HTP, *O*-methylation of dopamine or deamination of 5-HT and [^{14}C]-β-PEA were calculated from non-linear regression analysis using the GraphPad Prism statistics software package (22). Geometric means are given with 95% confidence limits and arithmetic means are given with S.E.M.. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student's *t* test for unpaired comparisons. A P value less than 0.05 was assumed to denote a significant difference.

Drugs

Drugs used were: L-3,4 dihydroxyphenylalanine (Sigma Chemical Company, St. Louis, Mo, USA), dopamine hydrochloride (Sigma), 5-hydroxytryptamine hydrochloride (Sigma), β-phenylethylamine hydrochloride (Sigma), [^{14}C]-β-phenylethylamine hydrochloride (NEN Chemical; 50 Ci $mmol^{-1}$), pargyline hydrochloride (Sigma), tolcapone (kindly donated by late Professor Mosé Da Prada; Hoffman La Roche, Basle, Switzerland).

Results

AAAD activity

Incubation of homogenates of Caco-2 cells and rat jejunal epithelial cells with L-DOPA (50 to 5,000 μM) resulted in a concentration-dependent formation of dopamine (figure 1). The V_{max} values for AAAD using L-DOPA as the substrate in rat jejunal epithelial cells were found to be significantly ($P<0.01$) higher than those observed in CACO-2 cells (table 1). In fact, AAAD in rat jejunal epithelial cells was approximately 6-fold that observed in Caco-2 cells. However, K_m values in Caco-2 cells (1.24±0.37 mM) were similar to those observed in rat jejunal epithelial cells (1.30±0.29 mM). Similar results were obtained when AAAD activity was evaluated using L-5HTP as substrate (figure 2), in rat jejunal epithelial cells V_{max} values were found to be 5-fold higher those in Caco-2 cells (table 1). The K_m values in Caco-2 cells (0.23±0.08 mM) were again similar to those observed in rat jejunal epithelial cells (0.09±0.03 mM).

COMT activity

No formation of 3-methoxytyramine was observed when homogenates of Caco-2 cells were incubated in the presence of increasing concentrations of dopamine (5 to 500 μM). By contrast, incubation of homogenates of rat jejunal epithelial cells with dopamine (50 to 2000 μM) resulted in a concentration-dependent formation of 3-methoxytyramine (figure 3); non-linear regression analysis revealed V_{max} and K_m values of 8.6 ±0.4 nmol mg protein^{-1} h^{-1} and 516±57 μM, respectively.

TABLE 1

Kinetic parameters (V_{max} and K_m) of AAAD, COMT, MAO-A and MAO-B activities in homogenates of CACO-2 cells and homogenates of rat jejunal epithelial cells. Values are arithmetic means ± S.E.M. (n=5).

		CACO-2 cells	Rat jejunal epithelial cells
AAAD (L-DOPA)	V_{max} (nmol mg protein^{-1} h^{-1})	22.5±2.6	127.3±11.4 *
	K_m (mM)	1.24±0.37	1.30±0.29
AAAD (L-5HTP)	V_{max} (nmol mg protein^{-1} h^{-1})	3.0±0.2	16.3±1.0 *
	K_m (mM)	0.23±0.08	0.09±0.03
COMT	V_{max} (nmol mg protein^{-1} h^{-1})	-	8.6±0.4
	K_m (μM)	-	516±57
MAO-A	V_{max} (nmol mg protein^{-1} h^{-1})	19.0±0.6	46.9±3.1 *
	K_m (μM)	147±22	383±90
MAO-B	V_{max} (nmol mg protein^{-1} h^{-1})	5.4±0.6	9.6±1.2 *
	K_m (μM)	19±6	38±13

Significantly different from corresponding values in Caco-2 cells (* $P<0.05$) using Student's t test.

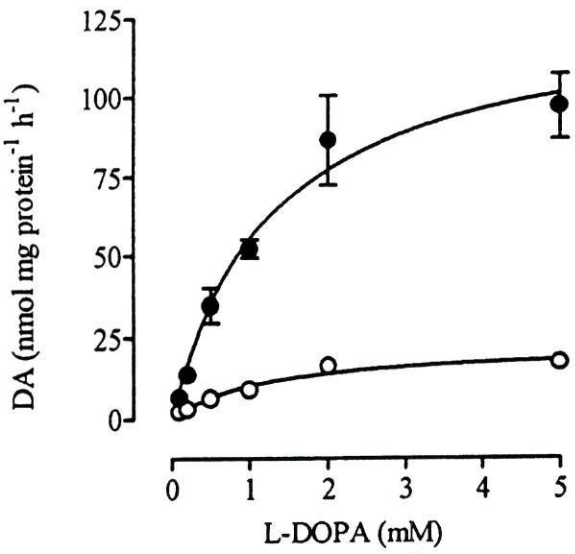


Fig. 1

Decarboxylation of L-DOPA (50 to 5,000 μM) in homogenates of CACO-2 cells homogenates (open circles) and rat jejunal epithelial cells (closed circles). The results are levels (in nmol mg protein^{-1} h^{-1}) of DA formed. Each point represents the mean of five experiments per group; vertical lines show S.E.M.

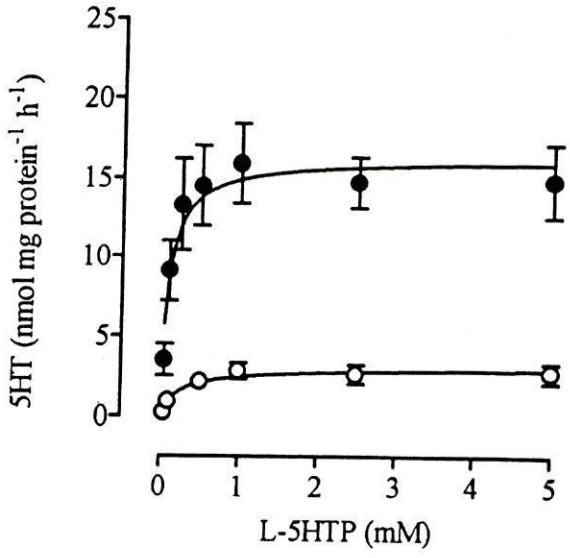


Fig. 2

Decarboxylation of L-5HTP (50 to 5,000 µM) in homogenates of CACO-2 cells homogenates (open circles) and rat jejunal epithelial cells (closed circles). The results are levels (in nmol mg protein^{-1} h^{-1}) of 5-HT formed. Each point represents the mean of five experiments per group; vertical lines show S.E.M.

MAO activity.

Rat jejunal epithelial cells were found to deaminate quite actively both 5-HT and [^{14}C]-ß-PEA. Figures 4A and 4B show the saturation curves obtained when homogenates of rat jejunal epithelial cells were incubated in the presence of increasing concentrations of 5-HT and [^{14}C]-ß-PEA, respectively. Deamination of 5-HT and [^{14}C]-ß-PEA by Caco-2 cells was also found to be dependent on the concentration of the substrate and was similar to that observed in rat jejunal epithelial cells (figure 4A and 4B); as shown in table 1, K_m values for MAO-A and MAO-B did not significantly differ between the two preparations. The V_{max} values for MAO-A and MAO-B are shown in table 1.

Figure 5 shows saturation curves obtained when homogenates of rat jejunal epithelial cells were incubated in the presence of increasing concentrations of DA, a common substrate for MAO-A and MAO-B. Deamination of DA by both jejunal epithelial cells and Caco-2 cells was dependent on the concentration of the substrate, but V_{max} values (in nmol/mg protein/h) were markedly higher in jejunal epithelial cells (127±9) than in Caco-2 cells (9±1). By contrast, K_m values (in µM) for DA did not significantly differ between the two preparations (jejunal epithelial cells = 22±4; Caco-2 cells = 31±4).

Transepithelial resistance

Rat jejunal preparations had a mean basal I_{sc} value of 19.8±2.2 µA/cm^2 (n=48) and tissue resistance was 151.0±5.8 Ω.cm^2 (n=48). On Snapwell filters, transepithelial electrical resistance (203.8±7.6 Ω.cm^2) of Caco-2 cells was accompanied by a small potential difference (0.22±0.01 mV) and by short-circuit current (2.2±0.5 µA/cm^2, n=34), both of which were ouabain sensitive (data not shown).

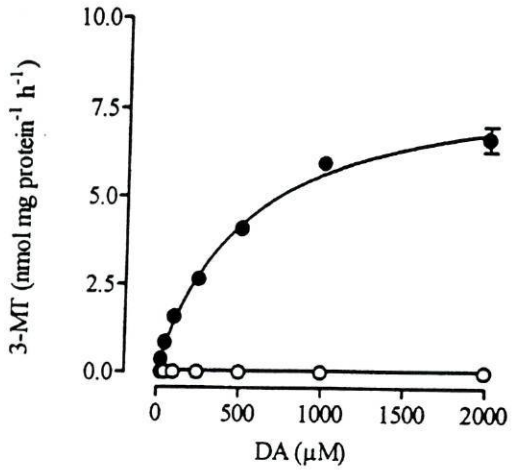


Fig. 3

O-methylation of increasing concentrations (5 to 2000 μM) of DA in homogenates of CACO-2 cells homogenates (open circles) and rat jejunal epithelial cells (closed circles). The results are levels (in nmol mg protein^{-1} h^{-1}) of 3-methoxytyramine formed from added dopamine. Each point represents the mean of five experiments per group; vertical lines show S.E.M.

Na^+,K^+-ATPase assay

Na^+,K^+-ATPase activity (in nmol Pi mg protein^{-1} min^{-1}) in isolated jejunal epithelial cells obtained from 60-day old rats (130±5) was 2.5-times higher than that in Caco-2 cells (51±1). By contrast, Na^+,K^+-ATPase activity in 20-day old rats (51±4 nmol Pi mg protein^{-1} min^{-1}) was similar to that observed in Caco-2 cells.

Discussion

The results presented here show that Caco-2 cells are endowed with the necessary synthetic and metabolic enzyme machinery to form and degrade DA and 5-HT, though significant differences do exist when data on Caco-2 cells are compared with that obtained in rat jejunal epithelial cells.

Previous studies have evaluated the ability of Caco-2 cells to take up L-DOPA and L-5HTP, the precursors for DA and 5-HT respectively, and found that these cells have an efficient saturable uptake systems for both substrates (7,23). The results presented here show that Caco-2 cells are able to decarboxylate L-DOPA and L-5-HTP and form DA and 5-HT, and that the affinity of AADC for both substrates is similar to that in rat jejunal epithelial cells. In fact, K_m values obtained for AADC activity in Caco-2 cells were similar to those in the rat jejunal epithelial cells. On the other hand, it is interesting to observe that in both types of cells the affinity AADC for L-5HTP and L-DOPA differs markedly, as indicated by differences in K_m values. Similar findings have already been described in several other tissues (3,16,24-27). Furthermore, purified rat renal AADC has also been demonstrated to preferentially decarboxylate L-DOPA over L-5-HTP (16). Similar results were reported by Sumi et al. (28) using human AADC expressed in COS cells. Different arrangements in the aromatic rings of L-DOPA and L-5HTP appear to be the

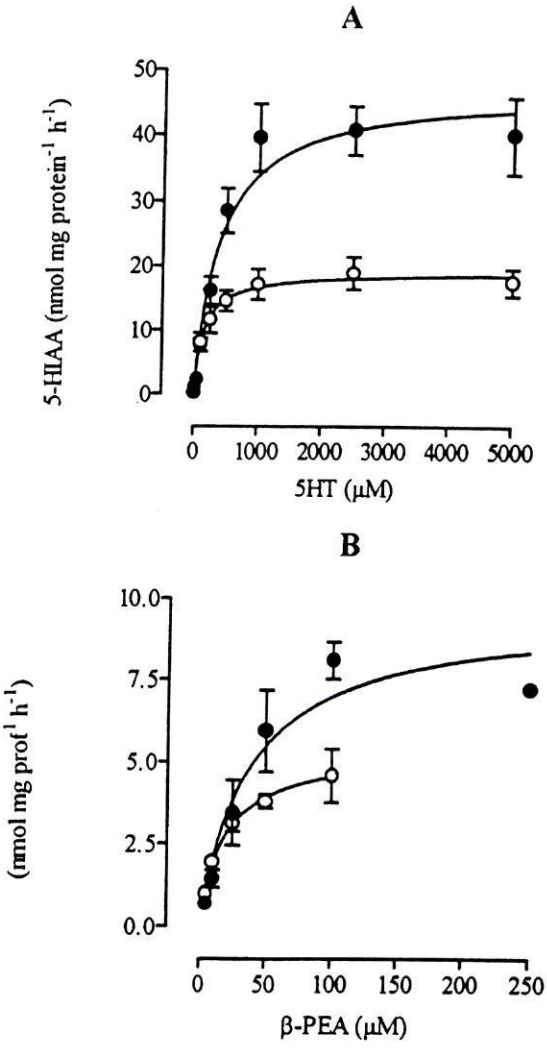


Fig. 4

Part A shows type A monoamine oxidase activity (deamination of 5-HT; 50 to 5000 μM) and part B shows type B monoamine oxidase activity (deamination of [^{14}C]-β-PEA; 5 to 250 μM) in homogenates of CACO-2 cells homogenates (open circles) and rat jejunal epithelial cells (closed circles). The results are levels (in nmol mg protein^{-1} h^{-1}) of product formed from added substrate. Each point is the mean of 5 experiments per group; vertical lines indicate S.E.M..

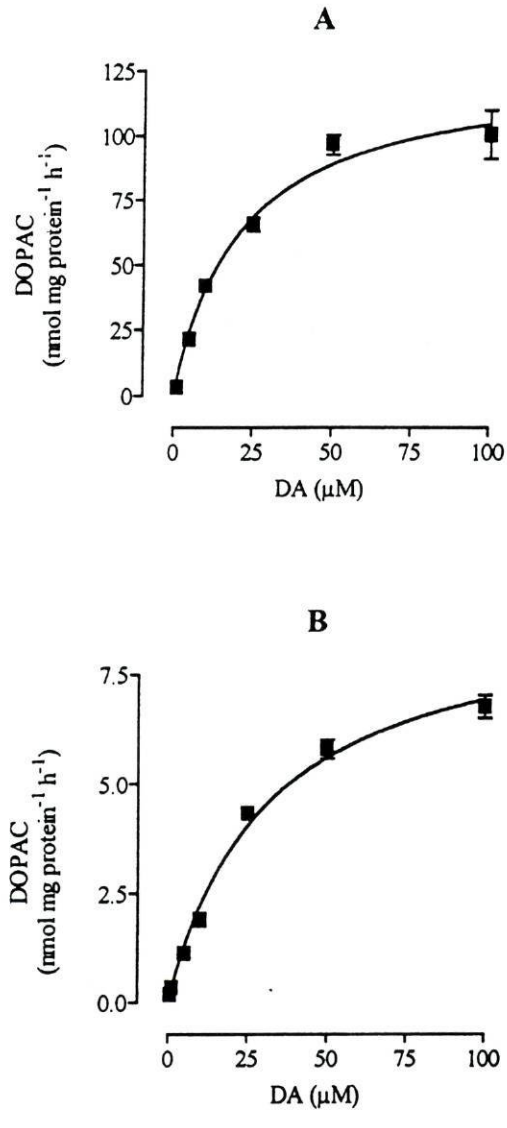


Fig. 5

Deamination of dopamine (DA; 1 to 100 μM) in homogenates of jejunal epithelial cells (A) and Caco-2 cells (B). The results are levels (in nmol mg protein^{-1} h^{-1}) of product formed from added substrate. Each point is the mean of 5 experiments per group; vertical lines indicate S.E.M..

sensitivity of Isc and potential difference to ouabain (data from (13) and that presented here). In fact, Caco-2 cells have considerable Na^+-K^+ATPase activity, though it was lower than that measured in epithelial cells from 60-day old rats (51±1 vs 130±5 nmol Pi mg protein^{-1} min^{-1}). In this respect, it is interesting to note that 20-day old rats express lower levels of Na^+-K^+ATPase α_1 isoform and are endowed with lower Na^+-K^+ATPase activity than 60-day old rats (9,35). It is quite possible that the lower Na^+-K^+ATPase activity in Caco-2 cells may have to do with the immature functional profile of these cells, as suggested by Grasset et al. (13).

In conclusion, Caco-2 cells in culture are endowed with the synthetic and metabolic machinery needed to form and degrade DA and 5-HT. Though, COMT activity could not be detected in Caco-2 cells, the amounts of the enzymes AAAD, MAO-A, MAO-B and Na^+-K^+ATPase found to occur in this cell line are most probably quite enough to reproduce in *in vitro* conditions the environment in which the intestinal dopaminergic and 5-hydroxytryptaminergic systems operate.

Acknowledgement

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Factores que modulam a resposta da dopamina sobre o transporte epitelial.

a) Dopamine-dependent inhibition of jejunal Na^+,K^+-ATPase during high-salt diet in young but not in adult rats.

Am. J. Physiol. **275 (6)**: G1317-G1323

b) Effect of salt intake on jejunal dopamine, Na^+,K^+-ATPase activity and electrolyte transport.

Acta Physiol. Scand. **168**: 225-231

c) Food intake abolishes the response of rat jejunal Na^+,K^+-ATPase to dopamine.

J Nutr. **130(4)**: 877-881

Dopamine-dependent inhibition of jejunal Na^+-K^+-ATPase during high-salt diet in young but not in adult rats

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Vieira-Coelho, M. Augusta, Vera A. Lucas Teixeira, Yigael Finkel, Patricio Soares-da-Silva, and Alejandro M. Bertorello. Dopamine-dependent inhibition of jejunal Na^+-K^+-ATPase during high-salt diet in young but not in adult rats. *Am. J. Physiol.* 275 (*Gastrointest. Liver Physiol.* 38): G1317–G1323, 1998.—During high-salt diet endogenous dopamine (DA) reduces jejunal sodium transport in young but not in adult rats. This study was designed to evaluate whether this effect is mediated, at the cellular level, by inhibition of Na^+-K^+-ATPase activity. Enzyme activity was determined in isolated jejunal cells by the rate of [γ-^{32}P]ATP hydrolysis. Cells were obtained from weanling and adult rats fed either with high- or normal-salt diet. In 20-day-old but not in 40-day-old rats Na^+-K^+-ATPase activity was significantly reduced during high-salt diet. This inhibition was abolished by a blocker of DA synthesis. The decreased activity was associated with a decreased α_1-subunit at the plasma membrane. During high-salt diet there was an increase in DA content in jejunal cells from 20-day-old rats, associated with a parallel decrease in 5-hydroxytryptamine, compared with normal-salt diet. In 40-day-old rats, however, the catecholamine level remained unchanged during high-salt diet. Incubation of isolated jejunal cells with DA resulted in a dose-dependent inhibition of Na^+-K^+-ATPase activity in 20- but not in 40-day-old rats. We conclude that during high-salt diet, jejunal Na^+-K^+-ATPase in 20-day-old rats is inhibited, and this effect is likely to be mediated by locally formed DA.

sodium-potassium-adenosinetriphosphatase synthesis; serotonin; L-3,4-dihydroxyphenylalanine

WE HAVE RECENTLY SHOWN that sodium absorption and catecholamine content in the jejunal mucosa of young rats were higher than in mature animals (14). In addition, young animals during high-salt diet experienced a decrease in sodium absorption with a parallel increase in tissue levels of dopamine (DA), whereas in adult animals there were no significant changes in sodium absorption, nor in catecholamine content of the jejunal mucosa (6, 14). That study suggested that during high-salt diet, an increase in the jejunal levels of DA associated with a decrease in the norepinephrine (NE) content was responsible for the control of sodium absorption in the jejunal mucosa of young animals. Adult (40-day-old) animals did not have a significant change in jejunal sodium absorption or in catecholamines levels during high-salt diet. The lack of any change in the jejunal function in response to high-salt diet coincided with the period in which the renal function has reached maturation (23, 24), thus suggesting complementary functions between the intestine and the kidney during development.

Sodium and water homeostasis is a critical step during adaptation to early life; although nephrogenesis is completed at birth, renal tubular function continues to develop postnatally (23, 24). During early postnatal life the kidney has a limited capacity to regulate fluids and electrolyte homeostasis, leading to high sodium excretion and often to negative sodium balance and hyponatremia (30, 31). Intestinal function is also determined by a developmental process that has a great impact not only during the uptake of nutrients but, equally importantly, in maintaining electrolytes and water metabolism (17, 32). Therefore, understanding the cellular mechanisms controlling this process is of paramount importance.

In transporting epithelia, vectorial movement of sodium is accomplished by means of the Na^+-K^+-ATPase located at the basolateral plasma membrane and several sodium transport mechanisms localized at the apical domain of the cell (25). In the renal epithelia (proximal and distal tubules) of mature animals, DA decreases Na^+-K^+-ATPase activity during high-salt diet, which is believed to contribute to increased urinary sodium excretion (3). Thus the present study was designed to investigate whether DA-dependent inhibition of sodium absorption across the jejunal mucosa of young animals is associated with decreased Na^+-K^+-ATPase activity.

EXPERIMENTAL PROCEDURES

All experiments were performed in Sprague-Dawley rats (BK Universal, Sollentuna, Sweden), aged 20 (weaning) and 40 (adult) days, weighing 37.1 ± 1.2 g (n = 8) and 170 ± 3.4 g (n = 8), respectively. Animals were kept in air-conditioned animal quarters, in litters of four animals per litter. Adult rats were fed ordinary solid rat chow (BK Universal); young rats were given the same chow but softened by mixing it with water. Tap water was provided ad libitum. The high-salt diet group received as drinking fluid 0.9% saline instead of tap water. The 20-day-old rats on high-salt diet were given saline for 4 days, whereas the 40-day-old rats received saline for 7 days before the study. We have previously examined Na^+-K^+-ATPase activity in response to high salt (3, 20) and found no differences between 2 and 7 days of administration of high-salt diet. The daily sodium intake averaged 0.5 and 5.0 mmol/100 g body wt in normal- and high-salt diet groups, respectively.

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To determine the role of endogenous DA, rats were injected intraperitoneally (0.1 ml) with benserazide (10 mg/kg body wt; Roche, Basel, Switzerland), an inhibitor of aromatic L-amino acid decarboxylase (AADC), 1 h before the animals were killed. This treatment was sufficient to cause complete inhibition of the peripheral DA-converting enzyme for up to 4 h after administration (10). Control animals were injected with the same volume of vehicle (sterile water).

Cell isolation. The method for cell isolation was as described previously (18, 22) with minor modifications. Briefly, the animals were killed by decapitation under anesthesia, and the abdominal cavity was immediately opened to excise a jejunal segment ~10 cm in length. The selected segment was placed on an ice-cold glass plate, cut in smaller segments of ~1.5 cm in length, and rinsed free from blood and intestinal contents with saline (0.9% NaCl). The tissue fragments were everted with fine forceps and incubated for 45 min in 5 ml warm (37°C) and gassed (95% O_2-5% CO_2) Hanks' solution with 0.06% collagenase type I (Sigma Chemical, St. Louis, MO). At the end of the incubation period the preparation was gently vortexed to allow the epithelial cells to detach. The fragments were then removed from the solution, and the medium containing the detached cells was centrifuged (200 *g*, 4°C) for 4 min. The cell pellet formed was resuspended in Hanks' solution. Cell viability was estimated by the trypan blue (0.04%, 1 min) exclusion method, and the percentage of viable cells (excluding the dye) was >90%, as determined by hemocytometer counting.

Determination of Na^+-K^+-ATPase activity. Na^+-K^+-ATPase activity in isolated cells in suspension was determined as described previously (2). Briefly, after cell isolation 10-μl aliquots (protein content ~5–15 μg protein) were transferred to the Na^+-K^+-ATPase assay medium (final volume 100 μl) containing (in mM) 50 NaCl, 5 KCl, 10 $MgCl_2$, 1 EGTA, 50 Tris-HCl, 10 Na_2ATP (Sigma), and [^{32}P]ATP (Amersham; specific activity 3,000 Ci/mmol) in tracer amounts (3.3 nCi/μl). Cells were transiently exposed to a thermic shock (10 min at −20°C) to render the plasma membrane permeable to ATP. The samples were then incubated at 37°C for 15 min. The reaction was terminated by rapid cooling to 4°C and addition of TCA-charcoal (5–10%). After the charcoal phase was separated (centrifugation at 14,000 rpm for 5 min), an aliquot from the supernatant was taken and the liberated ^{32}P was counted. Na^+-K^+-ATPase activity was calculated as the difference between test samples (total ATPase activity) and samples assayed in a medium devoid of Na^+ and K^+ and in the presence of 2 mM ouabain (ouabain-insensitive ATPase activity). Protein determination was performed according to Bradford using a conventional dye reagent (Bio-Rad, Richmond, CA).

Assay of tissue catecholamines. Segments of jejunum were opened longitudinally with fine scissors, rinsed free from blood and intestinal contents with cold saline (0.9% NaCl), and the jejunal mucosa was removed with a scalpel. The mucosae thus removed were blotted onto filter paper, weighed, placed in 1 ml 0.2 M perchloric acid, and stored frozen at −20°C. The assay of L-3,4-dihydroxyphenylalanine (L-DOPA), NE, DA, and 5-hydroxytryptamine (5-HT) was performed by means of HPLC with electrochemical detection (EC) as previously described (13). Briefly, aliquots of 500 μl of perchloric acid in which the tissue had been kept were placed in 5-ml conical-based glass vials with 50 mg alumina, and the pH of the sample was immediately adjusted to 8.6 by addition of Tris buffer. Mechanical shaking for 10 min was followed by centrifugation, and the supernatant was discarded. The adsorbed catecholamines were then eluted from the alumina with 200 μl of 0.2 M perchloric acid on Spin-X microfilter tubes (Costar, Badhoevedorp, The Netherlands); 50 μl of the eluate were injected into an HPLC-EC system. The detection was performed electrochemically by means of a thin-layer cell with a glassy carbon working electrode, an Ag/AgCl reference electrode, and an amperometric detector (Gilson model 141; Gilson Medical Electronics, Villiers Le Bel, France). The detector cell was operated at 0.75 V. The current produced was monitored using the Gilson 712 HPLC integration software connected to a personal computer system. The mobile phase was a degassed solution of 0.1 mmol/l citric acid, 0.5 mmol/l sodium octyl sulfate, 0.1 mol/l sodium acetate, 0.17 mmol/l ethylenediaminetetraacetic acid, 1 mmol/l dibutylamine, 12% methanol (vol/vol), pH 3.5, which was pumped at a rate of 1.0 ml/min. Standard solutions of L-DOPA, NE, DA, 5-HT, and dihydroxybenzylamine (internal standard) were injected at different concentrations, and peak height increased linearly. The lower limits for detection of L-DOPA, NE, DA, and 5-HT ranged from 350 to 500 fmol.

Western blot analysis. Jejunal cells were isolated from 20- and 40-day-old rats as previously described. The cells were homogenized, and aliquots (50 μg protein) were analyzed by SDS-PAGE using the Laemmli buffer system as described (1). Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) in a buffer containing 25 mM Tris-HCl, 192 mM glycine, SDS, 0.1% (wt/vol), and 20% methanol (vol/vol). Transferring was performed at 1 Amper during 3 h using a Transphor system (Hoaffer, San Francisco, CA). Protein identification was carried out using a monoclonal antibody against the α_1-subunit (21; courtesy of Dr. M. Caplan) and the α_2 and α_3 with a monoclonal antibody kindly provided by Dr. K. J. Sweadner. Immunoreactivity was detected with an enhanced chemiluminescence detection kit (Amersham) used exactly as recommended by the manufacturer. Measurements were performed with multiple exposures to ensure that signals were within the linear range of the film. Scans were performed using a Scan Jet IIc scanner (Hewlett-Packard, Palo Alto, CA). Each band was scanned two times in different regions, the scans were averaged, and the area of the peak minus the background (in arbitrary units) was quantified.

Statistical analysis. The data were analyzed using Student's *t*-test. Values are means ± SE. $P < 0.05$ was considered significant. A significant difference between one control and two experimental groups was determined using ANOVA with the Newman-Keuls posttest method (29)

The accumulation of L-DOPA in the jejunal mucosa after inhibition of AADC was calculated from a semilog plot of L-DOPA levels against time of inhibition; the slope of accumulation was calculated using linear regression. The fractional accumulation of L-DOPA (k) was then obtained from the expression k = slope/0.434 (6).

RESULTS

Na^+-K^+-ATPase activity was determined in isolated jejunal cells from 20- and 40-day-old rats. Basal enzyme activity, examined under optimal (maximal velocity) substrate conditions, was 300 ± 85 (n = 8) and 526 ± 51 (n = 5) nmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$, respectively. Administration of the high-salt diet resulted in a significant decrease in Na^+-K^+-ATPase activity in 20- but not 40-day-old rats (Fig. 1). This decrease in 20-day-old rat Na^+-K^+-ATPase activity observed during high-salt diet was blunted when benserazide, an inhibitor of AADC, was given 1 h before the experiments.

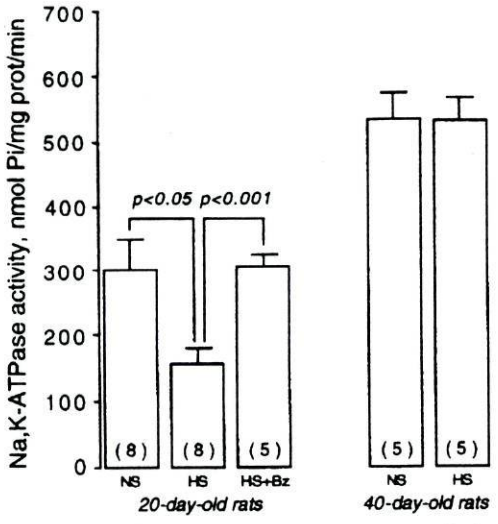


Fig. 1. Jejunal Na^+-K^+-ATPase activity in 20- and 40-day-old rats fed normal-salt (NS), high-salt (HS), or high-salt diet-benserazide (HS-Bz). Each bar represents mean ± SE of determinations performed independently and in duplicate. Number in bars represents experiments (no. animals) performed in each group.

The presence of different Na^+-K^+-ATPase isoforms in the jejunal cells has not been previously characterized. Thus we first determined which of the isoforms are present in jejunal cells from 20- and 40-day-old rats (Fig. 2) and whether this pattern was affected by high-salt diet. Isolated rat striatum was used as a positive control. Whereas all three isoforms were present in the striatal membranes, the jejunal cell plasma membrane exhibited the presence of only the Na^+-K^+-ATPase α_1-isoform (Fig. 2). In addition, plasma membranes isolated from jejunal cells from 20-day-old rats on high-salt diet showed a significant reduction (% of control 74.2 ± 4.8, $n = 4$) in the α_1-subunit abundance compared with age-matched controls receiving normal saline, whereas in 40-day-old animals high salt did not affect the α_1-subunit abundance.

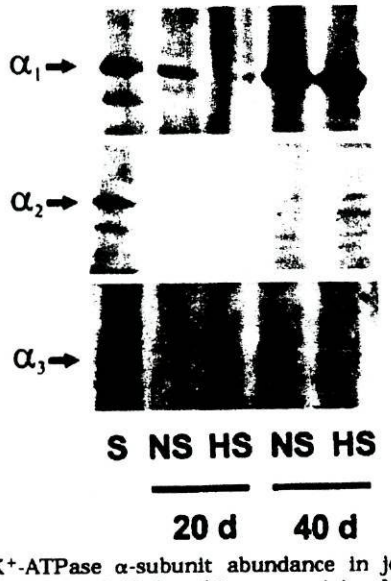


Fig. 2. Na^+-K^+-ATPase α-subunit abundance in jejunal plasma membrane from 20- and 40-day-old rats receiving NS or HS diet. Each lane contains equal amount of protein (18.4 μg) except for Western blots with α_3-isoform antibody (9.2 μg). Western blots were repeated 4 times (animals). S, striatum.

Table 1. *Levels of L-DOPA, NE, DA, and 5-HT in jejunal mucosa of 20- and 40-day-old rats receiving normal-salt diet*

Condition	L-DOPA	NE	DA	5-HT
20-Day-old	217 ± 45*	755 ± 183*	110 ± 8*	9,659 ± 557*
40-Day-old	86 ± 29	35 ± 6	41 ± 19	3,440 ± 1,142

Values are means ± SE; n = 6–12 animals/group. L-DOPA, L-3,4-dihydroxyphenylalanine; NE, norepinephrine; DA, dopamine; 5-HT, 5-hydroxytryptamine. * Significantly different from corresponding values in 40-day-old rats ($P < 0.05$, Newman-Keuls method).

Levels of L-DOPA, NE, DA, and 5-HT were determined in the jejunal mucosa of 20- and 40-day-old rats fed normal- and high-salt diets. Basal levels of all monoamines in the jejunal mucosa of 20-day-old rats were higher than in 40-day-old rats (Table 1), in agreement with results reported in a previous study (14). In 20-day-old rats administration of high-salt diet resulted in a significant increase (~57%) in the tissue level of DA, whereas 5-HT content was significantly decreased by ~27% (Fig. 3). The tissue content of NE did not change, regardless of the diet administered. Benserazide treatment induced a significant ($P < 0.05$) increase in L-DOPA (from 113 ± 18.8 to 752 ± 165 pmol/g) and abolished the increase in DA during high-salt diet (Fig. 3, *left*) but did not induce a significant change in the tissue levels of 5-HT (Fig. 3, *right*). In 40-day-old rats high-salt diet did not significantly affect the levels of either DA or 5-HT (Fig. 4). During inhibition of AADC with benserazide (Fig. 5), the rate constant of accumulation of L-DOPA in the jejunal mucosa of young rats ($k = 0.0288 \pm 0.0030$, $n = 6$) was found to be similar ($P = 0.09$) to that observed in adult rats ($k = 0.0356 \pm 0.0021$, $n = 7$); the total amount of L-DOPA accumulated during AADC inhibition was, however, considerably higher in young rats (932 ± 39 pmol/g) than in adult rats (220 ± 33 pmol/g).

We next examined the possibility that DA may affect fluid transport across the intestinal epithelium by

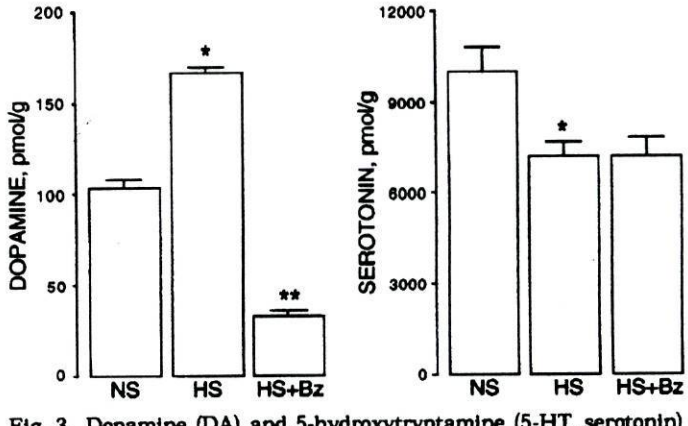


Fig. 3. Dopamine (DA) and 5-hydroxytryptamine (5-HT, serotonin) levels in jejunal epithelial cells of 20-day-old rats in NS, HS, and HS-Bz groups. Each bar represents mean ± SE of 5 determinations performed in duplicate. * $P < 0.05$ from corresponding value in NS group. ** $P < 0.001$ from corresponding value in HS group.

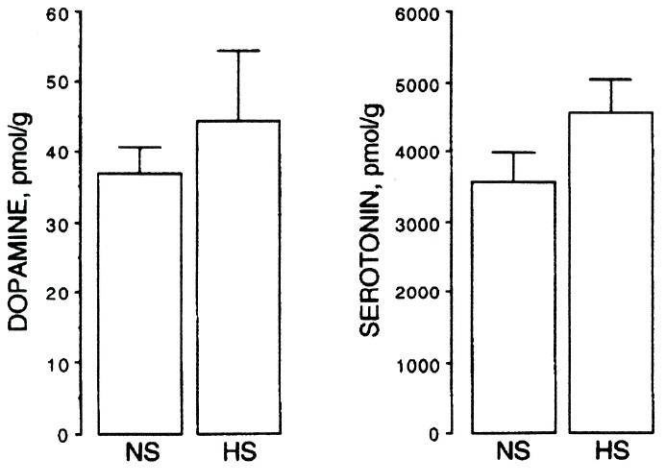


Fig. 4. DA and 5-HT levels in jejunal epithelial cells of 40-day-old rats on NS and HS diet. Each bar represents mean ± SE of 5 determinations performed in duplicate.

inhibiting the Na^+-K^+-ATPase activity. In isolated cells from 20-day-old animals, in vitro incubation with DA resulted in a concentration-dependent inhibition of Na^+-K^+-ATPase activity (Fig. 6). The EC_{50} for DA-induced inhibition of Na^+-K^+-ATPase was ~100 nM. DA (1 μM) failed to inhibit Na^+-K^+-ATPase activity in isolated cells from 40-day-old rats (% of control 93.1 ± 3.8, $n = 7$, not significant).

Because the high-salt diet was not only associated with increased DA levels but also with a decrease in the cellular content of 5-HT, it was next evaluated whether the inhibitory action of DA was affected by 5-HT (Fig. 7). The inhibitory effect of DA was prevented by 1 and 10 μM 5-HT.

DISCUSSION

High-salt diet in young animals is associated with a decrease in sodium and water absorption in the small intestine (14). The results presented here suggest that at the cellular level the decreased sodium absorption (vectorial transport) could be the result of decreased Na^+-K^+-ATPase activity. Moreover, control of the Na^+-K^+-ATPase catalytic activity is likely to be dependent on endogenous DA tissue levels.

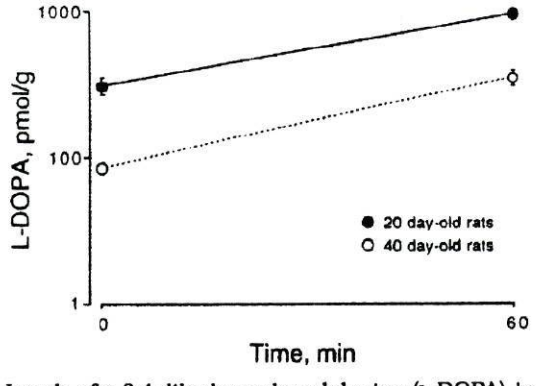


Fig. 5. Levels of L-3,4-dihydroxyphenylalanine (L-DOPA) in jejunal mucosa of 20- and 40-day-old rats at *time 0* and 60 min after administration of benserazide (10 mg/kg). Results are shown for observations of accumulation of L-DOPA. Each point represents mean of 8 animals/group; SE values were <10% of corresponding means.

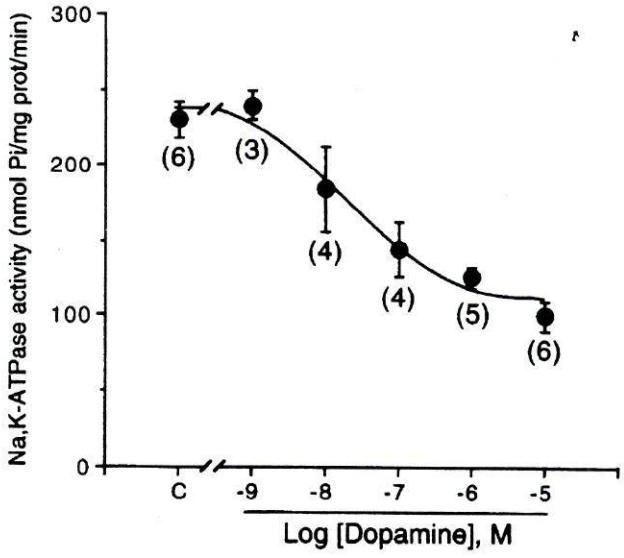


Fig. 6. Na^+-K^+-ATPase activity in jejunal cells, which were isolated from weanling rats and incubated in vitro with DA. Cells were incubated with different concentrations of DA for 15 min at room temperature. Each value represents mean ± SE of triplicate determinations. Number of experiments performed for each concentration is in parentheses.

Tissue catecholamines are important regulators of jejunal cell function (7). In a previous report we have demonstrated that the ability of young animals to maintain sodium balance during high-salt diet depends on the tissue levels of DA (14). Their ability to reduce sodium and water absorption during high-salt diet is blunted by benserazide (14). The data presented here suggest that the effect of DA could be mediated by inhibition of Na^+-K^+-ATPase activity. Young rats on a high-salt diet [transport less sodium across the jejunal epithelium (14)] demonstrate lower Na^+-K^+-ATPase activity and higher DA tissue levels than young ani-

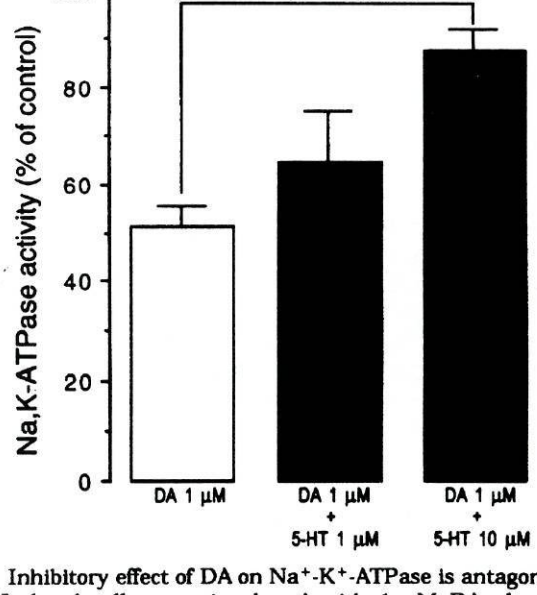


Fig. 7. Inhibitory effect of DA on Na^+-K^+-ATPase is antagonized by 5-HT. Isolated cells were incubated with 1 μM DA alone or in presence of 1 and 10 μM 5-HT. Na^+-K^+-ATPase activity was expressed as percentage of control. Each bar represents mean ± SE of 5 experiments.

mals on normal-salt diet. The reduction in Na^+-K^+-ATPase activity during high-salt diet could be prevented by pretreatment with benserazide, an inhibitor of DA synthesis, suggesting a causal relationship between endogenous DA and inhibition of the sodium pump. The possibility that DA may regulate Na^+-K^+-ATPase activity was further supported by experiments demonstrating that enzyme activity was reduced in jejunal cells incubated in vitro with DA and that this effect was only present in jejunal cells isolated from young but not adult rats. The finding that exogenous DA produced an effective inhibition on Na^+-K^+-ATPase activity in isolated cells from the jejunal mucosa is an additional argument favoring the view that inhibition of intestinal sodium absorption by DA may be dependent on an interaction with the enzyme.

At the cellular level inhibition of Na^+-K^+-ATPase activity by DA could be the result of a reduced number of pump units in the plasma membrane, a mechanism perhaps analogous to the one present in renal epithelial cells (8, 9). This view is supported by the finding that plasma membranes isolated from jejunal cells of young rats on a high-salt diet showed a parallel decrease in the Na^+-K^+-ATPase α_1-subunit abundance compared with age-matched rats on a normal-salt diet.

Na^+-K^+-ATPase activity was determined under optimum substrate concentrations (sodium, potassium, and ATP), thus excluding the possibility that the inhibitory effect of DA could be secondary to changes in sodium permeability. However, the latter does not exclude the possibility that during in vivo conditions there also exists a concomitant decrease in apical sodium permeability, as reported for the renal epithelia (4, 5); a simultaneous decrease in apical sodium permeability and Na^+-K^+-ATPase activity would result in decreased vectorial transport without changes in intracellular sodium.

In contrast to data obtained from young rats, adult rats were found to show higher Na^+-K^+-ATPase activity. In addition, contrariwise to young rats, changes in neither Na^+-K^+-ATPase activity nor Na^+-K^+-ATPase α_1-subunit abundance were found to occur in adult rats during a high-salt diet. Following the rationale used for young animals that associates sodium load, Na^+-K^+-ATPase activity, and DA levels, it might be suggested that Na^+-K^+-ATPase activity in adult rats behaved differently because jejunal cells have less ability to synthesize DA and are less sensitive to the amine; altogether, this would result in a decreased dopaminergic tonus on the sodium pump. Several observations point in this direction. First, the basal levels of DA and L-DOPA, the amine precursor, in adult rats were one-half those in young rats. Second, exogenous DA failed to produce inhibition of Na^+-K^+-ATPase in jejunal cells of adult rats. This, however, does not explain why adult rats behave differently from young rats when challenged with a high-salt diet. Despite low levels of endogenous L-DOPA and DA in the jejunal mucosa of adult rats during normal-salt diet, the rate of L-DOPA utilization (given from the L-DOPA/DA ratios) was similar to that observed in young rats. This contrasts with the results obtained during high-salt diet, in which the rate of L-DOPA utilization in young animals was higher than in adult animals (Table 2). It appears therefore that during high-salt diet, the link between the stimulus (high-sodium intake) and the ability of the jejunal cells to increase DA synthesis is not present at an older age. However, this reduced ability of adult rats to synthesise DA may not be related to a decrease in AADC activity, because after being challenged with benserazide the rate of accumulation of L-DOPA is similar in both groups of animals (Fig. 5, see also Ref. 6). This may indicate that the decreased ability to synthesize DA during high-salt diet is related to a reduced availability of L-DOPA to the intracellular milieu and therefore to AADC. The cellular uptake of L-DOPA, at least in renal tubular epithelial cells, has an important sodium-dependent component (26), and a possible explanation for this reduced ability of adult rats to synthesize DA could reside in the appearance of resistance to sodium-dependent mechanisms as animals become older. Although one may attribute the failure of adult animals to inhibit Na^+-K^+-ATPase activity to decreased ability to synthesize DA, it is interesting to note that in young animals the jejunal content of L-DOPA was higher than in adult animals despite the fact that they received a similar diet. On the other hand, the finding that exogenous DA is devoid of an inhibitory effect on Na^+-K^+-ATPase activity in adult rats may suggest a developmental change in the response that coincides with the maturation of the renal function and furthermore with the ability of DA to inhibit Na^+-K^+-ATPase activity in renal epithelial cells (15).

Another aspect of importance concerns the role of 5-HT, by far the most abundant amine at the level of the intestinal epithelia and a well-known intestinal secretagogue (9, 18). Although there are differences between species and between different areas of the digestive tract (jejunum, ileum, and colon), the main effect of 5-HT is a potent stimulation of intestine fluid and electrolyte secretion. This appears to result from an increase in the electrogenic secretion of chloride ions together with an inhibition of electroneutral absorption of sodium chloride (16, 33). The finding that high-salt diet in 20-day-old rats was associated with a decrease in 5-HT levels and Na^+-K^+-ATPase activity, and because in rat renal tubules 5-HT was found to stimulate the sodium pump (27, 28), it was hypothesised that at

Table 2. *Rate of L-DOPA utilization in jejunal mucosa of 20- and 40-day-old rats receiving normal- and high-salt diet*

Condition	L-DOPA/DA Ratio	
	20-Day-old	40-Day-old
Normal-salt diet	1.45 ± 0.59	1.56 ± 0.50
High-salt diet	0.60 ± 0.05*	1.98 ± 0.30†

Values are means ± SE; n = 5 animals/group. *Significantly different from corresponding value in normal-salt diet ($P<0.05$). †Significantly different from corresponding value in 20-day-old rat ($P<0.05$).

the intestinal level an association between 5-HT levels and Na^+-K^+-ATPase activity could also be present. However, the decrease in Na^+-K^+-ATPase activity during high-salt diet was abolished by benserazide, and the levels of 5-HT were not even modified. This only suggests that 5-HT, in contrast to DA, resides in a storage compartment (enterochromaffin cells; 12), which is fairly stable and not so easily modified by the acute administration of benserazide. As mentioned previously, in the kidney 5-HT was found to stimulate Na^+-K^+-ATPase activity, and the coincubation of 5-HT agonist with DA was found to revert the inhibitory effect of the latter (27). In the present study, the inhibitory action of maximal doses of DA on Na^+-K^+-ATPase activity was significantly prevented by coincubation with 5-HT, suggesting the presence of a functional antagonism between the two amines in the control of enzyme activity. To our knowledge this is the first time that 5-HT in the intestine is described to exert an effect on Na^+-K^+-ATPase. It is therefore difficult to integrate this information with the evidence that 5-HT decreases net sodium absorption and stimulates net chloride secretion, which necessarily needs further study.

In conclusion, the results of this study indicate that the reduced sodium absorption observed in young rats during high-salt diet is probably due to decreased Na^+-K^+-ATPase activity. This study also demonstrated that the rate of utilization of L-DOPA in jejunal cells of adults rats on high-salt diet is lower than in young rats, and this may be due to a decreased availability of the substrate to the intracellular milieu rather than a specific deficiency in AADC activity.

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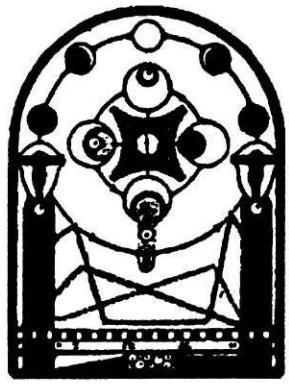
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Effect of salt intake on jejunal dopamine, Na^+,K^+-ATPase activity and electrolyte transport

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ABSTRACT

The present study addresses the question of the relevance of salt intake on jejunal dopamine, Na^+,K^+-ATPase activity and electrolyte transport. Low salt, but not high salt, intake for 2 weeks increased dopamine levels in the jejunal mucosa accompanied by a marked decrease in L-3,4-dihydroxyphenylalanine tissue levels. By contrast, in rats fasted for 72 h the effect of refeeding for 24 h with a low salt diet failed to change dopamine tissue levels, although it significantly increased those of L-3,4-dihydroxyphenylalanine. By contrast, high salt intake markedly increased the tissue levels of both dopamine and L-3,4-dihydroxyphenylalanine, without changes in dopamine/L-3,4-dihydroxyphenylalanine tissue ratios. Tissue levels of both L-3,4-dihydroxyphenylalanine and dopamine in control conditions (normal salt intake for 2 weeks) were markedly higher ($P < 0.05$) than in rats submitted to 72 h fasting plus 24 h refeeding. The effect of fasting for 72 h followed by 24 h refeeding was a marked decrease in jejunal Na^+,K^+-ATPase activity, particularly evident for rats fed a normal salt and high salt diets during the refeeding period. Basal short circuit current was similar in rats fed a normal salt diet for 2 weeks and 24 h, and the type of diet failed to alter basal short circuit current after refeeding with normal, low and high salt diets. On the other hand, the effect of prolonged low salt intake was a marked decrease in jejunal Na^+,K^+-ATPase activity and basal short circuit current, whereas high salt intake failed to alter enzyme activity and basal short circuit current. In rats fed for 2 weeks a high salt diet ouabain was found to be more potent in reducing jejunal short circuit current than in rats fed normal and low salt diets. The effect of furosemide was more marked in rats fed for 2 weeks high and low salt diets than in animals receiving a normal salt intake. Dopamine (up to 1 μmol L^{-1}) was found not to alter Na^+,K^+-ATPase and basal short circuit current in jejunal epithelial sheets, in rats fed with normal, low and high salt diets for 2 weeks and 24 h.

Keywords dopamine, Na^+,K^+-ATPase, jejunum, salt intake, L-DOPA.

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The relationship between salt intake and high blood pressure has been investigated extensively in both humans and laboratory animals (Taubes 1998). The relative importance of the renal dopaminergic system in controlling natriuresis assumes particular relevance in view of the findings that salt-sensitive hypertensives may have a fault in renal dopamine production and this may be associated with salt sensitivity of their blood pressure (Kuchel & Kuchel 1991, Lee 1993). Furthermore, high blood pressure may be linked to a deficient stimulation of second messenger production by adenyl cyclase, phospholypase C and phospholypase A_2 (Jose & Felder 1996, Hussain & Lokhandwala 1998).

Similarities between the renal and intestinal autocrine/paracrine non-neuronal dopaminergic system have been described (Vieira-Coelho *et al.* 1997). Epithelial cells from both renal proximal tubules and the intestinal mucosa are endowed with (1) efficient mechanisms for L-3,4-dihydroxyphenylalanine (L-DOPA) uptake, (2) high aromatic L-amino acid decarboxylase activity, which easily converts intracellular L-DOPA to dopamine, (3) efficient enzyme systems for the metabolic degradation of newly formed dopamine, and (4) specific receptors for the amine, the activation of which leads to Na^+,K^+-ATPase inhibition and transepithelial sodium flux. In both systems the final effect is concordant with respect to sodium, leading to decreased sodium absorption in the intestine and increased sodium excretion in the kidney (Vieira-Coelho *et al.* 1997). A high salt (HS) intake has been found to constitute an important stimulus for the production of dopamine in rat jejunal epithelial cells and this is accompanied, in 20-day-old

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animals, by a decrease in sodium intestinal absorption (Finkel *et al.* 1994). This effect is accomplished, at the cellular level, by inhibition of Na^+,K^+-ATPase activity (Vieira-Coelho *et al.* 1998). The relative importance of this system in controlling sodium absorption assumes particular relevance in view of the findings that 40-day-old rats submitted to an HS intake have a fault in intestinal dopamine production during salt loading and intestinal Na^+,K^+-ATPase is insensitive to the inhibitory effects of dopamine, in contrast to that occurring in 20-day-old animals (Finkel *et al.* 1994, Vieira-Coelho *et al.* 1998). The lack of changes in the jejunal function in response to HS intake coincides with the period in which the renal function has reached maturation (Robillard *et al.* 1992), suggesting the occurrence of complementary functions between the intestine and the kidney during development.

It is not known, however, if the duration of exposure to different salt intake may influence the expression of dopamine effects upon mechanisms involved in jejunal electrolyte absorption. Therefore, the present study addressed the question of the relevance of salt intake on jejunal dopamine, Na^+,K^+-ATPase activity and jejunal electrolyte transport.

MATERIALS AND METHODS

Animals

All the experiments were performed on 60-day-old male Wistar (260–300 g) rats (Harlan-Interfauna, Barcelona, Spain). The rats were housed in air-conditioned animal quarters (22 ± 2 °C, 60 ± 10% humidity) and had free access to drinking water until the day of the experiment. Animals were killed by decapitation under ether anaesthesia. Rats were divided into two groups: (1) rats fed with normal salt (NS), low salt (LS) or high salt (HS) diets for 2 weeks; (2) animals fasted for 72 h and refed with NS, LS or HS diets for 24 h. NS and HS diets consisted of normal rat chow (Harlan-Interfauna, RMM type diet, Barcelona, Spain) plus tap water or 1% saline, respectively; animals on LS intake were fed with a sodium-deficient diet (0.01–0.02% sodium; diet TD 90228, Harlan Teklad, Madison, Wisconsin, USA).

Electrical transepithelial measurements

Rats were killed by decapitation and two jejunal segments located 10–15 cm distal from the pyloric sphincter were removed. Each segment (2 cm long) of jejunum was cut longitudinally along the mesenteric border, washed free of luminal contents and the tissue pinned mucosal side down on a dental wax block. The serosa and muscularis were stripped away by dissection to obtain the epithelial sheets, as previously described (Vieira-Coelho & Soares-da-Silva 1998). Two adjacent pieces were routinely prepared from a single jejunum. Rat epithelial sheets were mounted in Ussing chambers (window area 0.28 cm^2) filled with Krebs–Hensleit solution, gassed with 95% O_2 and 5% CO_2 and maintained at 37 °C. D-Glucose (10 mmol L^{-1}) was added to the serosal-side reservoir and an equimolar amount of mannitol was added to the mucosal-side reservoir. The Krebs–Hensleit solution contained (in mmol L^{-1}): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2; the pH was adjusted to 7.4 after gassing with 5% CO_2 and 95% O_2. The tissues were continuously voltage clamped to zero potential differences by application of external current, with compensation for fluid resistance, by means of an automatic voltage current clamp (DVC 1000, World Precision Instruments, Sarasota, Florida, USA). Transepithelial resistance (Ω cm^2) was measured by altering the membrane potential stepwise (±5 mV) and applying the Ohmic relationship. Epithelial layers were voltage clamped to zero potential difference and short-circuit current (I_{sc}) was continuously recorded as an index of electrogenic ion transport. I_{sc} is reported in μA cm^{-2} and negative values are consistent with cation absorption or anion secretion. The voltage/current clamp unit was connected to a PC via a BIOPAC MP1000 data acquisition system (BIOPAC Systems, Goleta, CA, USA). The data analysis was performed using Acq*Knowledge* 2.0 software (BIOPAC Systems, Goleta, CA, USA). Usually two preparations were mounted in chambers. After a 30- to 45-min preincubation period, by which time the potential difference had stabilized, furosemide or ouabain were added to the serosal-side reservoir only. In some experiments, dopamine was also added to the serosal-side reservoir; ascorbic acid (1 mmol L^{-1}) was present in the serosal bathing solution to reduce oxidation of dopamine.

Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity was measured by the method of Quigley & Gotterer (1969) and adapted in our laboratory with slight modifications. Briefly, isolated jejunal epithelial cells, obtained as previously described (Vieira-Coelho *et al.* 1998), were preincubated for 20 min at 37 °C. After the preincubation period, the jejunal epithelial cells were permeabilized by rapid freezing in dry ice–acetone and thawing. The reaction mixture, in a final volume of 1.025 mL, contained (in mmol L^{-1}) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium EDTA, 5 $MgCl_2$, NaN_3, 75

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tris(hydroxymethyl)aminomethane(tris) hydrochloride and 100 μL tubular and epithelial cell suspension (100 μg protein). The reaction was initiated by the addition of 4 mmol L^{-1} ATP. For determination of ouabain-sensitive ATPase, NaCl and KCl were omitted, and Tris–HCl (150 mmol L^{-1}) and ouabain (1 mmol L^{-1}) were added to the assay. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of 50 μL of ice-cold trichloroacetic acid. Samples were centrifuged (1500g), and liberated P_i in supernatant was measured by spectrophotometry at 740 nm. Na^+,K^+-ATPase activity is expressed as nanomoles P_i per milligram protein per minute and determined as the difference between total and ouabain-insensitive ATPase. The protein content in cell suspension (≈2 mg mL^{-1}), as determined by the method described by Bradford (1976) with human serum albumin as a standard, was similar in all samples.

Assay of monoamines

The assay of L-3,4-dihydroxyphenylalanine (L-DOPA) and dopamine was performed by means of high-pressure liquid chromatography, as previously described (Vieira-Coelho & Soares-da-Silva 1993). The detection was carried out electrochemically with a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141); the detector cell was operated at 0.75 V. The current produced was monitored using the Gilson 712 HPLC software. The lower limit for detection of L-DOPA and dopamine ranged between 350 and 500 fmol.

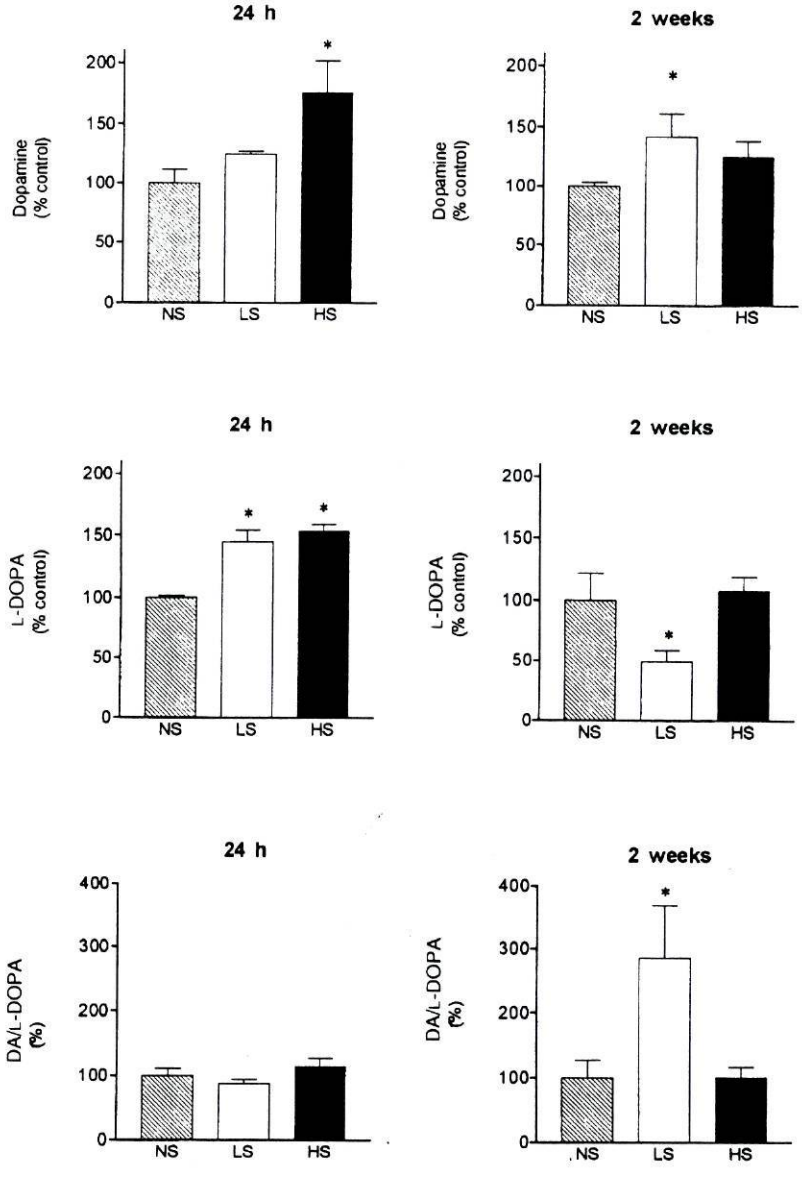


Figure 1 Levels of dopamine and L-DOPA and dopamine/L-DOPA tissue ratios in the jejunal mucosa of rats fed with LS, NS or HS diets for 24 h and 2 weeks. Columns represent means of four to five experiments per group; vertical lines indicate SEM. Significantly different (* $P < 0.05$) from animals fed with NS diet.

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Drugs

The compounds used were: dopamine hydrochloride, ouabain and furosemide, obtained from Sigma Chemical Company (St. Louis, MO, USA).

Statistics

Results are mean ± SEM of values for the indicated number of determinations. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student's *t*-test for unpaired comparisons. A *P*-value less than 0.05 was assumed to denote a significant difference.

RESULTS

As shown in Fig. 1, the effect of LS intake for 2 weeks was an increase in dopamine levels in the jejunal mucosa accompanied by a marked decrease in L-DOPA tissue levels; this resulted in that the dopamine/L-DOPA tissue ratio, a rough measure of dopamine rate of synthesis, became markedly increased. By contrast, HS intake for 2 weeks did not change the levels of dopamine and L-DOPA. In rats fasted for 72 h, the effect of refeeding for 24 h with an LS diet failed to change dopamine tissue levels, although it significantly increased those of L-DOPA. On the other hand, HS intake markedly increased the tissue levels of both dopamine and L-DOPA, without changes in dopamine/L-DOPA tissue ratios. Another major finding was that absolute tissue levels of both L-DOPA and dopamine in control conditions (NS diet for 2 weeks) were markedly higher ($P < 0.05$) than in rats submitted to 72 h fasting plus 24 h refeeding (L-DOPA, 268.9 ± 57.9 vs. 33.9 ± 0.4 pmol g^{-1}; dopamine, 52.8 ± 1.5 vs. 19.9 ± 2.3 pmol g^{-1}).

Figure 2 shows Na^+,K^+-ATPase activity in isolated jejunal epithelial cells in rats fed with NS, LS or HS diets for 2 weeks and 24 h; this figure also shows basal I_{sc} in jejunal epithelial sheets obtained from these animals. As can be observed in the figure, jejunal Na^+,K^+-ATPase activity in rats fasted for 72 h followed by 24 h refeeding with NS and HS diets was lower ($P < 0.05$) than in rats fed with the same diets for 2 weeks. Basal I_{sc} was similar in rats fed an NS diet for 2 weeks or 24 h, and the type of diet failed to alter basal I_{sc} after refeeding 24 h with NS, LS or HS diets. On the other hand, the effect of prolonged LS intake was a marked decrease in jejunal Na^+,K^+-ATPase activity and basal I_{sc}, whereas HS intake failed to alter enzyme activity and basal I_{sc}.

Figure 3 shows the effect of inhibitors of major intestinal transporters, Na^+,K^+-ATPase and Na^+–K^+–$2Cl^-$ (NKCC), respectively, ouabain and furosemide, alone or in combination upon I_{sc} in jejunal epithelial sheets from rats fed with NS, LS or HS diets for 2 weeks and 24 h. In rats fed for 2 weeks on HS diet ouabain was found to be more potent in reducing jejunal I_{sc} than in rats fed NS and LS diets. The effect of furosemide was more marked in rats

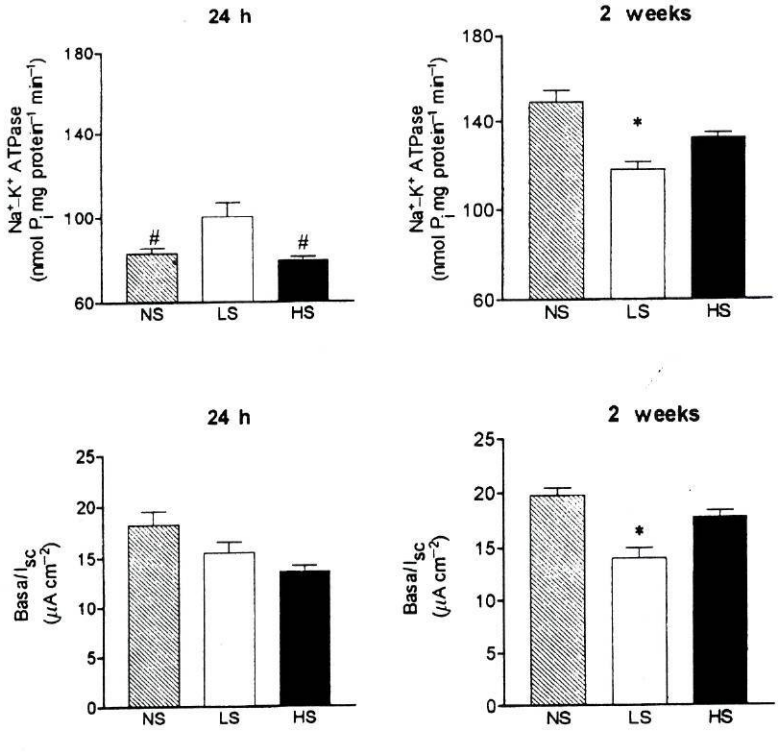


Figure 2 Na^+,K^+-ATPase activity and basal short circuit current (I_{sc}) in jejunal epithelial cells and jejunal epithelial sheets, respectively, of rats fed with LS, NS or HS diets for 24 h and 2 weeks. Columns represent means of five to eight experiments per group; vertical lines indicate SEM. Significantly different from animals fed with NS diet for 2 weeks(*$P <$ 0.05) or corresponding values in animals fed with NS and HS diets for 2 weeks (#$P < 0.05$).

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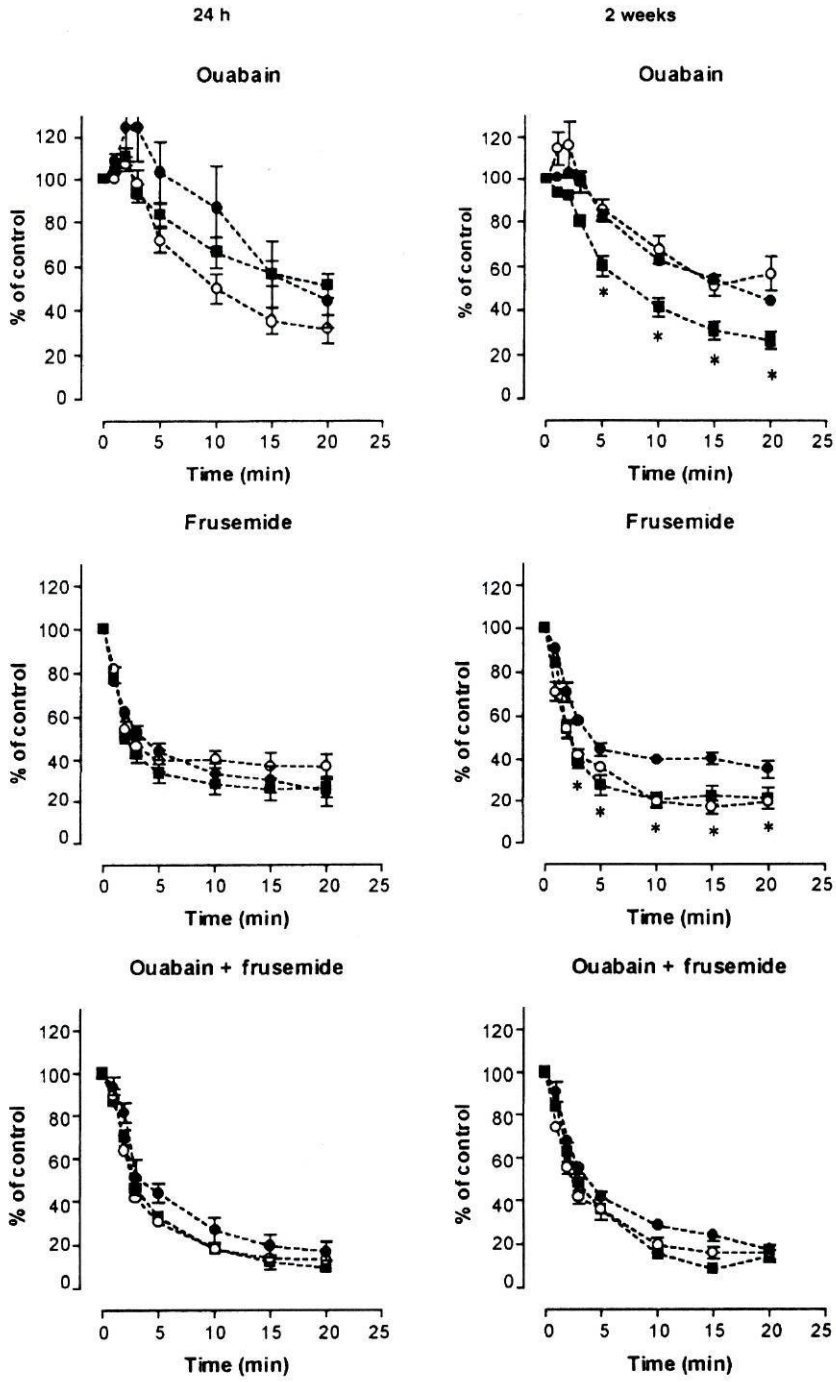


Figure 3 Effect of ouabain and furosemide, alone or in combination, upon basal short circuit current (I_{sc}) in jejunal epithelial sheets of rats fed with LS (○), NS (●) or HS (■) diets for 24 h and 2 weeks. Symbols represent means of five to eight experiments per group; vertical lines indicate SEM.

fed for 2 weeks HS and LS diets than in animals receiving an NS intake. The effect of ouabain plus furosemide was identical in NS, LS and HS groups. The effects of ouabain and furosemide, alone or in combination, were identical in rats fed NS, LS or HS diets for 24 h.

Dopamine (up to 1 μmol L^{-1}) was found not to alter Na^+,K^+-ATPase and basal I_{sc} in jejunal epithelial sheets, either in rats fed for 2 weeks or 24 h NS, LS and HS diets (data not shown).

DISCUSSION

The results presented here show that salt intake alters, in a time-dependent manner, levels of dopamine and of its precursor L-DOPA in the jejunal mucosa, and major processes involved in jejunal ion transport. The evidence supporting this functional link between availability of dopamine and transepithelial movement of sodium is, however, circumstantial, as the result of inhibition of amine formation and receptor blockade was not

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evaluated. More important, perhaps, is the finding that food availability appears to be determinant for the maintenance of stable levels of dopamine and its precursor L-DOPA in the jejunal mucosa, this being independent of salt intake. In fact, fasting for 72 h followed by 24 h refeeding produced a drastic decrease in dopamine and L-DOPA. As the more relevant reduction concerned that of L-DOPA, it may be argued that this effect is related to a decrease in the supply of L-DOPA containing nutrients in the diet (Kaufman *et al.* 1989).

LS intake for 2 weeks markedly increased dopamine levels in the jejunal mucosa, this being accompanied by an increase in dopamine/L-DOPA tissue ratios, an indication of enhanced rate of dopamine synthesis, and significant decreases in Na^+,K^+-ATPase activity and basal I_{sc}. These results may suggest the presence of an enhanced dopaminergic tonus during prolonged LS intake, but its relationship with decreases in Na^+,K^+-ATPase activity and changes in jejunal ion transport is not linear. This is particularly evidenced by the lack of effect of exogenous dopamine upon Na^+,K^+-ATPase and basal I_{sc}, which is in agreement with previous reports (Vieira-Coelho *et al.* 1998, Vieira-Coelho & Soares-da-Silva 1998). It might be then speculated that the relationship between enhanced dopaminergic tonus and low Na^+,K^+-ATPase activity and reduced jejunal ion transport would involve the participation of another process yet to be identified. Alternatively, these putative effects of dopamine upon Na^+,K^+-ATPase and jejunal ion transport observed during LS intake may be concerned with long- rather than short-term adaptations. The classic view is that HS intake increases the formation of dopamine and LS intake does the opposite (Kuchel & Kuchel 1991, Lee 1993). Furthermore, the decrease in renal Na^+,K^+-ATPase activity following HS intake is associated with increases in the formation of dopamine (Bertorello *et al.* 1988, Seri *et al.* 1990). This is observed at the kidney level and in the jejunum of 20-day-old rats. In fact, older animals (Sprague–Dawley and Wistar) have been demonstrated not to respond to HS intake with an increase in dopamine formation and reduced Na^+,K^+-ATPase activity (Bertorello *et al.* 1993, Finkel *et al.* 1994, Vieira-Coelho *et al.* 1998). It would be logical to have in operation a local mechanism preventing the intestinal absorption of high amounts of sodium during HS intake: sodium would promote the formation of dopamine (facilitation of L-DOPA uptake and enhanced activity of aromatic L-amino acid decarboxylase), and then the newly formed amine would inhibit Na^+,K^+-ATPase activity through the activation of specific receptors. It is possible this is no longer required in adult animals, where this type of phenomenon is fully functional at the kidney level, and coincides with the period in which the renal function has reached maturation (Robillard *et al.* 1992). However, this does not explain the result why prolonged LS intake results in an increase in dopamine formation. On the other hand, from a conceptual point of view it is acceptable that an LS intake would be accompanied by a decrease in Na^+,K^+-ATPase activity and jejunal ion transport. The same rationale applies to the experimental condition in which animals are submitted to a prolonged period of fasting (72 h). Fasting has been described to be accompanied by a decrease in Na^+,K^+-ATPase activity (Murray & Wild 1980, Lucas-Teixeira *et al.* 1999). The recovery of Na^+,K^+-ATPase activity to initial levels appears, however, to depend on the duration of the fasting and the refeeding periods. In fact, we have previously reported that 48 h fasting produced a marked reduction in Na^+,K^+-ATPase activity, and a complete recovery in enzyme activity was attained at 48 h refeeding (Lucas-Teixeira *et al.* 1999).

The results on the effects of ouabain and furosemide, alone or in combination, upon jejunal I_{sc}, complement those on Na^+,K^+-ATPase activity, in the sense they add information concerning the activity of another major transporter, the NKCC. In rats on LS intake for 2 weeks, the reduction in Na^+,K^+-ATPase activity is logically accompanied by no marked differences on the I_{sc} response to ouabain. On the other hand, the result of an enhanced response to furosemide, an indication of enhanced activity of the NKCC co-transporter, is in agreement with previous reports (Carey *et al.* 1994). By contrast, the enhanced response to ouabain and furosemide in rats given an HS diet for 2 weeks would agree with an increase in activity of the NKCC co-transporter accompanied by an increase in sodium absorption, which apparently is not yet detected by an increase in Na^+,K^+-ATPase activity.

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Food Intake Abolishes the Response of Rat Jejunal Na^+,K^+-ATPase to Dopamine[1]

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ABSTRACT The aim of the present study was to evaluate whether the sensitivity of jejunal Na^+,K^+-ATPase to inhibition by dopamine (DA) in young rats is related to the type of food (breast milk vs. solid) or reflects a developmental adaptation. When 18-d-old rats were separated from their dams and fed solid food (the same used to feed adult rats) for 2 d, intestinal Na^+,K^+-ATPase activity was significantly greater than that of breast-fed pups of the same age (20 d) (127 ± 8 vs. 52 ± 4 nmol Pi ·mg.protein^{-1} · min^{-1}; $P < 0.05$).Activity in rats fed solid food was insensitive to inhibition by 1 μmol/L DA. Na^+,K^+-ATPase activity in 60-d-old rats (117.4 ± 4.2 nmol Pi · mg protein^{-1} · min^{-1}) was also higher ($P < 0.05$) than in breast-fed rats, and DA (1 μmol/L) did not inhibit enzyme activity. The B_{max} value for binding of [^{3}H]-Sch 23390 in 20-d-old breast-fed rats did not differ from that in age-matched rats fed a solid food for 2 d and or that in 60-d-old rats. Levels of DA, but not L-3,4-dihydroxyphenylalanine and amine metabolites, in the jejunal mucosa of 20-d-old rats that had eaten solid food for 2 d were 60% lower than in age-matched rats, breast-fed rats, and not different from those in the jejunal mucosa of 60-d-old rats fed the solid food. We conclude that in adult rats, in contrast to in young rats, DA does not inhibit jejunal Na^+,K^+-ATPase activity, and food intake in young rats plays an important role in the development of the insensitivity of Na^+,K^+-ATPase activity to DA. J. Nutr. 130: 000–000, 2000.

KEY WORDS: • *dopamine* • *jejunum* • *rats* • *Na^+,K^+-ATPase* • *food intake*

The current view of the intestinal dopaminergic system is that of a local nonneuronal system constituted by epithelial cells of intestinal mucosa rich in aromatic L-amino acid decarboxylase (AADC)[3] activity and using circulating or luminal L-3,4-dihydroxyphenylalanine (L-DOPA) as a source for dopamine (DA) (Vieira-Coelho et al. 1997). DA is particularly abundant in the mucosal cell layer (Eaker et al. 1988; Esplugues et al. 1985), and studies on the formation of DA from exogenous L-DOPA along the rat digestive tract showed that the highest AADC activity is located in the jejunum (Vieira-Coelho and Soares-da-Silva 1993). Because the DA produced in this area is in close proximity to epithelial cells which contain receptors for the amine, it has been hypothesized that DA may act as a paracrine or autocrine substance (Vieira-Coelho et al. 1997). A high salt (HS) intake has been found to constitute an important stimulus for the production of DA in rat jejunal epithelial cells, and this is accompanied, in 20-d-old animals, by a decrease in sodium intestinal absorption (Finkel et al. 1994). This effect is accomplished, at the cellular level, by inhibition of Na^+-K^+-ATPase activity (Vieira-Coelho et al. 1998). The relative importance of this system in controlling sodium absorption assumes particular relevance in view of the findings that 40-d-old rats subjected to a HS intake have a fault in intestinal DA production during salt loading, in contrast to that occurring in 20-d-old animals. The lack of changes in the jejunal function in response to HS intake coincides with the period in which the renal function has reached maturation (Robillard et al. 1992), suggesting the occurrence of complementary functions between the intestine and the kidney during development.

In transporting epithelia, vectorial movement of sodium is accomplished by means of the Na^+,K^+-ATPase located at the basolateral plasma membrane and several sodium transport mechanisms localized at the apical domain of the cell (Rodriguez-Boulon and Nelson 1989). The basal activity of this pump and its modulation, which will reflect intestinal function (absorption and secretion), can be influenced by different factors, such as absence or presence of food in the intestine, protein and salt content in the diet, and stage of the developmental process (Binder 1983). This has a great impact during the uptake of nutrients and in the maintenance of electrolyte homeostasis and water metabolism during development (Herbst and Suskind 1969, Younoszai et al. 1978).

The aim of the present study was to evaluate whether the sensitivity of Na^+,K^+-ATPase to inhibition by DA in young rats is related to the type of diet or reflects a developmental adaptation. For these purposes, young breast-fed rats were challenged with solid food, the same fed to adult rats.

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[3] Abbreviations used: AADC, aromatic L-amino acid decarboxylase; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindolacetic acid; HS, high salt; 5-HT, 5-hydroxytryptamine; HVA, homovanillic acid; L-DOPA, L-3,4-dihydroxyphenylalanine; 3-MT, 3-methoxytyramine; NE, norepinephrine.

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MATERIALS AND METHODS

Animals

All the experiments were performed in male Wistar rats (Harlan-Interfauna, Barcelona, Spain) that were 20-d-old (40–50 g) or 60-d-old (260–300 g). Rats were kept in air-conditioned animal quarters and had free access to drinking water until the day of the experiment. Rats were killed by decapitation under ether anesthesia. Young rats were divided in two groups: i) those separated from their dams and given the solid food for 2 d, and ii) breast-fed rats. Adult rats were fed the solid food. The solid food (rat maintenance diet, catalog number 9609) was obtained from Harlan-Teklad (Oxon, United Kingdom).

Cell isolation

The method of cell isolation was similar to that previously described (Vieira-Coelho et al. 1998) with minor modifications. The jejunum was isolated and divided in small fragments. These were everted with fine forceps and incubated for 45 min in 5 mL warm (37°C) and gassed (95% O_2 and 5% CO_2) Hanks' solution with 0.06% collagenase type I (Sigma Chemical Co., St. Louis, MO). At the end of the incubation period the fragments were removed from the solution, and the medium containing the detached cells was centrifuged (200 × *g*, 4°C) for 4 min, and the cell pellet was resuspended in Hanks' solution. Cell viability was estimated by the Trypan blue (0.04%; 1 min) exclusion method, and the percentage of viable cells (excluding the dye), determined by hemocytometer counting, was > 90%.

Na^+,K^+-ATPase activity

Na^+,K^+-ATPase activity was measured by the method of Quigley and Gotterer (1969) and adapted in our laboratory with slight modifications. Briefly, isolated jejunal epithelial cells, obtained as described above, were preincubated for 20 min at 37°C. After the preincubation period the jejunal epithelial cells were permeabilized by rapid freezing in dry ice-acetone and thawing. The reaction mixture contained (in mmol/L) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium EDTA, 5 $MgCl_2$, 6 NaN_3, 75 tris(hydroxymethyl)aminomethane(tris) hydrochloride and 100 μL cell suspension (100 μg protein). The reaction was initiated by the addition of 4 mmol/L ATP (25 μL). For determination of ouabain-sensitive ATPase, NaCl and KCl were omitted, and ouabain (1 mmol/L; 100 μL) or vehicle (water; 100 μL) was added to the assay. After incubation at 37°C for 15 min, the reaction was terminated by the addition of 50 μL of ice-cold trichloroacetic acid. Samples were centrifuged (1,500 × *g*), and liberated P_i (free phosphorus) in the supernatant was measured by spectrophotometry at 740 nm. Na^+,K^+-ATPase activity is expressed as nanomoles P_i per milligram protein per minute and determined as the difference between total and ouabain-insensitive ATPase. The protein concentration in cell suspensions (~2 g/L), as determined by the method described by Bradford (1976) with human serum albumin as a standard, was similar in all samples.

Radioligand binding

Membranes from intestinal mucosa were obtained from 20-d-old breast-fed rats, 18-d-old rats separated from their dams and given the solid food for 2 d, and 60-d-old rats fed solid food. After killing, a segment of jejunum (5–10 cm) was removed, opened longitudinally along the mesenteric border and rinsed free from the alimentary contents with cold saline (9 g/L NaCl), and the jejunal mucosa was removed with a scalpel. The mucosa thus obtained was homogenized in 10 mmol/L Tris-HCl, pH 7.4, containing 250 mmol/L sucrose, 1 mmol/L PMSF, 1 mmol/L EDTA and 5 mg/L each of leupeptin and pepstatine, with a Potter-Elvehjem Teflon homogenizer, and centrifuged (20,000 × *g*, 20 min, 4°C). Pellets were resuspended to a concentration of 2 g protein · L^{-1} in 10 mmol · L^{-1} Tris-HCl, pH 7.4 with 5 mmol/L $MgCl_2$ and 250 mmol/L sucrose and stored aliquoted at −80°C. Membranes were thawed at room temperature, centrifuged (20,000 × *g*, 20 min, 4°C) and resuspended in binding buffer (in mmol/L: 50 Tris-HCl, 120 NaCl, 5 KCl, 2 $CaCl_2$ and 1 $MgCl_2$, pH = 7.4). Saturation experiments were performed in four replicates 96-well EIA/RIA plates (Costar) in a final volume of 0.2 mL respective binding buffer containing 0.1–10 nmol/L [^{3}H]-Sch 23390 a 100–200 μg membrane protein. Nonspecific binding was determin in the presence of 10 μmol/L of unlabeled Sch 23390. After a 30-m incubation at 30°C in a shaking water bath, assays were terminated vacuum filtration through glass fiber filter mats with the Brandel cell Harvester (Brandel, Gaithersburg, MD). Filters were wash three times with 200 μL of cold 50 mmol/L Tris-HCl pH 7.4, dri and impregnated with MeltiLex A (Wallac, Finland) and radioacti ity measured in a Microbeta counter (model 1450; Wallac, Finlanc

Assay of monoamines

The assays for DA, norepinephrine, 5-hydroxytrptamine (5-HT and metabolites were performed by means of HPLC, as previous described (Soares-da-Silva et al. 1996). The detection was carried o electrochemically with a glassy carbon electrode, an Ag/AgCl refe ence electrode and an amperometric detector (Gilson model 141 the detector cell was operated at 0.75 V. The current produced w monitored using the Gilson 712 HPLC software. The lower limit f detection of L-DOPA, DA, 3,4-dihydroxyphenylacetic ac (DOPAC), 3-methoxytyramine (3-MT), homovanillic acid (HVA norepinephrine, 5-HT and 5-hydroxyindolacetic acid (5-HIAA ranged between 350 to 1,000 fmol.

Drugs

The compounds used were DA hydrochloride, 5-HT hydrochlorid ouabain and pargyline hydrochloride, obtained from Sigma Chemic Company. Quinerolane, SKF 83566, SKF 38393 and (S)-Sulpiride we obtained from Research Biochemicals International (RBI, Natick, MA The radioligand [^{3}H]-Sch 23390 ([N-methyl-^{3}H]R[+]-7-chloro-2,3,4,5 tetrahydro-3-methyl-1-phenyl-1H-3-benzazepine-8-ol, specific activi 2600–3200 GBq/mmol was purchased from New England Nuclear (Bo ton, MA). Tolcapone was kindly donated by late Professor Mosé D Prada (Hoffman La Roche, Basel, Switzerland).

Statistics

Results are means ± SEM for the indicated number of determina tions. [^{3}H]-Sch 23390 saturation parameters, B_{max} and K_D, we obtained with nonlinear iterative curve-fitting algorithms using th GraphPad Prism statistics software package (Motulsky et al. 1994 Statistical analysis was performed by one-way ANOVA followed b Student's *t* test for unpaired comparisons. A *P*-value < 0.05 denote a significant difference.

RESULTS

Basal jejunal Na^+,K^+-ATPase activity in 20-d-old breast fed rats was considerably lower ($P < 0.05$) than that i age-matched rat fed solid food for 2 d (**Fig. 1**). This dramati difference in jejunal Na^+,K^+-ATPase activity was accompa nied by loss of sensitivity to the inhibitory effects of DA. DA (1 μmol/L) significantly inhibited jejunal Na^+,K^+-ATPas activity in breast-fed 20-d-old rats, but not in aged-matche rats fed solid food (**Fig. 2**). The inhibitory effect of DA (μmol/L) on jejunal Na^+,K^+-ATPase activity in breast-fe 20-d-old rats was abolished by pretreatment with the selectiv D_1 receptor antagonist SKF 83566 (1 μmol/L), but not by th selective D_2 receptor antagonist S-sulpiride (1 μmol/L). Th selective D_1 receptor agonist SKF 38393 (10 nmol/L) inhib ited jejunal Na^+,K^+-ATPase activity in breast-fed 20-d-ol rats, but not in aged-matched rats fed solid food. Basal jejuna Na^+,K^+-ATPase activity in 60-d-old rats was 2.5-fold that o 20-d-old breast-fed rats, but not different from that in 20-d-ol rats fed solid food for 2 d (Fig. 1). In 60-d-old rats, DA (μmol/L), the selective D_1 receptor agonist SKF 38393 (1 nmol/L) and the selective D_2 receptor agonist quinerolane (1

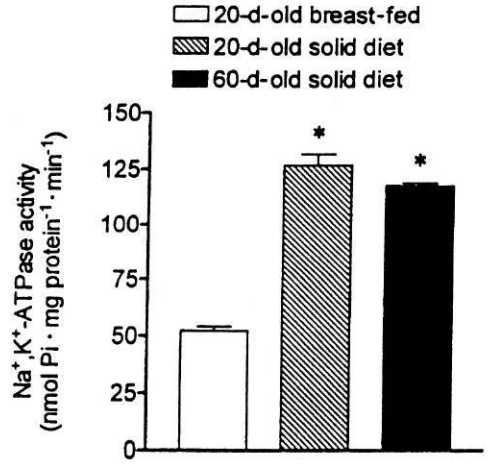


FIGURE 1 Basal jejunal Na^+, K^+-ATPase activity in 20-d-old breast-fed rats and age-matched rats fed solid food for 2 d and in 60-d-old rats. Columns represent means of four rats per group; vertical lines indicate SEM Significantly different from corresponding values for 20-d-old breast-fed rats (* $P < 0.05$).

nmol/L) did not inhibit jejunal Na^+,K^+-ATPase activity (data not shown).

Saturation experiments with [^{3}H]-Sch 23390 (0.1–10 nmol/L) performed in membranes from jejunal epithelial cells revealed the presence of a single class of receptors, with an

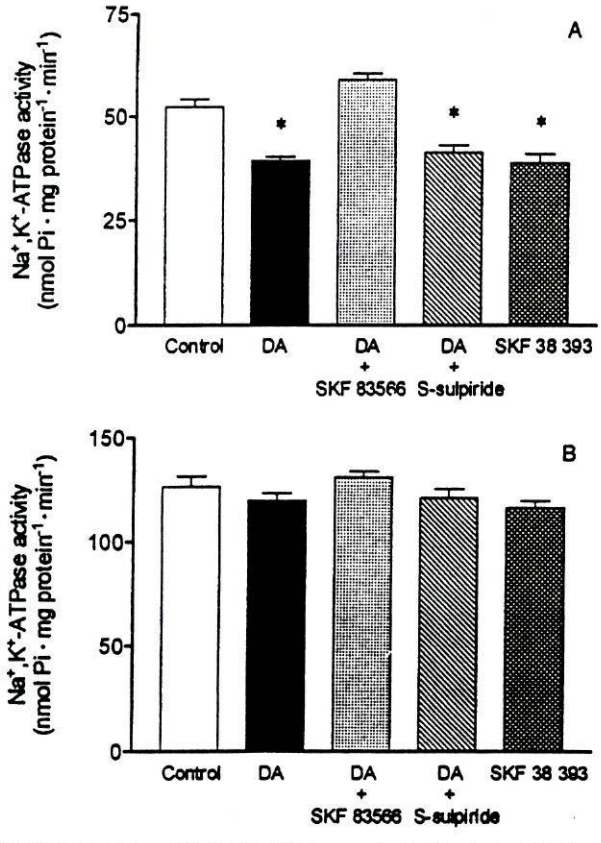


FIGURE 2 Jejunal Na^+, K^+-ATPase activity in 20-d-old (A) breast-fed rats and (B) age-matched rats feed for 2 d solid food in the absence and the presence of dopamine (DA) (1 μmol/L), DA (1 μmol/L) + SKF 83 566 (1 μmol/L), DA (1 μmol/L) + S-sulpiride (1 μmol/L) and SKF 38 393 (10 nmol/L). Columns represent means of four rats per group; vertical lines indicate SEM Significantly different from corresponding values for control (* $P < 0.05$).

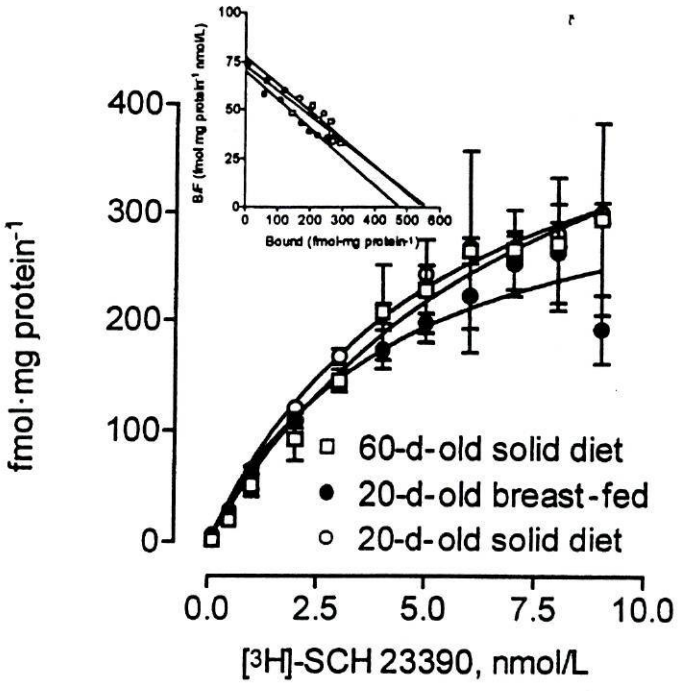


FIGURE 3 Specific binding of [^{3}H]-Sch23390 (0.1–9 nmol/L) to membranes from intestinal mucosa of 20-d-old breast-fed rats or fed solid food, and 60-d-old rats. The inset graph represents a Scatchard plot with amount bound (fmol · mg protein^{-1}) in ordinates and the abscissa represents the ratio amount bound/free ligand (fmol · mg protein^{-1} · nmol/L). Symbols represent means of five experiments with four replicate determinations and vertical lines show SEM

apparent K_D in the low nanomolar range (Fig. 3). There were F3 no significant differences in B_{max} or K_D among 20-d-old breast-fed rats, 20-d-old rats fed solid food or 60-d-old rats fed solid food (Table 1). The kinetic parameters of the D_1 binding site reported here for adult rats differ from those described in the literature (Marmon et al. 1993). K_D values are of similar magnitude, but B_{max} values are markedly higher in the present study (539.3 vs. 1.37 fmol.mg protein^{-1}). The most likely explanation for this apparent discrepancy is that Marmon et al. used the entire intestinal wall, whereas we used isolated epithelial cells from the intestinal mucosa. This suggests that D_1 receptors may be preferentially located in the mucosal cells and not homogeneously distributed across the intestinal wall.

Levels of DA, but not L-DOPA or amine metabolites, in the jejunal mucosa of 20-d-old rats fed solid food for 2 d were 60% lower than in age-matched, breast-fed rats. Levels of norepinephrine and 5-HT did not differ between the two groups

TABLE 1

Apparent K_D and B_{max} values for dopamine D_1 receptor binding sites labeled with [^{3}H]-Sch 23390 in membranes from jejunal epithelial cells of 20- and 60-d-old rats[1]

	B_{max}, fmol · mg protein^{-1}	K_D, nmol/L
20-d-old breast-fed	370.6 ± 63.1	4.6 ± 1.7
20-d-old solid food	508.1 ± 32.6	6.1 ± 0.8
60-d-old solid food	596.3 ± 87.8	8.7 ± 2.2

Values are means ± SEM of five rats per group (four replicate determinations per rat).

TABLE 2

Concentrations of L-DOPA, DA, DOPAC, 3-MT, HVA, NE, 5-HT and 5-HIAA in the jejunal mucosa of 20- and 60-d-old rats[1,2]

	20-d-old rats, breast-fed	20-d-old rats, solid food	60-d-old rats, solid food
		pmol/g	
L-DOPA	495 ± 43*	394 ± 120	311 ± 48
DA	210 ± 24*	80 ± 26**	114 ± 22
DOPAC	4 ± 1	11 ± 2**	6 ± 1
3-MT	239 ± 48*	64 ± 7**	37 ± 6
HVA	66 ± 7*	69 ± 4	41 ± 3
NE	465 ± 98*	840 ± 176	1303 ± 130
5-HT	5419 ± 234	4520 ± 384	5067 ± 685
5-HIAA	7323 ± 701*	2366 ± 701**	846 ± 321

[1] Values means ± SEM, $n = 8$.

[2] Significantly different from corresponding values for 60-d-old rats (* $P < 0.05$) and 20-d-old breast-fed rats (** $P < 0.05$). L-DOPA, L-3,4-dihydroxyphenylalanine; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; 3-MT, 3-methoxytyramine; HVA, homovanillic acid; NE, norepinephrine; 5-HT, 5-hydroxytryptamine; 5-HIAA, 5-hydroxyindolacetic acid.

(Table 2). Levels of DA in the jejunal mucosa of 60-d-old rats were lower than those in 20-d-old breast-fed rats and not different from those in 20-d-old rats fed with the solid diet.

DISCUSSION

The results presented here demonstrate that DA inhibits jejunal Na^+,K^+-ATPase in young, breast-fed Wistar rats through activation of D_1 receptors, but not in adult animals. This inhibitory effect is completely absent when young rats are fed solid food for 2 d and is accompanied by a marked increase in basal jejunal Na^+,K^+-ATPase activity. These results confirm previous observations from our group obtained in 20-d-old Sprague-Dawley rats, where DA produced a concentration-dependent inhibition of Na^+,K^+-ATPase activity, indicating that this effect is not dependent on the rat strain. The finding that the inhibitory effect of DA was completely antagonized by SKF 83566, but not by S-sulpiride, strongly suggests that this effect is mediated via the activation of D_1 receptors; this is further supported by the finding that the selective D_1 receptor agonist mimicked the effect of DA. This is consistent with results obtained in rat renal proximal tubular epithelial cells where DA inhibits Na^+,K^+-ATPase activity via the activation of D_1 DA receptors (Kansra et al. 1997). On the other hand, these results further indicate that this loss of sensitivity to DA may not be an age-dependent phenomenon, since the type of food available markedly affected the response of Na^+,K^+-ATPase to DA.

Because basal Na^+,K^+-ATPase activity in 20-d-old rats fed solid food for 2 d was higher than in breast-fed rats, we hypothesized that the loss of sensitivity to DA in the former rats was related to the high level of enzyme activity. However, in adult, food-deprived rats, which have lower basal Na^+,K^+-ATPase activity than fed rats, DA still was not inhibitory (Lucas-Teixeira et al. 1999). In fact, a low basal Na^+,K^+-ATPase activity after food deprivation for 48 h is consistent with a previous report (Murray and Wild 1980), with the low activity being completely reverted by refeeding. As previously described in 40-d-old Sprague-Dawley rats (Vieira-Coelho et al. 1998), DA did not inhibit Na^+,K^+-ATPase in 60-d-old Wistar rats. These differences appear not to be related differences in the density of DA receptors, since the densit D_1 binding sites did not differ in young and adult rats.

Differences in basal Na^+,K^+-ATPase activity betw young breast-fed rats and age-matched rats fed solid food r be related to different salt or protein contents of the d (maternal milk vs. solid food). Both sodium and amino ac affect renal Na^+,K^+-ATPase activity (Bertorello et al. 19 Jakobsson et al. 1990). However, basal Na^+,K^+-ATPase tivity in young rats fed HS was lower than in rats fed no s without differences in sensitivity to inhibition by DA (Vie Coelho et al. 1998). This change in basal Na^+,K^+-ATP activity during HS intake was completely reverted by pretre ment with benserazide, a AADC inhibitor, suggesting tha was related to the enhanced availability of DA. In fact, intake was demonstrated in 20-d-old rats to increase the f mation of DA in the jejunal mucosa (Finkel et al. 19 Vieira-Coelho et al. 1998). The finding that DA levels in t jejunal mucosa of breast-fed rats were higher than in rats f the solid diet suggests that the high Na^+,K^+-ATPase activ may be related to low inhibitory dopaminergic tonus upon t enzyme. Another observation supporting the view of low junal dopaminergic tonus in 20-d-old rats fed solid food is th the low levels of DA were not accompanied by a change in t density of D_1 binding sites. This apparently conflicts with t result that jejunal Na^+,K^+-ATPase activity in 20-d-old ra fed the solid food was not sensitive to exogenous DA or l receptor stimulation. Perhaps the solid food contains an u known substance which impairs the response of Na^+,K ATPase to DA and simultaneously alters the availability endogenous DA.

The intestinal nonneuronal dopaminergic system and i effects on the regulation of electrolyte transport, especial sodium absorption, have been recently described (Finkel et a 1994, Vieira-Coelho et al. 1997and 1998). There are simil properties in this autocrine/paracrine intestinal system and th kidney nonneuronal dopaminergic system (Soares-da-Silv 1994), where diuretic and natriuretic effects of DA are we known (Aperia 1994, Jose et al. 1992, Lee 1993, Lokhandwa and Hegde 1991). Epithelial cells from both renal proxima tubules and the intestinal mucosa are endowed with i) efficien mechanisms for L-DOPA uptake, ii) high AADC activit which easily converts intracellular L-DOPA to DA, iii) eff cient enzyme systems for the metabolic degradation of newly formed DA and iv) specific receptors for the amine, th activation of which leads to Na^+,K^+-ATPase inhibition an transepithelial sodium flux. In the two systems, the final effec on sodium is the same, there is a decrease in sodium absorptio in the intestine and an increase in sodium excretion in th kidney. Because defective responses to DA receptor activatio might lead to sodium retention and increased blood pressure these nonneuronal dopaminergic systems are physiologicall relevant (Hussain and Lokhandwala 1998, Jose et al. 1998).

In conclusion, DA inhibits jejunal Na^+,K^+-ATPase i young breast-fed Wistar rats through activation of D_1 recep tors, but not in adult rats or in young rats fed solid food for 2 d The lack of DA sensitivity is accompanied by markedly ele vated basal jejunal Na^+,K^+-ATPase activity.

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Efeito da activação de receptores α-adrenérgicos sobre o transporte epitelial: factores que modulam essa resposta.

a) Food deprivation increases α_2-adrenoceptor-mediated modulation of jejunal epithelial transport in young and adult rats.

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d Deprivation Increases α_2-Adrenoceptor–Mediated Modulation of unal Epithelial Transport in Young and Adult Rats[1]

V. Lucas-Teixeira, M. A. Vieira-Coelho, M. P. Serrão and P. Soares-da-Silva[2]

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ABSTRACT This study examined the effect of food deprivation on the jejunal response to α_2-adrenoceptor activation in young (20-d-old) and adult (60-d-old) rats, using short-circuit (I_{sc}) measurements in the absence or presence of furosemide (1 mmol/L). The effect of α_2-adrenoceptor stimulation by 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK 14,304; 0.3–3000 nmol/L) was a concentration-dependent decrease in I_{sc} with similar half-maximal effective concentration (EC_{50}; 12.3 ± 1.1 vs. 9.6 ± 1.1 nmol/L) and maximal effect (E_{max}; 70.6 ± 6.9 vs. 80.6 ± 4.5% of reduction) values in adult food-deprived and fed rats. The effect of UK 14,304 on I_{sc} in fed and food-deprived rats was markedly ($P < 0.05$) attenuated by furosemide (1 mmol/L). E_{max} values for UK 14,304 in 20-d-old food-deprived rats were higher ($P < 0.05$) than those observed in fed rats (93.3 ± 3.3 vs. 67.0 ± 11.3% of reduction), without differences in EC_{50} values. The effect of UK 14,304 on I_{sc} in 20-d-old fed rats was completely abolished by furosemide (1 mmol/L). In food-deprived young rats, the effect of UK 14,304 was also markedly ($P < 0.05$) antagonized by furosemide, but not completely abolished. Specific [^{3}H]-rauwolscine binding in membranes from jejunal epithelial cells revealed the presence of a single class of binding sites, with an apparent K_D in the low nmol/L range. In 20-d-old food-deprived rats, specific [^{3}H]-rauwolscine binding was markedly increased, and this was reversed by refeeding. Na^+,K^+-ATPase activity in isolated jejunal epithelial cells from 60-d-old fed rats was twice that in 20-d-old fed rats [117 ±/vs. 52 ±/nmol free inorganic phosphorus/(mg protein·min)]. Food deprivation in adult rats, but not in 20-d-old rats, was accompanied by a significant decrease in Na^+,K^+-ATPase activity. In both young and adult rats (fed and food-deprived), UK 14,304 did not affect Na^+,K^+-ATPase activity. In conclusion, food deprivation in 20-d-old rats enhanced the response to α_2-adrenoceptor stimulation. This effect, which depends primarily on the stimulation of a furosemide-sensitive antisecretory mechanism, is suggested to result from increases in the number of jejunal epithelial α_2-adrenoceptors. J. Nutr. 130: 000–000, 2000.

1415

KEY WORDS: • *rat jejunum* • *α_2-adrenoceptors* • *$Na^+,K^+,2Cl^-$-co-transporter* • *Na^+K^+-ATPase*

he growth and differentiation of the intestinal mucosa are ndent upon signals derived from luminal contents, includ- lietary nutrients and digestive secretions (Johnson 1988). se factors, related to enteral nutrition, play a key role in modulation of gastrointestinal function. It is not only the ence of food, but also the absence of luminal contents in gastrointestinal tract that has the capacity to influence stinal function, comprising both absorptive and secretory hanisms. Although the factors directly responsible for such nges have not been detailed, it is believed that the effect is not to systemic influences associated with fasting condi- s, but to the reduction in contact between the intestinal osa and luminal contents (Carey and Cooke 1992, Carey l. 1994). Food deprivation has been shown to change stinal mucosal structure as well as function (Johnson 1988, liamson 1978). Studies performed in rats deprived of food a period of 24–48 h (Young and Levin 1990a and 1990b) ground squirrels (48–72 h) (Carey 1992) showed that the jejunum and ileum became hyperactive to a variety of stimuli that elicit intestinal secretion, despite the decreasing cell population of crypts and villi. In addition, the intestine of food-deprived animals is endowed with enhanced absorptive capacity, most likely as the result of an increase in enterocyte maturity and decrease in cell proliferation (Thompson and Debnam 1986, Young and Levin 1990a).

The driving force for fluid absorption in the mammalian small intestine is the active transport of Na^+, and Cl^-, which may be electrically silent (Frizzell et al. 1979) or which may involve electrogenic Na^+ transport (Esposito 1984). In addition, there is active secretion of Cl^- accompanied by Na^- and water (Rao and Fields 1983). Studies on the neurohumoral control of intestinal transport showed that catecholamines stimulate electrolyte absorption and inhibit anion secretion in the small and large intestines (Field and McColl 1973, Sellin and DeSoignie 1984). The proabsorptive and antisecretory effect of catecholamines has been attributed to the activation of α-adrenoceptors. Liu and Coupar (1997) suggested that the α_2-adrenoceptors involved in the antisecretory actions in the rat intestinal epithelium are of the α_{2D} or α_{2A} subtype. All of these observations are supported by radioligand studies that

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monstrated the presence of α_2-adrenoceptors in enterocyte embranes (Chang et al. 1982 and 1983, Cotterell et al. 1984, akaki et al. 1983, Tanaka and Starke 1979).

During postnatal development, rats gradually shift from a w carbohydrate, high fat and low salt intake to one that is igh carbohydrate, low fat and high salt (Buddington and iamond 1989). To digest and absorb nutrients properly, the nall intestine adapts by regulating membrane properties, enme activities and transporter activities at levels capable of rocessing diets typically consumed at each developmental tage (Buddington and Diamond 1989, Meddings and Theisen 989). Na^+,K^+-ATPase is one of the enzymes localized at the asolateral enterocyte membrane that perform a very impornt role in intestinal physiology by setting the ionic gradients ecessary for absorptive and secretory mechanisms. The basal ctivity and modulation of this ionic pump has been shown in ats/depend on several factors: 1) dietary salt level, 2) age of he rats and 3) stage of development (Binder 1983, Finkel et l. 1994, Lucas-Teixeira et al. 2000, Vieira-Coelho et al. 1998). In addition to the sodium pump, other membrane ransporters are involved in the vectorial movement of ions cross the intestinal epithelium; for example, the $Na^+,K^+,2Cl^-$ co-transporter (NKCC)[3] is very important for establishing the Cl^- gradient necessary for its secretion at the apical membrane (Binder 1983, Esposito 1984, Frizzell et al. 1979).

The modulation by α_2-adrenoceptors of jejunal electrolyte transport has not yet been analyzed in detail in young rats (20 d old) vs. adult rats (60 d old) in fed and food-deprived states. This study reports that the effect of α_2-adrenoceptor activation on the short-circuit current (I_{sc}) is mediated through a furosemide-sensitive pathway and therefore depends on the ionic gradients generated by the NKCC co-transporter, and that this effect is affected by food deprivation.

MATERIALS AND METHODS

Animals. All of the experiments were performed in male Wistar rats (Harlan-Interfauna, Barcelona, Spain), 20 d old (40–50 g) and 60 d old (260–300 g). Adult rats were divided in two groups as follows: *1*) rats fed a standard nonpurified diet [rat maintenance diet (fat 2.2%, protein, 15.0%, fiber, 5.2%), catalog number 9609 obtained from Harlan-Teklad, Oxon, UK] and 2) rats deprived of food for 48 h. Young rats were also divided in two groups as follows: *1*) breast-fed rats that were kept with their dams and 2) rats deprived of food for 24 h. All rats consumed tap water ad libitum. The Scientific Review Board of the Foundation for Science and Technology (Portugal) approved the experimental protocol.

Preparation of stripped jejunal epithelial sheets. Rats were killed by decapitation while under ether anesthesia, and three jejunal segments located 10–15 cm distal from the pyloric sphincter were removed. Each segment (2 cm long) of jejunum was cut longitudinally along the mesenteric border, washed free of luminal contents and the tissue pinned mucosal side down on a dental wax block. The serosa and muscularis were stripped away by dissection to obtain the epithelial sheets, as described previously (Nellans et al. 1974). Three adjacent pieces were routinely prepared from a single jejunum.

Experimental procedure. Epithelial sheets were mounted in Ussing chambers (window area 0.28 cm^2) equipped with water-jacketed gas lifts, bathed on both sides with 10 mL of Krebs-Hensleit solution, gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. D-Glucose (10 mmol/L) was added to the serosal-side reservoir and an equimolar amount of mannitol to the mucosal-side reservoir. The Krebs-Hensleit solution contained the following (mmol/L): NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $MgSO_4$, 1.2; the pH was adjusted to 7.4 after gassing with 5% CO_2 and 95% O_2. Tissues were voltage-clamped continuously to zero potential differences by application of external current, with compensation for fluid resistance, by means of an automatic voltage current clamp (DVC 1000, World Precision Instruments, Sarasota, FL). Transepithelial resistance ($\Omega \cdot cm^2$) was determined by altering the membrane potential stepwise (± 5 mV) and applying the Ohmic relationship. Changes in I_{sc} ($\mu A/cm^2$) were measured continuously as an index of electrogenic ion transfer. The voltage/current clamp unit was connected to a PC via a BIOPAC MP1000 data acquisition system (BIOPAC Systems, Goleta, CA). Data analysis was performed using AcqKnowledge 2.0 software (BIOPAC Systems).

After a 30- to 45-min preincubation period with vehicle (dimethylsulfoxide, 100 μL) or furosemide (1 mmol/L), by which time the potential difference had stabilized, 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK 14,304) was added to the serosal-side reservoir. Agonist concentration-response curves were constructed in a cumulative manner; each new concentration was added as soon as the potential difference response to the prior concentration reached its nadir.

Na^+,K^+-ATPase assay. Na^+,K^+-ATPase activity in isolated jejunal epithelial cells was measured by the method of Quigley and Gotterer (1969) and adapted in our laboratory with slight modifications. The method of cell isolation was similar to that described previously (Lucas-Teixeira et al. 2000). Isolated epithelial cells were incubated for 15 min at 37°C, and were then permeabilized by rapid freezing in dry ice/acetone and thawing. The reaction mixture, in a final volume of 1.025 mL, contained (mmol/L) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium EDTA, 5 $MgCl_2$, 6 NaN_3, 75 Tris(hydroxymethyl)aminomethane(Tris) hydrochloride and 100 μL cell suspension (100 μg protein). The reaction was initiated by the addition of 4 mmol/L ATP (25 μL). For determination of ouabain-insensitive ATPase, NaCl and KCl were omitted, and ouabain (1 mmol/L; 100 μL) or vehicle (water; 100 μL) was added to the assay. After incubation at 37°C for 20 min, the reaction was terminated by the addition of 50 μL of ice-cold trichloroacetic acid. Samples were centrifuged (1500 × g), and liberated inorganic phosphorus (P_i) in the supernatant was measured by spectrophotometry at 740 nm. Na^+,K^+-ATPase activity is expressed as nmol P_i/(mg protein · min) and determined as the difference between total and ouabain-insensitive ATPase. The protein content in cell suspension (~1.5 g/L), as determined by the method described by Bradford (1976) with human serum albumin as a standard, was similar in all samples.

Radioligand binding. Membranes from intestinal mucosa were obtained from 20- and 60-d-old rats and prepared according to Nakaki et al. (1983). After killing, a segment of jejunum (5–10 cm) was removed, opened longitudinally along the mesenteric border, rinsed free from the alimentary contents with cold saline (9 g/L NaCl) and the jejunal mucosa removed with a scalpel. The jejunal mucosa was homogenized in 10 mmol/L Tris-HCl, pH 7.4, containing 250 mmol/L sucrose, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA and 5 mg/L each of leupeptin and pesptatin. The cell suspension was homogenized on ice with a glass-teflon Potter-Elvehjem homogenizer and then centrifuged at 2600 × *g* for 15 min at 4°C. The resulting supernatant was centrifuged at 100,000 × *g* for 60 min at 4°C. The final pellet was resuspended to a concentration of 1 mg protein/mL in binding buffer (50 mmol/L Tris-HCl, pH 7.4, at 25°C with 5 mmol/L EDTA). The binding assay was performed according to Mohuczy-Dominiak and Garg (1993) with minor modifications. Saturation experiments were performed in four replicates in 96-well ELISA/RIA plates (Costar) in a final volume of 0.2 mL binding buffer containing 0.1–200 nmol/L [^{3}H]-rauwolscine and 100 μg membrane protein. Nonspecific binding was determined in the presence of 10 μmol/L phentolamine. After a 45-min incubation at 25°C in a shaking water bath, assays were terminated by vacuum filtration through glass-fiber filter mats with the Brandel 96-cell Harvester (Brandel, Gaithersburg, MD). Filters were washed three times with 200 μL of cold 50 mmol/L Tris-HCl, pH 7.4, dried and impregnated with MeltiLex A (Wallac, Pharmacia, Finland) and radioactivity measured in a Microbeta counter (model 1450; Wallac).

[3] Abbreviations used: EC_{50}, half-maximal effective concentration; E_{max}, maximal effect; I_{sc}, short-circuit current; NKCC, $Na^+,K^+,2Cl^-$ co-transporter; P_i, free inorganic phosphorus; UK 14,304, 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine.

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ugs. The compounds used were the following: furosemide and in from Sigma Chemical (St. Louis, Mo); UK 14,304 from rch Biochemicals International (RBI, Natick, MA); and ethyl-^{3}H]-rauwolscine (specific activity 2.81 TBq/mmoL) from med Amersham (Buckinghamshire, U.K.).

nalysis of data. Half-maximal effective concentration (EC_{50}) s for the effect of UK 14,304 on I_{sc}, and B_{max} and K_D values for auwolscine saturation curves, were obtained with nonlinear ive curve-fitting algorithms using the GraphPad Prism statistics are package (Motulsky et al. 1994). Arithmetic means are given SEM. Statistical analysis was performed by one-way ANOVA ved by the Newman-Keuls test for multiple comparisons. A ue < 0.05 denoted a significant difference.

RESULTS

asal I_{sc} in jejunal preparations from 60-d-old rats deprived od for 48 h (17.3 ± 2.1 μA/cm^2) was significantly higher ($P < 0.05$) than that in fed rats (10.6 ± 2.1 μA/cm^2). Tissue resistance was also significantly different ($P < 0.05$) between food-deprived and fed rats, 71.6 ± 5.2 $\Omega \cdot cm^2$ ($n = 25$) and 51.2 ± 3.2 $\Omega \cdot m^2$ ($n = 25$), respectively. The effect of α_2-adrenoceptor stimulation by UK 14,304 (0.3–3000 nmol/L), applied from the basolateral side, was a concentration-dependent decrease in I_{sc} (**Fig. 1**), with similar EC_{50} and maximal effect (E_{max}) values in adult fed and food-deprived rats (**Table 1**). F1 T1

Furosemide was used to evaluate the ionic mechanisms responsible for I_{sc} changes in basal conditions and during α_2-adrenoceptor stimulation in jejunal preparations from fed and food-deprived rats. The addition of furosemide (1 mmol/L), a blocker of the NKCC co-transporter (Frizzell et al. 1979, Kuo and Shanbour 1980), to the fluid bathing the serosal side induced a time-dependent decrease in basal I_{sc}, which attained its maximum at 20 min and was found to be similar in fed (44 ± 11% reduction) and food-deprived (49 ± 12% reduction) rats. The effect of UK 14,304 on I_{sc} in both fed and food-deprived rats was significantly attenuated, but not abolished by furosemide (1 mmol/L) (**Fig. 2**). F2

Basal I_{sc} in 20-d-old fed rats (19.2 ± 4.2 μA/cm^2, $n = 12$) was not different from that observed in 20-d-old rats subjected to 24 h of food deprivation (23.1 ± 5.4 μA/cm^2, $n = 11$). Similarly, tissue resistance did not differ between fed and food-deprived rats, 57.2 ± 5.3 $\Omega \cdot cm^2$ ($n = 12$) and 50.1 ± 3.8 $\Omega \cdot cm^2$ ($n = 12$), respectively. As observed in adult rats, the addition of UK 14,304 to the serosal side (0.3 to 3000 nmol/L) decreased jejunal I_{sc} in young rats (Fig. 1). In contrast to the observation in adult rats, the E_{max} in the 20-d-old food-deprived rats was greater than that observed in fed rats (Fig. 1 and Table 1). Despite differences in E_{max} values for UK 14,304 between fed and food-deprived rats, no significant differences were observed for the EC_{50} values (Table 1).

In 20-d-old rats, furosemide was also used during basal and α_2-adrenoceptor–stimulated conditions. The addition of furosemide (1 mmol/L) to the serosal side induced a time-dependent decrease in basal I_{sc}, which attained its maximum at 20 min and was found to be similar in fed (48 ± 9% reduction) and food-deprived (64 ± 12% reduction) rats. The effect of UK 14,304 on I_{sc} in 20-d-old fed rats was completely abolished by furosemide (1 mmol/L) (**Fig. 3**). In 20-d-old food-deprived rats, this effect was also markedly antagonized by furosemide, but not completely abolished (Fig. 3). F3

To determine whether the enhanced response to UK

TABLE 1

EC_{50} and E_{max} values for UK 14,304 (0.3–3000 nmol/L) in 60- and 20-day old fed and food deprived rats[1]

	EC_{50}	E_{max}
	nmol/L	%
60-d-old rats		
Fed	9.6 ± 1.1	80.6 ± 4.5
Food-deprived	12.3 ± 1.1	70.6 ± 6.9
20-d-old rats		
Fed	8.5 ± 1.2	67.0 ± 11.3
Food-deprived	10.9 ± 1.1	93.3 ± 3.3*

1 Values are means ± SEM (fed, $n = 7$; food-deprived, $n = 6$).
* Significantly different from corresponding values in fed rats ($P < 0.01$) using the Newman-Keuls multiple comparison test. EC_{50} half-maximal effective concentration; E_{max}, maximal effect; UK 14,304, 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine.

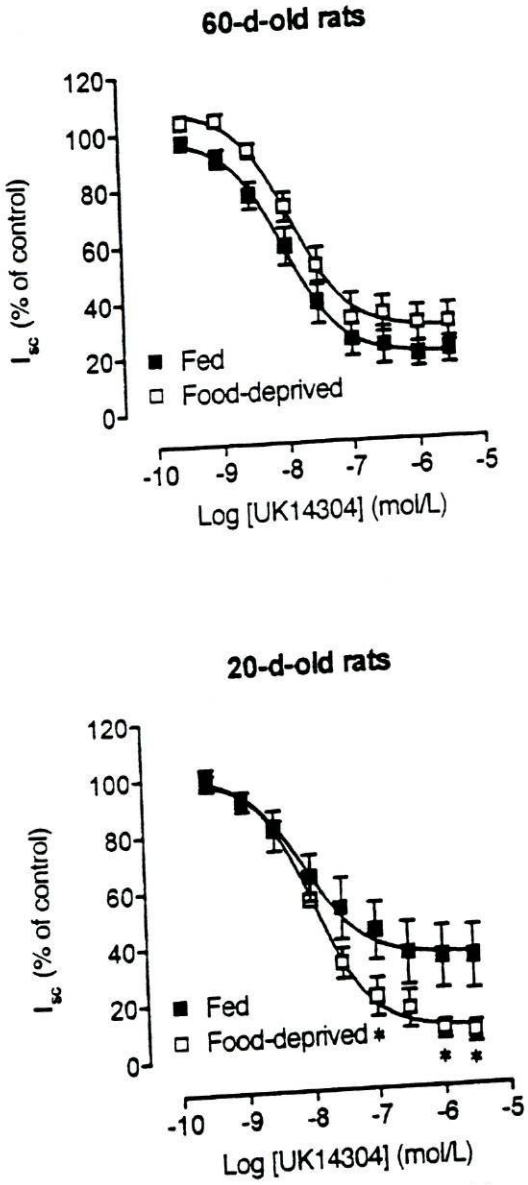


FIGURE 1 Concentration-response curves for 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK 14,304) on short-circuit current (I_{SC}) in voltage-clamped rat jejunal epithelial sheets obtained from (*A*) 60-d-old fed and 48-h food-deprived rats and (*B*) 20-d-old breast-fed and 24-h food-deprived rats. Symbols represent means of four rats per group and vertical lines show SEM. *Significantly different from corresponding values for fed rats ($P < 0.05$) using the Newman-Keuls multiple comparison test.

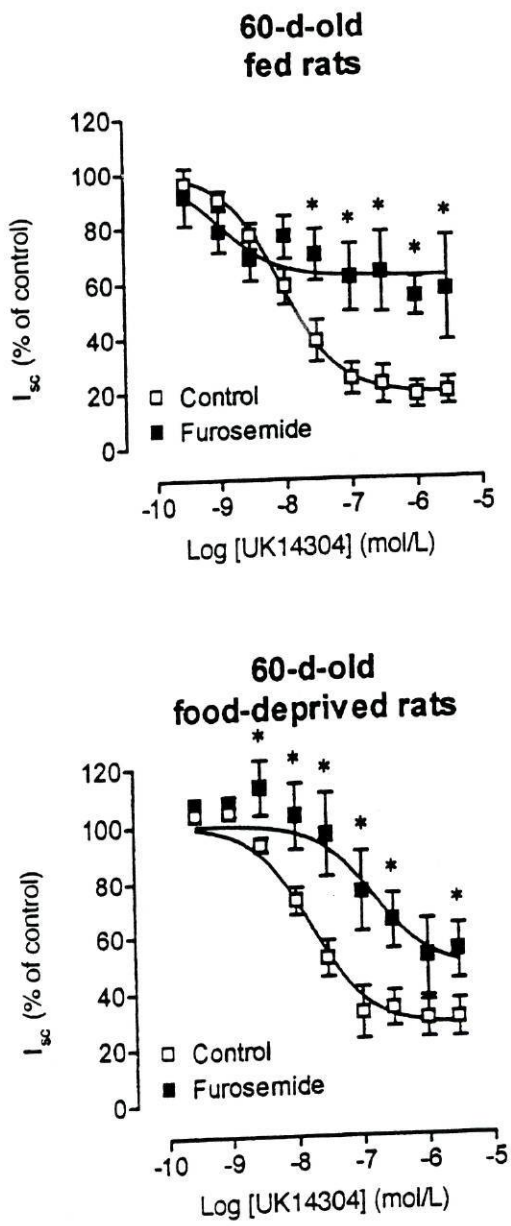


FIGURE 2 Effect of 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK 14,304; 1 μmol/L) on short-circuit current (I_{SC}) in voltage-clamped rat jejunal epithelial sheets obtained from 60-d-old (*A*) fed and (*B*) 48-h food-deprived rats in the absence and the presence furosemide (1 mmol/L). Symbols represent means of four rats per group and vertical lines show SEM. *Significantly different from corresponding control values ($P < 0.05$) using the Newman-Keuls multiple comparison test.

14,304 in 20-d-old food-deprived rats was related to upregulation of α_2-adrenoceptors, the abundance of α_2-adrenoceptor binding sites in membranes from the jejunal mucosa was determined. Figure 4 shows the results of saturation experiments with [^{3}H]-rauwolscine (0.1–200 nmol/L) performed in membranes from the jejunal mucosa. Nonlinear analysis revealed the presence of a single class of binding sites, with an apparent K_D in the low nmol/L range. In 20-d-old food-deprived rats, specific binding of [^{3}H]-rauwolscine was markedly increased; this was reversed by refeeding. B_{max} and K_D values for [^{3}H]-rauwolscine specific binding were similar to those in fed rats (Table 2).

Na^+,K^+-ATPase activity [in nmol Pi/(mg protein·min)] in isolated jejunal epithelial cells from 60-d-old fed rats was twice that in 20-d-old fed rats (Table 3). Food deprivation in adult rats was accompanied by a significant decrease in Na^+,K^+-ATPase activity, whereas in 20-d-old rats, food deprivation did not affect sodium pump activity (Table 3). In both young and adult rats (fed and food-deprived), UK 14,304 did not affect Na^+,K^+-ATPase activity (Table 3).

DISCUSSION

The findings reported here in 20- and 60-d-old rats agree with the view that α_2-adrenoceptors modulate jejunal ion secretion through modulation of the ionic gradients generated by the NKCC co-transporter, as evidenced by a furosemide-sensitive UK 14,304–mediated decrease in jejunal I_{sc}. Moreover, food deprivation in young rats resulted in an enhancement of the maximal response to UK 14,304 accompanied by

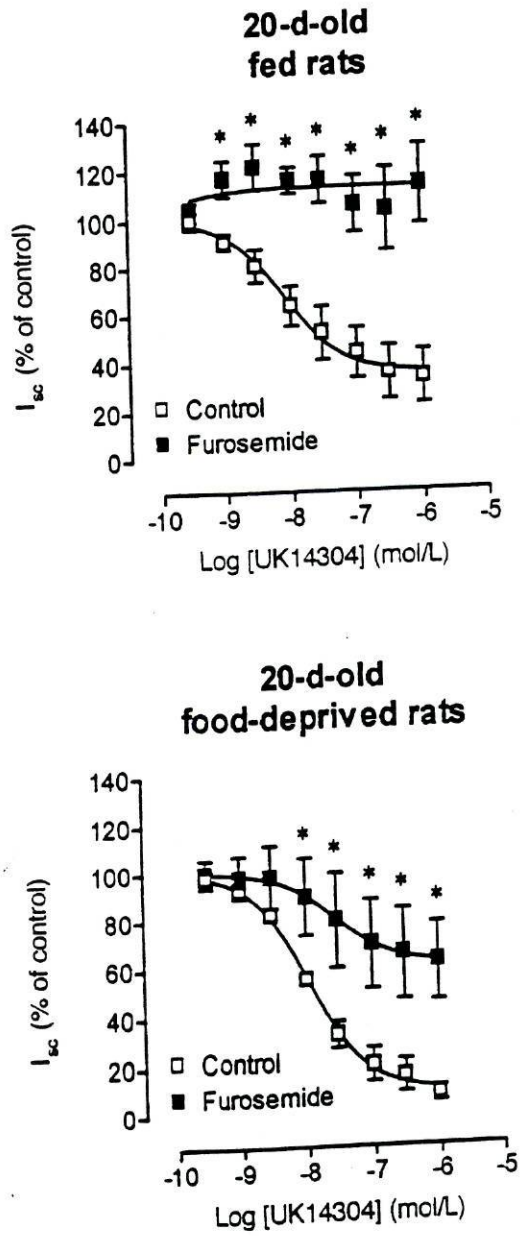
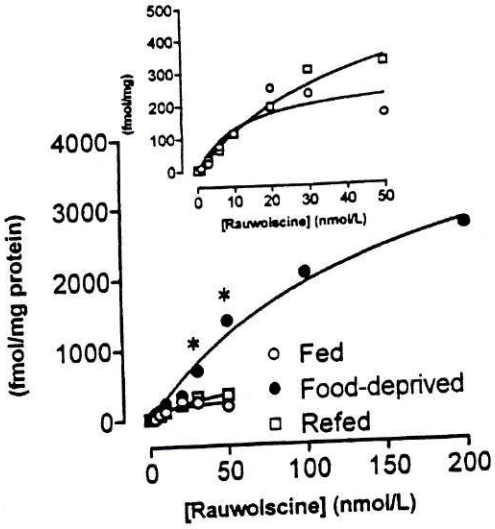


FIGURE 3 Effect of 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK 14,304; 1 μmol/L) on short-circuit current (I_{SC}) voltage-clamped rat jejunal epithelial sheets obtained from 20-d-old (*A*) breast-fed and (*B*) 24-h food-deprived rats in the absence and the presence of furosemide (1 mmol/L). Symbols represent means of four rats per group and vertical lines show SEM. *Significantly different from corresponding control values ($P < 0.05$) using the Newman-Keuls multiple comparison test.



GURE 4 Specific binding of [^{3}H]-rauwolscine (0.1–200 nmol/L) embranes from intestinal mucosa of 20-d-old breast-fed, 24-h deprived and refed rats. The inset shows saturation curves for st-fed and refed rats. Symbols represent means of five experis with four replicates and vertical lines show SEM. *Significantly ent from corresponding values in fed rats ($P < 0.01$) using the man-Keuls multiple comparison test.

ncrease in [^{3}H]-rauwolscine binding. These results raise possibility that, in young rats, differences in basal adrenc tonus may contribute to changes in intestinal electrolyte sport during food deprivation.

Multiple transport pathways involved in Na^+ and Cl^- sfer are present in the jejunum (Binder, 1983, Esposito, 4, Frizzell et al. 1979). These include transporters, exngers, pumps and channels responsible for the electroneuabsorption of NaCl and electrogenic secretion of Cl^-. In experimental set-up, a decrease in basal I_{sc} can be due to ecrease in sodium absorption, a decrease in chloride or arbonate secretion or both [for details see Vieira-Coelho Soares-da-Silva (1998)]. To determine the ionic mechams involved in the α_2-adrenoceptor–stimulated conditions, osemide (1 mmol/L), a NKCC co-transporter inhibitor, was led to the serosal side of the jejunal epithelium.

In 60-d-old fed rats, basal I_{sc}, which reflects electrogenic ion vement across the jejunum, was found to be significantly ver than that in food-deprived rats, suggesting a more active ctrolyte transport in a food-deprived state. Because food privation decreases basal Na^+,K^+-ATPase activity, other mps or transporters may be responsible for these changes duced by food deprivation. In fact, food deprivation in adult

TABLE 2

pparent K_D and B_{max} values for α_2-adrenoreceptor binding es labeled with [^{3}H]-rauwolscine in membranes from jejunal ithelial cells of 20-d-old fed, food-deprived and refed rats[1]

	Fed	Food-deprived	Refed
ax, fmol/mg protein	283 ± 78	4885 ± 676*	662 ± 142
, nmol/L	12 ± 9	153 ± 37	45 ± 16

1 Values are means ± SEM, n = 5 rats per group (four replicates per t).

* Significantly different from corresponding values in fed rats (P 0.01) using the Newman-Keuls multiple comparison test.

TABLE 3

Na^+, K^+-ATPase activity in isolated jejunal epithelial obtained from fed and food-deprived 60- and 20-d-old rats in the absence and the presence of UK 14,304 (1 μmol/L)[1]

	60-d-old rats		20-d-old rats	
	Fed	Food-deprived	Fed	Food-deprived
	nmol P_i (mg protein · min)			
Control	117.4 ± 4.2	94.1 ± 4.9*	51.8 ± 5.2#	54.7 ± 4.3
UK 14,304	112.5 ± 5.0	92.8 ± 6.5*	54.6 ± 4.7#	63.3 ± 11.6

1 Values are means ± SEM, n = 4 rats per group (three replicates per rat).

*# Significantly different from corresponding fed values or values for 60-d-old fed rats ($P < 0.05$) using the Newman-Keuls multiple comparison test. UK 14,304, 5-bromo-*N*-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine; P_i, free inorganic phosphorus.

AQ: 4

animals has been shown to induce changes in the structure of intestinal mucosa, with a decrease in cell proliferation and a consequent increase in enterocyte maturity (Johnson 1988, Williamson 1978). All of these factors change membrane physical properties. The decrease in resistance observed in the jejunum of food-deprived adult rats is in agreement with previous findings by Young and Levin (1990a); these authors found a gradual decrease in tissue resistance with increasing duration of the fasting period. Despite differences in basal I_{sc} and tissue resistance, the addition of UK 14,304 (0.3–3000 nmol/L) to the serosal side induced a concentration-dependent decrease, with EC_{50} and E_{max} values that did not differ between fed and food-deprived adult rats. In both fed and food-deprived rats, the effect of UK 14,304 was partially antagonized by furosemide, suggesting that the effect of UK 14,304 may depend in part on the activation of the NKCC co-transporter. The observation that the Na^+,K^--ATPase activity, measured in isolated jejunal epithelial cells from fed and food-deprived rats, was insensitive to UK 14,304 supports the view that UK 14,304–induced changes in I_{sc} might depend on the stimulation of the NKCC co-transporter.

AQ: 3

In 20-d-old rats, in contrast to adult rats, there were no differences in basal I_{sc} values between fed and food-deprived rats. It is possible that 24 h of food deprivation is not sufficient to change basal electrolyte transport. Also, no change was observed for tissue resistance. As observed in adult rats, the addition to the serosal side of UK 14,304 induced a concentration-dependent decrease in I_{sc}. In young rats, the response to α_2-adrenoceptor stimulation was markedly potentiated, without changes in EC_{50} values. At least two possibilities can be raised to explain the potentiation in the UK 14,304 response in the 24-h food-deprived young rats. Because EC_{50} values were not different between fed and food-deprived rats, an increase in receptor affinity for the agonist can be excluded. Thus, increases in E_{max} values for UK 14,304 may result from either an increase in receptor number, an increase in the number of transporter units or their sensitivity to modulation by α_2-adrenoceptor agonists. Radioligand binding experiments performed in membranes from the jejunal mucosa from 20-d-old fed and food-deprived rats showed an increase in the number of binding sites in the membranes of the latter. Refeeding was accompanied by a reduction in receptor density to a level similar to that observed in fed rats. Again, to determine the ionic pathways involved in the UK 14,304 response, furosemide (1 mmol/L) was added to the serosal side

of the intestinal epithelium. In contrast to the effect observed in adult fed rats, furosemide abolished the UK 14,304–induced decrease in I_{sc} in young fed rats. However, the inhibitory effect of furosemide on UK 14,304–induced I_{sc} changes was less pronounced in the jejunum of food-deprived rats. On the other hand, this difference is consistent with the more pronounced UK 14,304–induced decrease in I_{sc} in food-deprived rats, which ultimately may be related to differences in NKCC activity between the fed and food-deprived rats. The findings that basal Na^+,K^+-ATPase activity was not increased in food-deprived rats and that UK 14,304 did not alter enzyme activity in either fed or food-deprived rats further support the hypothesis that NKCC is the major ionic pathway involved in the response to α_2-adrenoceptor activation.

In conclusion, food deprivation in 20-d-old rats enhances the jejunal electrolyte transport response to α_2-adrenoceptor activation. This effect, which appears to be dependent primarily on the ionic gradients generated by the NKCC co-transporter mechanism, is likely the result of an increase in the number of jejunal epithelial α_2-adrenoceptors. By contrast, in adult rats, the response to α_2-adrenoceptor stimulation is not increased by food deprivation despite the differences in basal I_{sc}, tissue resistance and basal Na^+,K^+-ATPase activity.

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Factores que modulam a resposta da 5-HT sobre o transporte epitelial.

a) Response of jejunal ATPase-Na^+,K^+ to 5-hydroxytryptamine in young and adult rats: effect of fasting and refeeding .

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Response of jejunal Na^+, K^+-ATPase to 5-hydroxytryptamine in young and adult rats: Effect of fasting and refeeding

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ABSTRACT

The present study is aimed to evaluate the effects of 5-hydroxytryptamine (5-HT) upon jejunal Na^+,K^+-ATPase in young (20-day-old) and adult (60-day-old) rats, and determine the effect of food intake on the response of the sodium pump to the amine. Basal Na^+,K^+-ATPase activity in jejunal epithelial cells from young rats was twice that in adult animals and responded to 5-HT with stimulation. In adult rats, fasting reduced by 25% basal jejunal Na^+,K^+-ATPase activity, whereas in young rats, no such change was observed. The sensitivity of jejunal Na^+,K^+-ATPase to 5-HT in young fasted rats was similar to that observed in fed animals. The effect of refeeding in young rats was a 2-fold increase in jejunal Na^+,K^+-ATPase activity, this being accompanied by insensitivity to 5-HT. In adult rats, refeeding was accompanied by an increase in jejunal Na^+,K^+-ATPase activity. It is concluded that the stimulatory effect of 5-HT upon jejunal Na^+,K^+-ATPase activity is a phenomenon dependent on both age and type of diet. In young rats, it is the food intake that plays an important role in development of insensitivity of Na^+,K^+-ATPase to stimulation by 5-HT, while in adult animals fasting or fasting followed by refeeding does not play a major role in regulating its sensitivity to the amine.

Keywords food intake, 5-hydroxytryptamine, jejunum, Na^+,K^+-ATPase.

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In transporting epithelia, vectorial movement of sodium is accomplished by means of the Na^+,K^+-ATPase located at the basolateral plasma membrane and several sodium transport mechanisms localized at the apical domain of the cell (Rodriguez-Boulon & Nelson 1989). The basal activity of this pump and its modulation, which will reflect intestinal function (absorption and secretion), can be influenced by different factors, such as absence or presence of food in the intestine, protein and salt content in the diet and stage of the developmental process (Binder 1983). This has a great impact during the uptake of nutrients and in the maintenance of electrolyte homeostasis and water metabolism during development (Herbst & Suskind 1969, Younoszai *et al.* 1978).

Dopamine is particularly abundant in the mucosal cell layer (Esplugues *et al.* 1985, Eaker *et al.* 1988) and locally formed dopamine has been hypothesized to act as a paracrine or autocrine substance regulating intestinal sodium absorption (Finkel *et al.* 1994). This effect is accomplished at the cellular level by inhibition of Na^+,K^+-ATPase activity (Vieira-Coelho *et al.* 1998). The relative importance of this system in controlling sodium absorption assumes particular relevance in view of the fact that the dopamine is no longer active in inhibiting jejunal Na^+,K^+-ATPase activity when animals reach adulthood (40–60 days of age) (Finkel *et al.* 1994, Vieira-Coelho *et al.* 1998). Another interesting aspect is concerned with the fact that when 20-day-old breast-fed rats are challenged with rat maintenance solid diet, jejunal Na^+,K^+-ATPase activity increases by 2-fold (to a level similar in adult rats) and is no longer sensitive to inhibition by dopamine (Lucas-Teixeira *et al.* 1999). 5-Hydroxytryptamine (5-HT) is another local agent which may have some importance in regulating jejunal Na^+,K^+-ATPase and its sensitivity to dopamine. In fact, 5-HT counteracts the inhibitory effect of dopamine upon jejunal Na^+,K^+-ATPase activity and 5-HT levels in young rats are twice those in adult rats. This antagonism between dopamine and 5-HT concerning sodium handling and Na^+,K^+-ATPase activity has also been observed at the kidney level (Soares-da-Silva *et al.* 1996a, b).

The present study is aimed to evaluate the effects of 5-HT upon jejunal Na^+,K^+-ATPase in young and adult rats and determine the effect of food intake on the response of the sodium pump to the amine. The tissue

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levels of 5-HT and its major metabolite (5-hydroxyindolacetic acid; 5-HIAA) were also measured and the 5-HIAA/5-HT tissue ratios were used as a rough measure of jejunal 5-HT rate of utilization.

MATERIALS AND METHODS

Animals

All the experiments were performed in male Wistar rats (Harlan-Interfauna, Barcelona, Spain), 20-day-old (40–50 g) and 60-day-old (260–300 g) rats. Animals were kept in air-conditioned animal quarters and had free access to drinking water until the day of the experiment. Adult animals were divided into three groups: (1) fed rats ($n = 6$) (2) animals fasted for 48 h ($n = 6$) and (3) animals fasted for 48 h followed by refeeding for 48 h ($n = 4$). Fed and refed rats were allowed *ad libitum* solid food (rat maintenance diet, catalogue number 9609 obtained from Harlan-Teklad, Oxon, UK). Young rats were also divided into three groups: (1) breast-fed rats ($n = 6$) (2) rats fasted for 24 h ($n = 6$) and (3) animals fasted for 24 h followed by refeeding for 24 h in the presence of their female progenitor ($n = 4$). The Scientific Review Board of the Foundation for Science and Technology (Portugal) approved the experimental protocol.

Cell isolation

The method of cell isolation was similar to that previously described (Vieira-Coelho *et al.* 1998) with minor modifications. The jejunum was isolated and divided in small fragments. These were everted with fine forceps and incubated for 45 min in 5 mL warm (37 °C) and gassed (95% O_2 and 5% CO_2) Hanks' solution with 0.06% collagenase type I (Sigma Chemical, St Louis, MO). The Hanks' medium had the following composition (mmol L^{-1}): NaCl 137, KCl 5, $MgSO_4$ 0.8, Na_2HPO_4 0.33, KH_2PO_4 0.44, $CaCl_2$ 0.25, $MgCl_2$ 1.0, Tris–HCl 0.15 and sodium butyrate 1.0, pH = 7.4. At the end of the incubation period, the fragments were removed from the solution and the medium containing the detached cells was centrifuged (200 ×*g*, 4 °C) for 4 min and the cell pellet was resuspended in Hanks' solution. Cell viability was estimated by the Trypan blue (0.04%, 1 min) exclusion method and the percentage of viable cells (excluding the dye), determined by haemocytometer counting was >90%.

Na^+, K^+-ATPase activity

Na^+, K^+-ATPase activity was measured by the method Quigley & Gotterer (1969) and adapted in our laboratory with slight modifications. Briefly, isolated jejunal epithelial cells, obtained as previously described (Vieira-Coelho *et al.* 1998), were incubated for 15 min at 37 °C with vehicle or 5-HT (10 μM). After the incubation period, the jejunal epithelial cells were permeabilized by rapid freezing in dry ice-acetone and thawing. The reaction mixture in a final volume of 1.025 mL, contained (in mmol L^{-1}) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium EDTA, 5 $MgCl_2$, 6 NaN_3, 75 tris(hydroxymethyl)aminomethane(tris) hydrochloride and 100 μL cell suspension (100 μg protein). The reaction was initiated by the addition of 4 mmol L^{-1} ATP (25 μL). For determination of ouabain-insensitive ATPase, NaCl and KCl were omitted, and ouabain (1 mmol L^{-1}; 100 μL) or vehicle (water; 100 μL) were added to the assay. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of 50 μL of ice-cold trichloroacetic acid. Samples were centrifuged (2600 × *g*), and liberated P_i in supernatant was measured by spectrophotometry at 740 nm. Na^+,K^+-ATPase activity is expressed as nanomoles P_i per milligram protein per minute and determined as the difference between total and ouabain-insensitive ATPase. The protein content in cell suspension (≈2 mg mL^{-1}) as determined by the method described by Bradford (1976) with human serum albumin as a standard was similar in all samples.

Assay of monoamines

The assays for 5-HT and 5-HIAA were performed by means of high-pressure liquid chromatography (HPLC), as previously described (Soares-da-Silva *et al.* 1996b). The detection was carried out electrochemically with a glassy carbon electrode, an Ag/AgCl reference electrode and an amperometric detector (Gilson model 141); the detector cell was operated at 0.75 V. The current produced was monitored using the Gilson 712 HPLC software. The lower limit for detection was ranged between 350 and 1000 fmol.

Drugs

The compounds used were 5-hydroxytryptamine hydrochloride, ouabain and pargyline hydrochloride, obtained from Sigma Chemical (St Louis, MO, USA).

Statistics

Results are mean ± SEM of values for the indicated number of determinations. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student's *t*-test for unpaired comparisons. A *P*-value less than 0.05 was assumed to denote a significant difference.

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RESULTS

Basal jejunal Na^+, K^+-ATPase activity in 60-day-old rats was found to be 2.5-fold that in 20-day-old rats under breast-feeding (117.4 ± 4.2 vs. 51.8 ± 3.5 nmol P_i (mg protein)$^{-1}$ min^{-1}; Fig. 1). This dramatic increase in jejunal Na^+,K^+-ATPase activity was accompanied by a 2-fold decrease in 5-HT tissue levels. This was also accompanied by an increase, although it did not attain statistical significance, in the 5-HIAA/5-HT tissue ratios, a rough measure of 5-HT utilization in the jejunal mucosa (Fig. 2). Jejunal Na^+,K^+-ATPase in 20-day-old rats, but not that in adult animals, responded to 5-HT with stimulation (Fig. 3).

As 20- and 60-day-old rats were on different alimentary regimens and solid diet is known to increase jejunal Na^+,K^+-ATPase activity (Lucas-Teixeira *et al.* 1999), it was decided to submit both groups of animals to a fasting period in order to reduce the influence of food upon the sodium pump. Young and adult rats were fasted for 24 and 48 h, respectively. As shown in Fig. 4, fasting was accompanied by a 25% decrease in Na^+,K^+-ATPase activity in adult rats, whereas in young animals, fasting failed to alter enzyme activity. Despite this decrease in Na^+,K^+-ATPase activity in adult rats, 5-HT was still devoid of stimulatory effect in adult rats

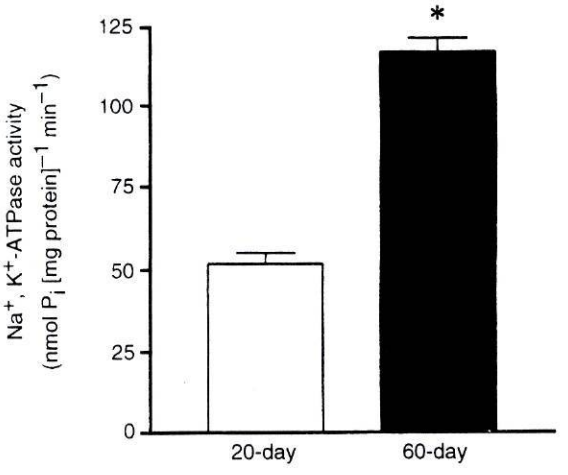


Figure 1 Basal jejunal Na^+, K^+-ATPase activity in 20-day-old breast-fed rats (open columns) and 60-day-old fed rats. Columns represent means of 7–11 experiments per group; vertical lines indicate SEM.

(Fig. 3). In 20-day-old animals, the stimulatory effect of 5-HT in fasted rats was similar to that observed in breast-fed rats (Fig. 3). The effect of refeeding in 60-day-old rats was a return of jejunal Na^+,K^+-ATPase activity to levels similar to those in fed rats (Fig. 4), being 5-HT still devoid of stimulatory effect upon the

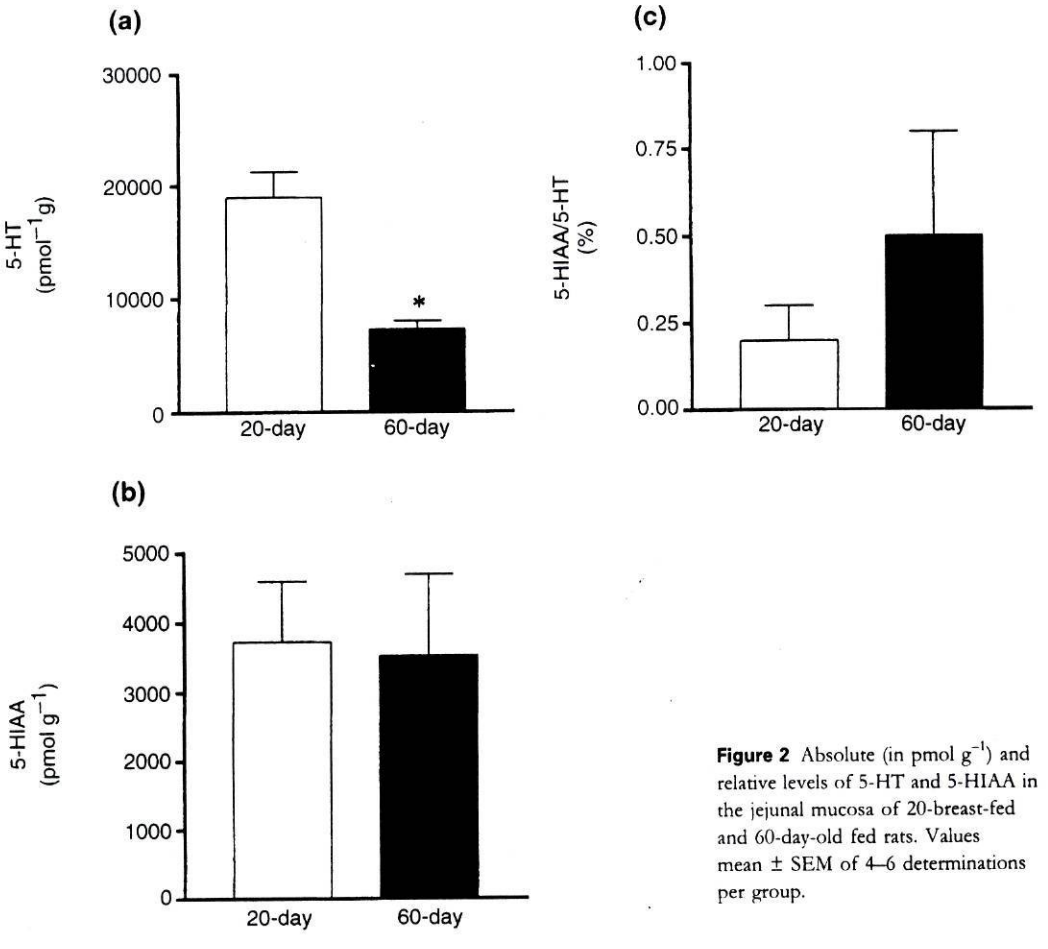


Figure 2 Absolute (in pmol g^{-1}) and relative levels of 5-HT and 5-HIAA in the jejunal mucosa of 20-breast-fed and 60-day-old fed rats. Values mean ± SEM of 4–6 determinations per group.

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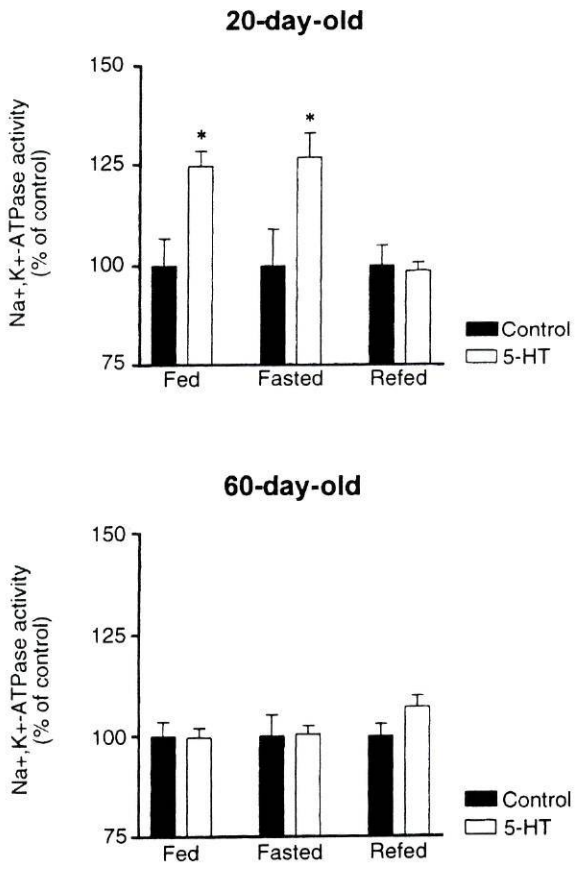


Figure 3 Jejunal Na^+,K^+-ATPase activity in 20- and 60-day-old breast or fed, fasted and refed rats in the absence (closed columns) and the presence of 5-HT (10 μM) (open columns). Columns represent means of 4–6 experiments per group; vertical lines indicate SEM. Significantly different from corresponding values for control ($^*P < 0.05$).

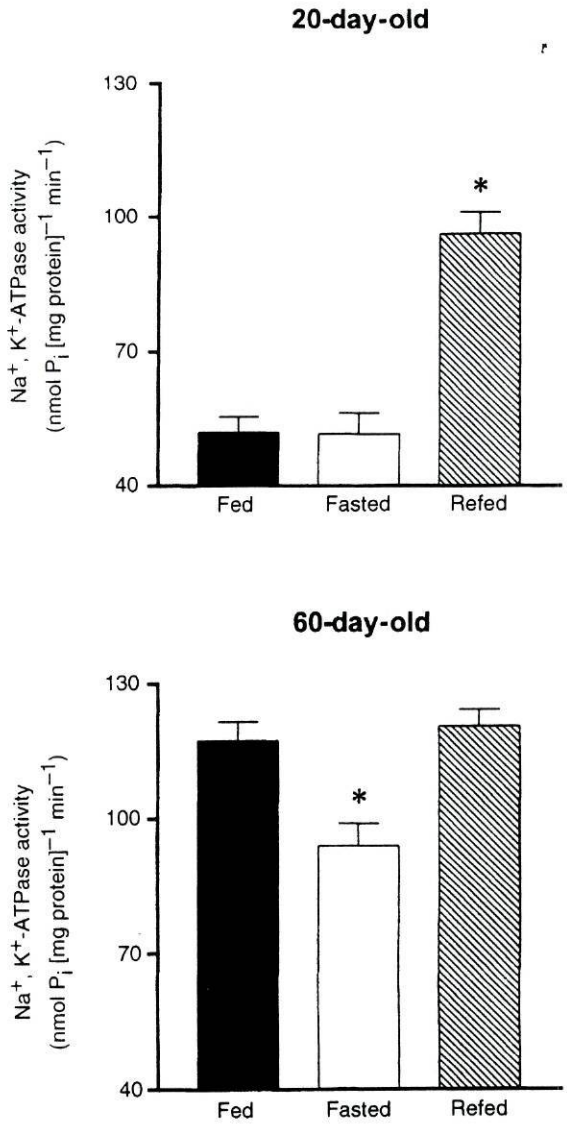


Figure 4 Basal jejunal Na^+,K^+-ATPase activity in 20- and 60-day-old rats under feeding (closed column), fasting (open column) and refeeding (hatched column) conditions. Columns represent means of 4–6 experiments per group; vertical lines indicate SEM. Significantly different from values for fed rats ($^*P < 0.05$).

enzyme (Fig. 3). By contrast, the effect of refeeding in 20-day-old rats was a 2-fold increase in jejunal Na^+,K^+-ATPase activity (Fig. 4), this being accompanied by complete loss of response to 5-HT (Fig. 3).

Basal levels of 5-HT during fasting and refeeding in both 20- and 60-day-old rats remained unchanged, although there were changes in 5-HIAA levels in 60-day-old animals (Fig. 5b). In these animals, fasting produced a marked increase in 5-HIAA/5-HT tissue ratios, indicating an enhanced utilization of 5-HT. By contrast, refeeding in 60-day-old rats was accompanied by a marked decrease in 5-HIAA levels and 5-HIAA/5-HT tissue ratios, an indication of a decrease in 5-HT utilization.

DISCUSSION

The results presented here demonstrate that 5-HT produces stimulation of jejunal Na^+,K^+-ATPase in breast-fed and fasted young Wistar rats, but not in fed or fasted adult animals. This stimulatory effect is completely absent when young rats are submitted to fasting plus refeeding, this being accompanied by a marked increase in basal jejunal Na^+,K^+-ATPase activity. These results confirm previous observations from our group obtained in Sprague–Dawley rats, where 5-HT was found to convert inhibition of Na^+,K^+-ATPase activity by dopamine to stimulation, indicating this is not dependent on the rat strain. This is also in agreement with results obtained in rat renal proximal tubular epithelial cells, where 5-HT stimulated Na^+,K^+-ATPase activity and counteracted the inhibitory effect of dopamine (Soares-da-Silva *et al.* 1996a).

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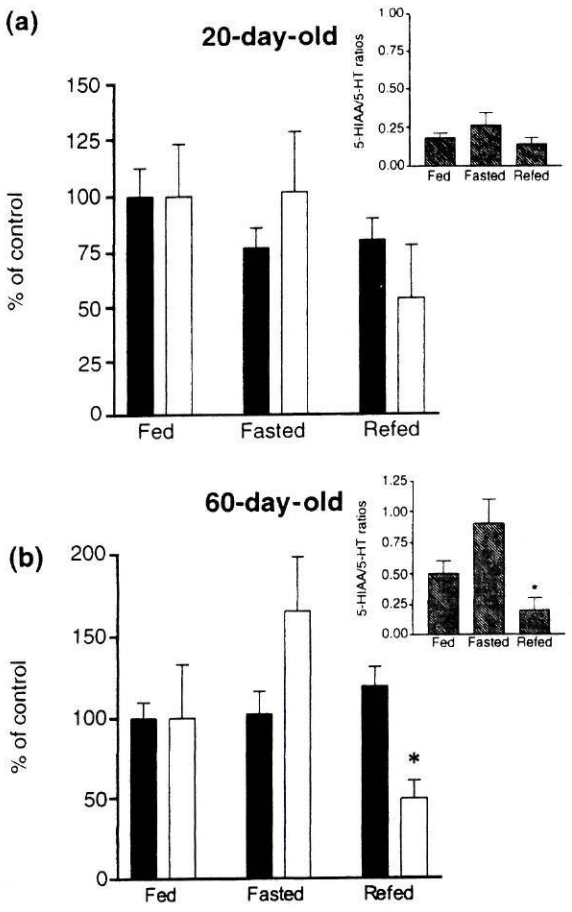


Figure 5 Effect of fasting and refeeding on absolute and relative tissue levels of 5-HT (closed columns) and 5-HIAA (open columns) in the intestinal mucosa of (a) 20- and (b) 60-day-old rats. Columns represent means of 4–6 experiments per group; vertical lines indicate SEM. Significantly different from values for fasted rats ($^*P < 0.05$).

On the other hand, the data presented here suggest that this loss of sensitivity to 5-HT may be not an age-dependent phenomenon, because the regimen of food intake and type of food available markedly affects the response of Na^+,K^+-ATPase to 5-HT. However, to a certain extent, these findings are in agreement with those of Grøndahl *et al.* (1996), who showed that the secretory response to 5-HT in pig small intestine decreases with increasing age and in the aboral direction.

As changing from breast feeding to solid diet markedly increased basal Na^+,K^+-ATPase activity (Lucas-Teixeira *et al.* 1999), it may be hypothesized that the loss of sensitivity to 5-HT had to do with the high level of enzyme activity. However, in adult rats, despite the marked reduction in basal Na^+,K^+-ATPase activity during fasting, the amine was still devoid of inhibitory effect. In fact, 48-h fasting was found to significantly reduce basal Na^+,K^+-ATPase activity, in agreement with a previous report (Murray & Wild 1980), and this was completely reverted by refeeding. The marked increase in Na^+,K^+-ATPase activity in 20-day-old rats under refeeding (with access to solid diet) may be related to different salt or protein content of the diet (maternal milk vs. rat maintenance solid diet). Both sodium and amino acid content are known to affect renal Na^+,K^+-ATPase activity (Bertorello *et al.* 1988, Jakobsson *et al.* 1990). However, in 20-day-old rats the effect of high salt (HS) intake was a decrease in basal Na^+,K^+-ATPase activity, without changes in sensitivity to inhibition by dopamine (Vieira-Coelho *et al.* 1998). This reduction in basal Na^+,K^+-ATPase activity during HS intake was completely reverted by pretreatment with benserazide, an AADC inhibitor suggesting that it was related to the enhanced availability of dopamine. In fact, HS intake was demonstrated in 20-day-old rats to increase the formation of dopamine at the level of the jejunal mucosa (Finkel *et al.* 1994, Vieira-Coelho *et al.* 1998). This supports the hypothesis that in young rats other food components, rather than sodium in the rat maintenance solid diet, may play a role in modulating Na^+,K^+-ATPase activity and its sensitivity to monoamines, such as dopamine and/or 5-HT. Several studies have reported that different dietary components, like fatty acids (Alam & Alam 1983), vanadium (Phillips *et al.* 1982), glutamine (Horvath *et al.* 1996), calcium levels (Blakeborough *et al.* 1990), protein content (Rebolledo-Varela *et al.* 1983) change Na^+,K^+-ATPase activity in both the kidney and the small intestine in different animal models and experimental conditions.

Another interesting observation concerns the changes in 5-HT and 5-HIAA levels during fasting and refeeding. In young rats, fasting and refeeding failed to affect the rate of utilization of intestinal 5-HT. Thus, one may hypothesise that the increase in Na^+,K^+-ATPase activity during refeeding in young rats would not be related to increased 5-HT stimulatory tonus. By contrast, in adult rats, the data presented here indicate a decrease in the rate of utilization of 5-HT during refeeding in adult rats. As jejunal Na^+,K^+-ATPase activity in these animals was insensitive to the stimulatory effects of the amine, it might be suggested that this would be independent of changes in jejunal 5-HT tonus. It is possible, however, that 5-HT might act upon other transporters, pumps or channels important in the modulation of certain mechanisms affected by fasting or refeeding.

In conclusion, although in young rats solid food intake plays an important role in the development of insensitivity of Na^+,K^+-ATPase to stimulation by 5-HT, in adult rats fasting or fasting followed by refeeding, in spite of altered basal jejunal Na^+,K^+-ATPase activity, does not play a major role in regulating the sodium pump sensitivity to 5-HT.

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Funcionamento do sistema dopaminérgico intestinal em situações patológicas.

a) Salt intake and sensitivity of intestinal and renal Na^+,K^+-ATPase on inhibition by dopamine in Spontaneous Hypertensive and Wistar-Kyoto rats.

Clin. Exp. Hypert.. "in press"

b) Intestinal dopaminergic activity in obese and lean Zucker rats: response to high salt intake.

(submetido para publicação)

SALT INTAKE AND SENSITIVITY OF INTESTINAL AND RENAL NA^+-K^+ ATPASE TO INHIBITION BY DOPAMINE IN SPONTANEOUS HYPERTENSIVE AND WISTAR-KYOTO RATS

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ABSTRACT

The present study evaluated the activity of jejunal Na^+-K^+-ATPase and its sensitivity to inhibition by dopamine in spontaneous hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats during low (LS), normal (NS) and high (HS) salt intake. Basal jejunal Na^+-K^+-ATPase activity in SHR on LS intake was higher than in WKY rats. Jejunal Na^+-K^+-ATPase activity in WKY rats, but not in SHR, on LS intake was significantly reduced (20% decrease) by dopamine (1 μM) and SKF 38393 (10nM), but not quinerolane (10 nM), this being antagonized the D_1 receptor antagonist (SKF 83566). Changing from LS to NS or HS intake in WKY rats increased basal jejunal Na^+-K^+-ATPase activity and attenuated the inhibitory effect of dopamine. In SHR, changing from LS to NS or HS intake increased basal jejunal Na^+-K^+-ATPase activity. Basal renal Na^+-K^+-ATPase activity in SHR on LS intake was similar to that in WKY rats and was insensitive to inhibition by dopamine . Changing from LS to NS or HS intake in WKY rats increased basal renal Na^+-K^+-ATPase activity without affecting the inhibitory effect of dopamine. In SHR, changing from LS to NS or HS intake failed to alter basal renal Na^+-K^+-ATPase activity. It is concluded that inhibition of jejunal Na^+-K^+ ATPase activity by D_1 dopamine receptor activation is dependent on salt intake in WKY rats, and SHR animals fail to respond to dopamine, irrespective of their salt intake.

Key words: Hypertension - sodium - dopamine - intestine - Na^+,K^+-ATPase

INTRODUCTION

Dopamine exerts natriuretic and diuretic effects by activating D_1-like receptors located at various regions in the nephron (1,2). At the level of the proximal tubule, the overall increase in sodium excretion produced by dopamine and D_1 receptor agonists results from inhibition of main sodium transport mechanisms at the basolateral and apical membranes, respectively Na^+-K^+ ATPase (3,4) and Na^+/H^+ exchanger (5). The physiological importance of the renal actions of dopamine mainly depends on the sources of the amine in the kidney and on the availability of this dopamine to activate the amine specific receptors. The proximal tubules, but not distal segments of the nephron, are endowed with a high aromatic L-amino acid decarboxylase (AADC) activity and epithelial cells of proximal tubules have been demonstrated to synthesise dopamine from circulating or filtered L-3,4-dihydroxyphenylalanine (L-DOPA) (4,6,7). This non-neuronal renal dopaminergic system appears to be highly dynamic and the basic mechanisms for the regulation of this system are thought to depend mainly on the availability of L-DOPA, its fast decarboxylation into dopamine and in precise and accurate cell outward amine transfer mechanisms (8). High blood pressure may be linked to abnormalities in the function of the renal dopaminergic system, such as decreased ability to

synthesise dopamine (9-11) and deficient coupling of dopamine receptors to effector mechanisms (12-17). Furthermore, recently mice lacking either D_{1A} or D_3 receptors were reported to develop hypertension (18,19).

Dopamine is also relatively abundant in the intestine mucosal cell layer (20,21) and studies on the formation of dopamine from exogenous L-DOPA along the rat digestive tract showed that the highest AADC activity is located in the jejunum (22). A high salt (HS) intake has been found to constitute an important stimulus for the production of dopamine in rat jejunal epithelial cells and this is accompanied, in 20-day old animals, by a decrease in sodium intestinal absorption (23). This effect is accomplished, at the cellular level, by inhibition of Na^+-K^+-ATPase activity (24). The relative importance of this system in controlling sodium absorption assumes particular relevance in view of the findings that 40-day old rats submitted to a HS intake have a fault in intestinal dopamine production during salt loading, in contrast to that occurring in 20-day old animals. The lack of changes in the jejunal function in response to HS intake coincides with the period in which the renal function has reached maturation (25), suggesting the occurrence of complementary functions between the intestine and the kidney during development. Furthermore, the similarity of effects of dopamine upon Na^+-K^+ ATPase in proximal renal tubules and jejunal epithelial cells strongly suggests that the dopaminergic influence upon sodium transport across epithelia assumes particular importance when mechanisms involved in maintenance of sodium balance are disturbed.

Because dopamine fails to inhibit renal Na^+-K^+-ATPase in hypertensives, and the intestinal dopaminergic system appears to be specially activated when the kidney fails to provide an optimal control over sodium balance, it was decided to evaluate the activity of jejunal Na^+-K^+-ATPase and its sensitivity to inhibition by dopamine in spontaneous hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats during low salt (LS), normal salt (NS) and HS intake. During the early phases of development of hypertension, SHR, in comparison to WKY rats, are endowed with an enhanced ability to synthesise dopamine at the kidney level (26). Therefore, it was decided to use in the present work animals in a fully developed hypertensive state, in which the activation of their renal dopaminergic systems is no longer upregulated (26-28). For the sake of comparison, the sensitivity of renal Na^+-K^+-ATPase activity to different salt diets and dopamine was also evaluated.

MATERIALS AND METHODS

SHR and WKY rats (Harlan-Inferfauna, Barcelona, Spain) 12 weeks old and weighing 310-340 g were selected after a 7 day period of stabilisation and following adaptation to blood pressure measurements. Animals were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 22±2 °C); fluid intake and food consumption were monitored daily throughout the study. SHR and WKY rats were fed with a low salt (LS) diet (0.01 to 0.02% sodium; diet TD 90228, Harlan Teklad, Madison, Wisconsin, USA) for 7 days and then were divided in three groups, and 1) maintained on LS intake, 2) fed a normal salt (NS) or 3) fed a high salt (HS) diet for 24 hours. NS and HS diets consisted of normal rat chow (Harlan-Interfauna, RMM type diet, Barcelona, Spain) plus tap water or normal rat chow plus 1% saline as drinking water, respectively. Blood pressure (systolic, SBP; diastolic, DBP) and heart rate were measured in conscious restrained animals, between 7.00 to 10.00 a.m., using a

photoelectric tail cuff pulse detector (LE 5000, Letica, Barcelona, Spain).

Na^+,K^+-ATPase activity was measured by the method Quigley and Gotterer (29) and adapted in our laboratory with slight modifications. Briefly, isolated rat jejunal epithelial cells and renal proximal tubules, obtained as previously described (24,30), were pre-incubated for 20 min at 37°C. After the pre-incubation period the jejunal epithelial cells and renal proximal tubules were permeabilized by rapid freezing in dry ice-acetone and thawing. The reaction mixture, in a final volume of 1.025 ml, contained (in mM) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium EDTA, 5 $MgCl_2$, NaN_3, 75 tris(hydroxymethyl)aminomethane(tris) hydrochloride and 100 µl tubular and epithelial cell suspension (100 µg protein). The reaction mixture also contained phentolamine (0.2 µM) and propranolol (1 µM) to prevent activation of α- and ß-adrenoceptors, respectivley, by dopamine. The reaction was initiated by the addition of 4 mM ATP. For determination of ouabain-sensitive ATPase, NaCl and KCl were omitted, and Tris-HCl (150 mM) and ouabain (1 mM) were added to the assay. After incubation at 37°C for 15 min, the reaction was terminated by the addition of 50 µl of ice-cold trichloroacetic acid. Samples were centrifuged (3,000 rpm), and liberated P_i in supernatant was measured by spectrophotometry at 740 nm. Na^+,K^+-ATPase activity is expressed as nanomoles P_i per milligram protein per minute and determined as the difference between total and ouabain-insensitive ATPase. The protein content in cell suspension (approximately 2 mg/ml), as determined by the method described by Bradford (31) with human serum albumin as a standard, was similar in all samples.

The assay of catecholamines and its metabolites in urine (L-DOPA, norepinephrine, dopamine, DOPAC, 3-MT and HVA), renal tissues and jejunal mucosa (L-DOPA, norepinephrine, dopamine and DOPAC) was performed by HPLC with electrochemical detection, as previously described (10). In brief, aliquots of 0.5 ml of acidified urine or plasma and 1.5 ml of perchloric acid in which tissues had been kept or acidified tissue homogenates were placed in 5 ml conical-based glass vials with 50 mg alumina and the pH of the samples adjusted to pH 8.6 by addition of Tris buffer. The adsorbed catecholamines were then eluted from the alumina with 200 µl of 0.2 M perchloric acid on Costar Spin-X microfilters; 50 µl of the eluate was injected into a high pressure liquid cromatograph (Gilson Medical Electronics, Villiers le Bel, France). The quantification of metanephrine in the homogenates was also performed by HPLC with electrochemical detection; 50 µl aliquots of filtered samples were directly injected into the chromatograph. For the quantification of HVA in urine samples, 50 µl of urine was diluted to 500 µl with perchloric acid (0.2 M), the sample filtered on Costar Spin-X microfilters and directly injected into the chromatograph. The lower limit of detection of L-DOPA, dopamine, norepinephrine, DOPAC, 3-MT and HVA ranged from 350 to 1000 fmol.

Urinary sodium and potassium were measured by flame photometry (model FML3) connected to a dilutor (model A 6241, Radiometer, Copenhagen, Denmark) and urine and plasma osmolality by means of an osmometer (Advanced Instruments, Inc., MA, USA, model 3 MO). Urinary and plasma creatinine and plasma urea were measured by a wavelength photometer (Hitashi Automatic Analizer, model 717, or a Beckman Analyzer II).

The compounds used were: dopamine hydrochloride, ouabain, phentolamine hydrochloride and DL-propranolol were obtained from Sigma Chemical Company

(St. Louis, Mo, U.S.A.); quinerolane, SKF 38393, SKF 83566 and (S)-Sulpiride were obtained from Research Biochemicals International (RBI, Natick, USA).

Results are means ± S.E.M. of values for the indicated number of determinations. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference.

RESULTS

Basal jejunal Na^+-K^+-ATPase activity in SHR on LS intake was markedly higher than in WKY rats (Fig. 1A), and was insensitive to the inhibitory effects of dopamine (Fig. 2). In fact, jejunal Na^+-K^+-ATPase activity in WKY rats on LS intake, despite being lower than in SHR, was significantly reduced by dopamine (1 μM). The effect of changing from LS to NS or HS intake in WKY rats was a significant increase in basal jejunal Na^+-K^+-ATPase activity (Fig. 1A), accompanied by a marked attenuation of the inhibitory effect of dopamine (Fig. 2).

In SHR, changing from LS to NS or HS intake was also accompanied by increases in basal jejunal Na^+-K^+-ATPase activity (Fig. 1A). Tissue levels (in pmol/g) of dopamine (SHR: LS = 37.2±3.9, NS = 31.0±3.3, HS = 29.0±5.5; WKY: LS = 34.6±0.4, NS = 38.4±5.2, HS = 34.0±3.7) and norepinephrine (SHR: LS = 89.2±14.1, NS = 62.1±11.1, HS = 72.6±14.9; WKY: LS = 94.1±32.0, NS = 116.8±39.3, HS = 110.4±71.2) did not differ between SHR and WKY rats, either on LS, NS or HS intake.

Figure 3, shows the effect of dopamine (1 μM) in the absence and in the presence of SKF 83566 (1 μM) or S-sulpiride (1 μM), SKF 38393 (10 nM) and quinerolane (1 μM) upon jejunal Na^+,K^+-ATPase activity in SHR and WKY rats on LS intake.

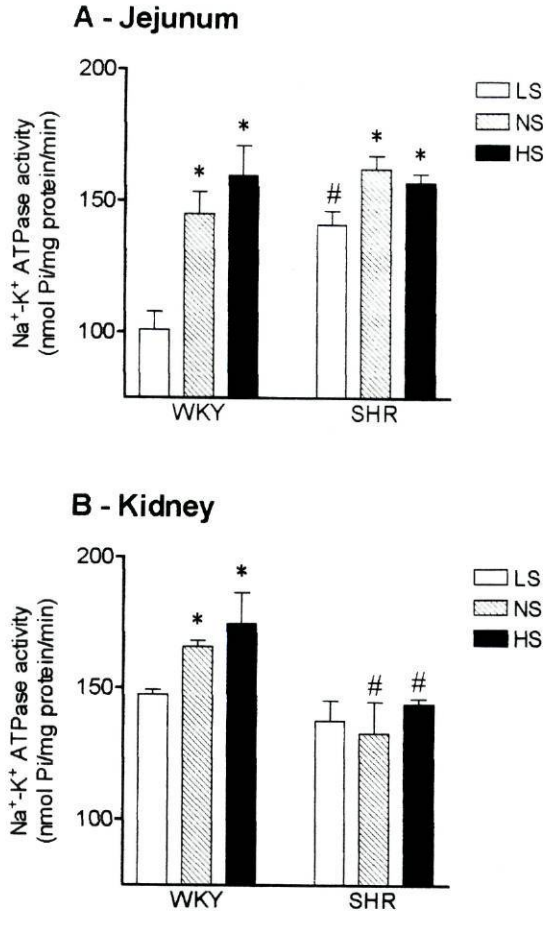


Figure. 1. Na^+,K^+-ATPase activity in (A) isolated jejunal epithelial cells and (B) isolated renal proximal tubules from WKY rats and SHR on LS, NS and HS intake. Columns represent means of four determinations per group and vertical lines show SEM. Significantly different from corresponding values during LS intake (* $P<0.05$) or corresponding values for WKY rats (# $P<0.05$).

As shown in this figure, dopamine and the selective D_1 receptor agonist (SKF 38393) or the selective D_2 receptor agonist (quinerolane) failed to affect jejunal Na^+,K^+-ATPase activity in SHR, whereas in WKY rats the effect of both dopamine and SKF 38393, but not quinerolane, was a

significant reduction in jejunal Na^+,K^+-ATPase activity. The inhibitory effect of dopamine upon jejunal Na^+,K^+-ATPase activity in WKY rats was completely prevented by the D_1 receptor antagonist (SKF 83566), whereas the selective D_2 receptor antagonist failed to revert the effect of dopamine.

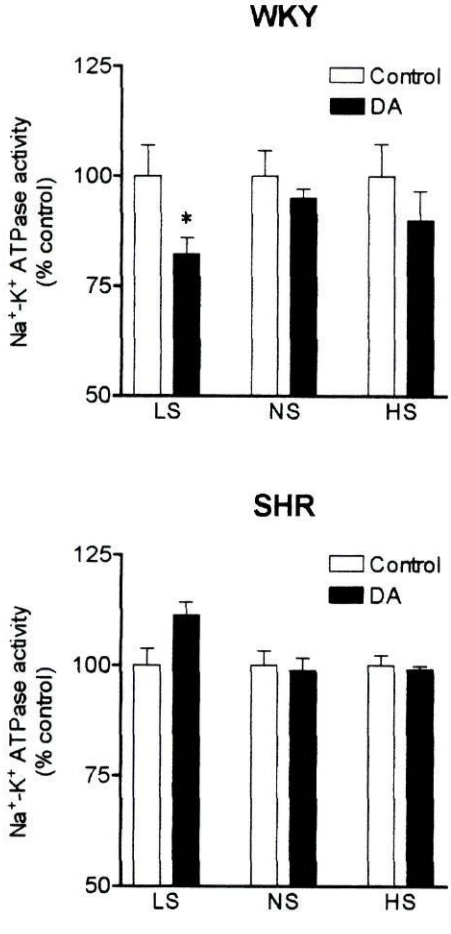


Figure. 2. Effect of dopamine (DA; 1 μM) on jejunal Na^+,K^+-ATPase activity from WKY rats and SHR on LS, NS and HS intake. Columns represent means of four determinations per group and vertical lines show SEM. Significantly different from corresponding control values (* $P<0.05$).

Basal renal Na^+-K^+-ATPase activity in SHR on LS intake was similar to that in WKY rats (Fig. 1 B) and was insensitive to the inhibitory effects of dopamine (Fig. 4).

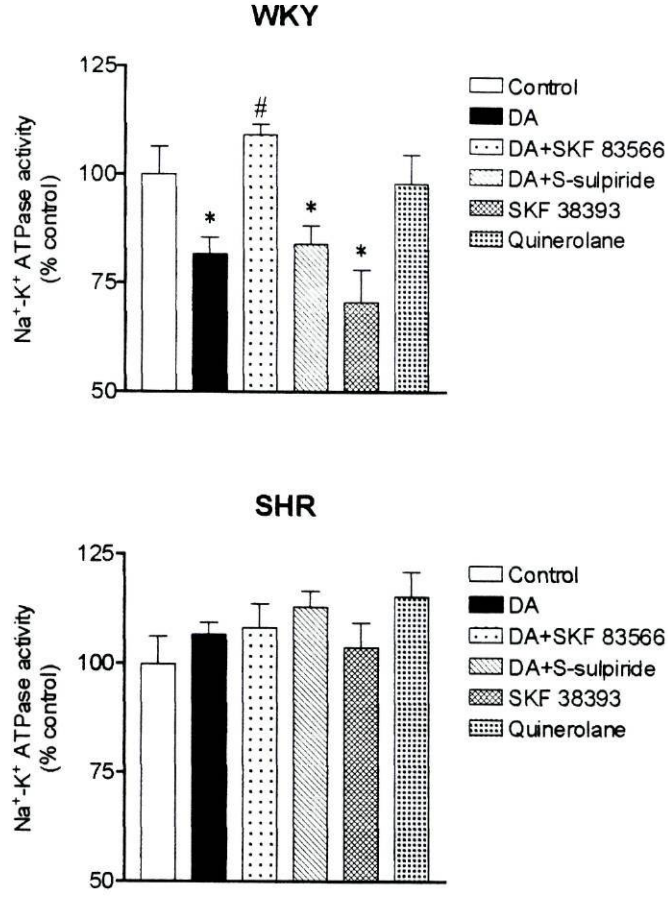


Figure. 3. Effect of dopamine (1 μM) alone and in the presence of SKF 83566 (1 μM) or S-sulpiride (1 μM), SKF 38393 (10 nM) and quinerolane (10 nM) on jejunal Na^+,K^+-ATPase activity from WKY rats and SHR on LS intake. Columns represent means of four determinations per group and vertical lines show SEM. Significantly different from corresponding control values (* $P<0.05$) or values for dopamine alone (# $P<0.05$).

The effect of changing from LS to NS or HS intake in WKY rats was a significant increase in basal renal Na^+-K^+-ATPase activity (Fig. 1B) without changes on the inhibitory effect of dopamine (Fig. 4). In SHR, the effect of changing from LS to NS

or HS intake failed to alter basal renal Na^+-K^+-ATPase activity. Contrary to that observed in the jejunum, dopamine (1 μM) was found to significantly reduce renal Na^+-K^+-ATPase activity in WKY rats either on LS, NS or HS intake.

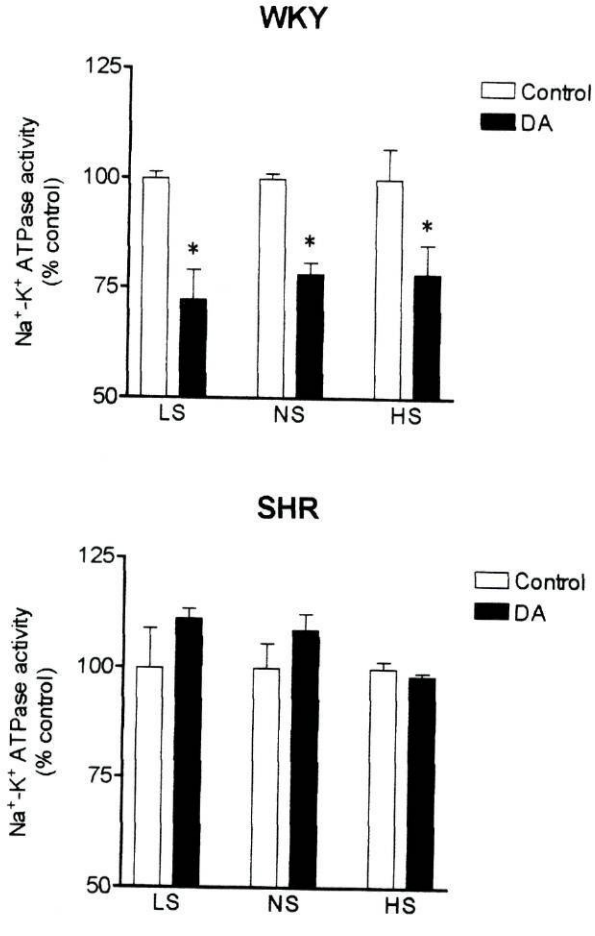


Figure. 4. Effect of dopamine (DA; 1 μM) on renal Na^+,K^+-ATPase activity from WKY rats and SHR on LS, NS and HS intake. Columns represent means of four determinations per group and vertical lines show SEM. Significantly different from corresponding control values (* $P<0.05$).

Body weight (BW, in g) and heart rate (HR, in beats per min) did not differ between SHR (BW=334±9; HR=330±3) and WKY rats (BW=312±8; HR=338±8), in contrast with blood pressure. Both systolic (S) and diastolic (D) blood pressure (in mm Hg) were significantly ($P<0.05$) higher in SHR (S=191±4; D=115±2) than in WKY rats (S=139±3; D=79±2). Feeding a LS diet for 7 days failed to affect blood pressure, heart rate and body weight. Similarly, changing from LS intake to NS or HS intake failed to affect these parameters. Liquid intake in both SHR and WKY rats on HS intake did not significantly differ from that in animals on LS or NS inake. By contrast, food intake was lower in both SHR and WKY rats on NS and HS intake (Table 1). Plasma levels of electrolytes (sodium, potassium and chloride), urea, creatinine and plasma osmolality were, however, similar in SHR and WKY rats either on LS, NS or HS intake (Table 1). The urine volume in SHR on LS and NS intake did not differ from that in WKY rats, and both groups of rats failed to respond with na increase in urine volume during HS intake (Table 1). The creatinine clearance was similar in SHR and WKY rats either on LS, NS or HS intake (Table 1). Urinary excretion of sodium and the fractional excretion of sodium in SHR on LS and NS intake did not differ from that in WKY rats (Table 1). As expected, urinary excretion of sodium and the fractional excretion of sodium during HS intake increased significantly in both SHR and WKY rats . Urinary excretion of potassium was similar in SHR and WKY rats either on LS, NS or HS intake. With the exception of DOPAC, the urinary excretion of L-DOPA, dopamine and amine metabolites (3-MT and HVA) and norepinephrine was similar in SHR and WKY rats (Table 2) either on LS, NS or HS intake. Similarly, tissues levels (in pmol/g) of L-DOPA (SHR: LS = 18.0±5.6, NS = 14.5±1.0, HS = 11.6±1.3; WKY: LS = 14.4±0.7, NS = 14.4±2.7, HS = 12.6±0.2), dopamine (SHR: LS = 52.8±4.6, NS = 56.0±5.3, HS = 51.7±3.9; WKY: LS = 50.3±4.2, NS = 63.1±6.1, HS = 46.5±2.5), DOPAC (SHR: LS = 13.6±0.7, NS =

18.1±1.1, HS = 18.9±2.1; WKY: LS = 15.5±2.6, NS = 15.8±4.7, HS = 9.3±0.1) and norepinephrine (SHR: LS = 625.6 ± 17.2, NS = 603.1 ± 51.8, HS = 619.7 ± 74.1; WKY: LS = 545.6±28.6, NS = 612.3±83.5, HS = 590.0±65.9) did not differ between SHR and WKY rats, either on LS, NS or HS intake.and WKY rats, either on LS, NS or HS intake.

DISCUSSION

At the intestinal level, previous studies have shown that the inhibitory effects of dopamine upon jejunal sodium absorption and Na^+-K^+ ATPase activity in rat jejunal epithelial cells are limited to animals under 20 days of age, adult animals being insensitive to the inhibitory effects of dopamine (23,24,32). Intestinal function has a great impact during early postnatal life not only for the uptake of nutrients but also in maintaining electrolytes and water metabolism (33,34). In fact, though nephrogenesis is completed at birth, renal tubular function continues to develop postnatally, and the kidney has a limited capacity to regulate fluids and electrolyte homeostasis (25).

The lack of effect of dopamine upon jejunal Na^+-K^+ ATPase activity in adult animals coincided with the period in which the renal function has reached maturation (23,24).

In 60-day old rats, the main effect of dopamine at the intestinal level of dopamine is mediated through α_2-adrenoceptors and leads to activation of Na^+-K^+-$2Cl^-$ co-transport (35). In 20-day old rats, dopamine is also able to stimulate Na^+-K^+-$2Cl^-$ co-transport via activation of α_2-adrenoceptors (EC_{50} = 7.0 μM dopamine), whereas inhibition of Na^+,K^+ ATPase activity results from activation of dopamine D_1 receptors (EC_{50} = 0.1 μM dopamine, in the presence of 0.2 μM phentolamine) (35).

The findings described here clearly show that inhibition of jejunal Na^+-K^+ ATPase activity by dopamine is dependent on salt intake in normotensives, and hypertensive animals failed to respond to dopamine, irrespective of their salt intake. Basal jejunal Na^+-K^+ ATPase activity in WKY rats on LS intake was also markedly lower than that in normotensive animals under NS or HS intake, this level of activity being similar to that observed in hypertensive rats. In WKY rats on LS intake, the effect of dopamine upon jejunal Na^+-K^+ ATPase activity is a D_1 dopamine receptor mediated event, as evidenced by the complete prevention by the selective D_1 receptor antagonist SKF 83566, but not the selective D_2 receptor antagonist S-sulpiride. Furthermore, the effect of dopamine was mimicked by the selective D_1 receptor agonist SKF 38393.

This is in full agreement with the evidence obtained in 20-day old rats (Wistar), in which the inhibitory effect of dopamine upon jejunal Na^+-K^+ ATPase activity was a D_1 receptor mediated event (36). The finding that basal jejunal Na^+-K^+ ATPase activity in SHR on LS intake was higher than in WKY rats, indicate that hypertensives might be endowed with enhanced absorptive capacities for sodium. This is in agreement with the findings reported by others on the increased Na^+/H^+ exchange in jejunal enterocytes from SHR (37).

Recently, it has been shown that changing from breast feeding to a normal rat chow produced a marked increase in jejunal Na^+-K^+ ATPase activity in 20-day old rats, this being accompanied by loss of sensitivity to the inhibitory effects of dopamine (38). This unresponsiveness to dopamine was not related to changes in D_1 receptor density, as

measured by means of [^{3}H]-Sch 23390 radioligand-binding studies (38).

TABLE 1

Body weight, metabolic balance and renal function in SHR and WKY rats on LS, NS or HS intake. Values are means ±S.E.M. (n=4).

	SHR		
	LS	**NS**	**HS**
Body weight (g)	333±6	320±7	322±15
Food intake (g)	15.0±0.9 *	7.0±1.7	8.3±3.4
Liquid intake (ml)	21.3±4.0	23.8±3.1	33.8±6.3
Plasma urea (mg/dl)	36.8±1.3	20.8±2.6	30.8±3.1
Plasma creatinine (mg/dl)	0.30±0.04	0.35±0.05	0.40±0.04
Plasma Na^+ (mmol/l)	150±1.5	149.0±3.7	149.8±4.6
Plasma K^+ (mmol/l)	4.8±0.4	5.5±0.3	5.4±0.3
Plasma Cl^- (mmol/l)	106.5±2.3	106.5±2.3	103.3±1.3
Plasma osmolality (mOsm/kg)	304.8±0.9	272.3±25.8	302.8±4.2
Urine volume	11.8±0.9	11.3±2.5	10.4±1.4
Creatinine clearance (ml/min)	1.97±0.14	1.76±0.26	1.40±0.11
Urinary Na^+ (mmol/kg/day)	0.35±0.03	0.35±0.07	9.07±1.38 *
Urinary K^+ (mmol/kg/day)	5.29±0.16	4.43±0.31	5.62±0.99
FE Na^+ (%)	0.028±0.003	0.030±0.005	1.017±0.213 *
FE K^+ (%)	13.3±1.1	11.3±2.6	17.7±4.0
	WKY		
	LS	**NS**	**HS**
Body weight (g)	314±8	302±7	309±10
Food intake (g)	12.3±0.9 *	8.0±1.4	7.8±1.3
Liquid intake (ml)	21.3±3.1	26.3±2.4	35.0±4.6
Plasma urea (mg/dl)	34.3±1.1	25.8±1.7	15.8±1.4
Plasma creatinine (mg/dl)	0.33±0.03	0.35±0.03	0.30±0.00
Plasma Na^+ (mmol/l)	151±1.2	151±2.3	146.8±2.5
Plasma K^+ (mmol/l)	4.8±0.2	5.2±0.3	4.4±0.2
Plasma Cl^- (mmol/l)	1046.0±0.8	104.3±2.1	101.5±1.8
Plasma osmolality (mOsm/kg)	334.3±23.6	354.3±49.7	312.8±10.5
Urine volume	11.0±1.2	13.5±3.1	14.9±2.9
Creatinine clearance (ml/min)	1.61±0.08	1.54±0.28	2.06±0.10
Urinary Na^+ (mmol/kg/day)	0.35±0.05	0.46±0.11	11.37±2.84 *
Urinary K^+ (mmol/kg/day)	4.74±0.40	4.74±1.00	7.49±0.75
FE Na^+ (%)	0.032±0.005	0.043±0.012	0.782±0.163 *
FE K^+ (%)	13.7±1.5	12.5±1.0	17.8±0.7

Significantly different from corresponding values in rats on NS intake (* $P<0.05$).

TABLE 2

Urinary excretion (nmol/kg body weight/day) of L-DOPA, dopamine, DOPAC, 3-MT, HVA and norepinephrine in SHR and WKY rats on LS, NS or HS intake. Values are means ±S.E.M. (n=4).

		SHR	
	LS	NS	HS
L-DOPA	0.5±0.2	0.5±0.3	0.2±0.1
Dopamine	26.8±8.4	30.5±9.6	22.4±3.7
DOPAC	39.3±9.3 *	26.3±4.4 *	19.1±5.3
3-MT	7.0±2.1	3.4±0.9	5.3±0.9
HVA	65.5±2.8	54.8±5.7	67.9±7.3
Norepinephrine	2.8±0.8	2.5±0.8	1.1±0.2
		WKY	
	LS	**NS**	**HS**
L-DOPA	0.2±0.0	0.3±0.2	n.d.
Dopamine	28.6±5.9	10.3±4.2	12.8±0.7
DOPAC	13.8±0.8	13.8±2.3	12.9±2.3
3-MT	3.1±1.2	3.1±0.3	2.9±0.7
HVA	59.5±12.6	40.1±8.6	52.4±6.7
Norepinephrine	1.7±0.2	1.2±0.3	1.2±0.1

- significantly different from corresponding values in WKY rats ($P<0.05$)

The results presented here suggest that unresponsiveness of jejunal Na^+-K^+ ATPase activity to dopamine in WKY rats on NS or HS intake may be related to increases in salt intake rather than food. In fact, food (protein) intake was significantly greater in rats on LS than in NS or HS intake. However, it is difficult to conceptualise a model in which increases in sodium delivery are accompanied by increases in jejunal Na^+-K^+ ATPase activity and loss of sensitivity to inhibition by dopamine.

This set of data suggests that the system apparently reacts to an increase in sodium delivery by increasing its capacities to absorb sodium. However, evidence has been provided that this intestinal dopaminergic system reacts appropriately when animals were submitted to uninephrectomy and salt load. In fact, uninephrectomy in adult rats was accompanied by a marked reduction in jejunal Na^+-K^+ ATPase activity and recuperation of the inhibitory effect by dopamine (39). Altogether, these results suggest that the type of food (eventually its sodium content) and systemic factors, such as those involved in keeping uninephrectomized rats within sodium balance, might influence jejunal Na^+-K^+ ATPase activity and its sensitivity to inhibition by dopamine. Within this context it is appropriate to stress on the presence, at the intestinal level, of a "natriuretic factor" that can sense the intake of salt and in some unknown way influence the kidneys to increase sodium excretion (40). Recent work suggests that this intestinal "natriuretic factor" may correspond to guanylin or uroguanylin (41,42). On the other hand, it should be underlined that the intestinal influence on renal sodium excretion is more pronounced in hypertensive than in normotensive rats (40,43). Considering

these two sets of data, it is stimulating to hypothesise on whether this enhanced activity of the intestinal "natriuretic factor" might correspond to a compensatory response to the failure to respond to dopamine, another natriuretic and sodium antiabsorptive factor, with inhibition of jejunal Na^+-K^+ ATPase activity.

The reason why SHR fail to respond to dopamine with inhibition of jejunal Na^+-K^+ ATPase activity is most probably a different one. Eventually, in these animals salt intake is not involved in the modulation of dopamine effects. In fact, it is quite likely that failure of dopamine to inhibit jejunal Na^+-K^+ ATPase activity may be due to the same deficient coupling of renal dopamine receptors to effector mechanisms (12-17). However, it is interesting to note that the effect of changing from LS to NS or HS intake was a significant increase in jejunal Na^+-K^+ ATPase activity, indicating that the enzyme in hypertensive animals is sensitive to salt as occurred in normotensive rats. This contrast with that observed at the kidney level, in which changing from LS to NS or HS intake increased renal Na^+-K^+ ATPase activity in WKY rats, but not in SHR. On one hand, this is in agreement with the evidence that SHR respond with enhanced natriuresis to salt load, in comparison with WKY rats; this is particularly true in the initial phase of development of hypertension (44). On the other hand, this is in contrast with that described by Aperia et al. (3), in which HS intake was accompanied by a decrease in renal Na^+-K^+ ATPase activity. Possible explanations for this apparent discrepancy are differences in strain of rats (Sprague-Dawley vs. WKY), duration of salt loading (7 days vs. 1 day) and age of animals (40- vs. 90-day old). Another important point concerns the role of endogenous dopamine in this adaptive response of renal Na^+-K^+ ATPase to salt loading. In fact, Aperia et al. (3) showed that the decrease in renal Na^+-K^+ ATPase activity could be reverted by benserazide, an inhibitor of AADC, suggesting that this response was mediated by endogenous dopamine. In the present study, we have found no evidence for an increase in renal dopamine production while changing from LS to NS or HS intake. This is agreement with that reported by other authors (26,28,45).

In conclusion, it is suggested that inhibition of jejunal Na^+-K^+ ATPase activity by dopamine is dependent on salt intake in WKY rats, and SHR failed to respond to dopamine, irrespective of their salt intake. In WKY rats on LS intake, the effect of dopamine upon jejunal Na^+-K^+ ATPase activity is a D_1 dopamine receptor mediated event. Increases in salt intake are accompanied by increases in jejunal Na^+-K^+ ATPase activity, of similar magnitude in both SHR and WKY rats.

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Intestinal dopaminergic activity in Obese and Lean zucker rats: Response to high salt intake[1]

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ABSTRACT The present study examined intestinal dopaminergic activity and its response to high salt (HS, 1% NaCl over a period of 24 hours) intake in obese (Ob) and lean (L) Zucker rats. Basal Na^+,K^+-ATPase activity (in nmol Pi/mg protein/min) in the jejunum of Ob rats on NS intake was higher than that observed in L rats under the same sodium regimen (ObNS = 111.3±6.0 vs LNS = 88.0±8.3). In both Ob and L rats basal sodium pump activity significantly increased with increases in salt intake (ObHS = 145.9±11.8; LHS= 108.8±6.7). SKF 38,393 (10 nM), a specific D_1–like dopamine receptor agonist, reduced jejunal Na^+,K^+-ATPase activity in Ob rats on HS intake, but failed to inhibit enzyme activity in Ob rats on NS intake and L rats on NS and HS intakes. Aromatic L-amino acid decarboxylase (AADC) activity in Ob rats was lower than that in L rats on NS intake. HS intake increased AADC activity in Ob rats, but not in L rats. During the NS intake jejunal monoamine oxidase (MAO) activity in Ob rats was similar to that in L rats. HS intake significantly decreased MAO activity in both Ob and L rats. Jejunal COMT in Ob rats on NS intake was higher than that in L rats under the same experimental conditions. HS intake reduced COMT activity in Ob rats only. It is concluded that inhibition of jejunal Na^+,K^+-ATPase activity through D_1 dopamine receptors is dependent on salt intake in Ob rats, whereas in L rats it failed to respond to the activation of D_1 dopamine receptors irrespective of their salt intake.

Key words: • *Sodium* • *dopamine* • *intestine* • *Na^+,K^+-ATPase*

Obesity is becoming a major health problem worldwide. Only in the USA more than half of the population is considered to be overweight (Wickelgren, 1998). Obesity is related to the development of a high number of other diseases, specifically the increased risk of heart disease (Alexander, 1985; Fletcher et al., 1986; Krieger and Landsberg, 1990; Landsberg, 1986), diabetes and hypertension (Berg, 1993).

Although the accepted relationship between obesity, diabetes and hypertension, the underlying mechanism is still not fully understood. Studies in diabetic patients have reported an unbalance in sodium homeostasis and an increase in sodium retention (O'Hare et al., 1986). This sodium retention is though to play a major role in the development of hypertension (Feldt-Rasmussen et al., 1987).

At the kidney level, local synthesized dopamine promotes natriuresis and diuresis via activation of D_1-like dopamine receptor present on the PCT cells (Jose et al., 1992; Lokhandwala and Amenta, 1991) and subsequent protein kinase C mediated

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inhibition of basolateral Na^+,K^+-ATPase (Felder et al., 1989; Kansra et al., 1995; Seri et al., 1990) and protein kinase A mediated inhibition of apical Na^+/H^+-exchanger (Felder et al., 1990). High blood pressure may be caused by an abnormal function in the renal dopaminergic system related to a decreased ability to synthesize dopamine (Kuchel and Shigetomi, 1992; Soares-da-Silva et al., 1995; Yoshimura et al., 1987) or to a deficient coupling of dopamine receptors to effector mechanisms (Chen et al., 1993; Felder et al., 1993; Hussain and Lokhandwala, 1997; Kansra et al., 1995; Kinoshita et al., 1989). Indeed, reduced dopamine-induced natriuresis and a defect on D_1-like dopamine receptor coupling with the intracellular transduction pathways have been reported in the PCT cells of hypertensive rats (for a review see (Hussain and Lokhandwala, 1998)). Such defects in renal dopamine receptor function contribute to sodium retention and development of hypertension.

At the intestinal level the dopamine synthesized in the epithelial cells, similarly to the kidney, is involved in the sodium balance by modulating the activity of the basolateral sodium pump via dopaminergic receptors. The similarities between the renal and intestinal autocrine/paracrine non-neuronal dopaminergic system described by certain authors, namely the efficient mechanisms for L-DOPA uptake, the presence of AADC that converts intracellular L-DOPA to dopamine, the efficient enzyme systems for the metabolic degradation of newly-formed dopamine, and the presence of specific dopamine receptors which activation leads to Na^+,K^+-ATPase inhibition (Vieira-Coelho et al., 1998) and transepithelial sodium flux modulation, is very important in sodium homeostasis. In both systems the final effect is concordant in respect to sodium, leading to decreased sodium absorption in the intestine and increased sodium excretion in the kidney (Rodriguez-Boulan and Nelson, 1989).

A high salt intake has been found to constitute an important stimulus for the production of dopamine in rat jejunal epithelial cells. Because the intestinal dopaminergic system appears to be specially activated when the kidney fails to provide an optimal control over sodium balance, and because obesity may also be related to changes in the nutrient absorption, it was decided to evaluate the status of the intestinal dopaminergic system in obese and lean rats during normal salt (NS) and high salt (HS) intake. The animal model used was the obese Zucker rat (Kurtz et al., 1989). These animals inherit obesity as an autossomal Mendelian recessive trait. When compared to the lean controls these animals are obese, hyperinsulinemic, hyperglycemic, diabetic (Boese et al., 1985; Kahn, 1978; Stern et al., 1972; York et al., 1972), hypertensive (Bray, 1977), and a show progressive glomerular sclerosis at the kidney level (Fiske et al., 1986) consistent with an impairment in renal function.

MATERIALS AND METHODS

Experimental protocol

Male obese and lean Zucker rats (Harlan Sprague-Dawley, Inc, Indianapolis, Ind) aged 11 to 12 weeks and weighing 450-500 g and 250-350 g, respectively, were selected after a 7 day period of stabilization. Animals were kept under controlled environmental conditions (12 h light/dark cycle and room temperature 22±2 °C); fluid intake and food consumption were monitored for 24 h under the two different salt intakes. All animals were fed *ad libitum* throughout the study with ordinary rat chow containing 0.4% sodium (Purina Mills, St. Louis, MO). Obese (n=12) and lean (n=12) rats were subdivided in 2 groups (6 in each group) according to their daily sodium

intake, i.e., normal salt (NS) and high salt (HS). Rats on NS intake received tap water, and their daily sodium intake averaged 0.5 mmol/100 g of body weight. Rats on a HS intake had 1.0 % (w/v) NaCl in their drinking water, and daily sodium intake averaged 5.0 mmol/100 g of body weight. All groups were maintained in metabolic cages for the duration of the study (24 h). The animals were housed in an AAALAC-accredited facility and the Institutional Animal Care and Use Committee approved all of the protocols.

After completion of this protocol, rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). Through an abdominal midline incision the jejunum was rapidly removed. Thereafter, the jejunum was cut longitudinally, rinsed free from blood and alimentary content and the mucosa isolated.

In vitro studies

In vitro studies included the assay of Na^+,K^+-ATPase activity and enzymes involved in the synthesis (AADC) and metabolism (MAO and COMT) of jejunal dopamine. For these studies, jejunal mucosa was obtained from the same 4 experimental groups of rats mentioned above.

Na^+,K^+-ATPase assay

Na^+,K^+-ATPase activity was measured by the method Quigley and Gotterer and (Quigley and Gotterer, 1969) adapted in our laboratory with slight modifications. Briefly, isolated renal and jejunal epithelial cells, obtained as previously described (Quigley and Gotterer, 1969), were pre-incubated for 20 min at 37°C. After the pre-incubation period the epithelial cells were permeabilized by rapid freezing in dry ice-acetone and thawing. The reaction mixture contained (in mM) 37.5 imidazole buffer, 75 NaCl, 5 KCl, 1 sodium EDTA, 5 $MgCl_2$, 6 NaN_3, 75 tris(hydroxymethyl)aminomethane(tris) hydrochloride and 100 µl cell suspension (100 µg protein). The reaction was initiated by the addition of 4 mM ATP (25 µl). For determination of ouabain-sensitive ATPase, NaCl and KCl were omitted, and ouabain (1 mM; 100 µl) or vehicle (water; 100 µl) were added to the assay. After incubation at 37°C for 15 min, the reaction was terminated by the addition of 50 µl of ice-cold trichloroacetic acid. Samples were centrifuged (1,500 x *g*), and liberated P_i (free phosphorus) in the supernatant was measured by spectrophotometry at 740 nm. Na^+,K^+-ATPase activity is expressed as nanomoles P_i per milligram protein per minute and determined as the difference between total and ouabain-insensitive ATPase. The protein content in cell suspension (approximately 2 mg/ml), as determined by the method described by Bradford (Bradford, 1976) with human serum albumin, as a standard was similar in all samples.

AADC Activity

AADC activity was determined in jejunal mucosa as previously described (Soares-Da-Silva et al., 1998). In brief, fragments of jejunal mucosa were homogenized, and aliquots of 500 µl of cell homogenate plus 400 µl of incubation medium were pre-incubated for 20 minutes. Thereafter, L-DOPA (100 to 10000 µM) was added to the medium for an additional 15 minutes; the final reaction volume was 1 ml. The incubation medium contained the following (in mM): NaH_2PO_4 0.35, Na_2HPO_4 0.15, $Na_2B_4O_7$ 0.11, and pyridoxal phosphate 0.2, pH 7.2; tolcapone (1 µM) and pargyline (100 µM) were also added to the medium to inhibit the enzymes COMT and MAO, respectively. The assay of dopamine was performed by high-performance liquid chromatography (HPLC) with electrochemical detection.

MAO Activity

MAO activity was determined in the jejunal mucosa as previously described (Fernandes and Soares-da-Silva, 1992). In brief, fragments of renal cortex and jejunal mucosa were homogenized, and aliquots of 500 µL of cell homogenate plus 400 µl of incubation medium were pre-incubated for 30 minutes. Thereafter, dopamine (1 to 500 µM) was added to the medium for an additional 20 minutes; the final reaction volume was 1 ml. The incubation medium contained the following (in mM): KH_2PO_4 67, Na_2HPO_4 67, pH 7.2; tolcapone (1 µM) was also added to the medium to inhibit the enzyme COMT. The assay of DOPAC was performed by HPLC with electrochemical detection.

COMT Activity

COMT activity was evaluated by the ability of tissue homogenates to methylate dopamine to 3-methoxytyramine (3-MT) (Vieira-Coelho and Soares-da-Silva, 1996). Aliquots of 100 µl of the homogenate were pre-incubated for 20 minutes with 100 µL of phosphate buffer containing (in mM): NaH_2PO_4 5, Na_2HPO_4 5, pH 7.8. The reaction mixture was incubated for 5 minutes with increasing concentrations of dopamine (50 to 5000 µM; 50 µl) in the presence of a saturating concentration of the methyl donor *S*-adenosyl-L-methionine (500 µM); the incubation medium also contained pargyline (100 µM), $MgCl_2$ (100 µM), and EGTA (1 mM). The assay of 3-MT was performed by HPLC with electrochemical detection.

Assay of Catecholamines

The assay of catecholamines and their metabolites in the jejunal mucosa was performed by HPLC with electrochemical detection. In brief, aliquots of 1 ml of perchloric acid, in which jejunal mucosa had been kept, were placed in 5-ml conical-base glass vials with 50 mg alumin, and the pH of the samples was adjusted to pH 8.6 by addition of Tris buffer. The adsorbed catecholamines were then eluted from the alumin with 200 µl of 0.2 M perchloric acid on Costar Spin-X microfilters; 50 µl of the eluate was injected into an HPLC (Gilson Medical Electronics, Villiers le Bel, France). Standard solutions and dihydroxybenzylamine (internal standard) were injected at different concentrations, and peak height increased linearly. The lower limit of detection of L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine (DA), norepinephrine (NE), epinephrine (AD), DOPAC, homovallinic acid (HVA), 5-hydroxytriptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) ranged from 350 to 1000 fmol.

Statistics

Results are expressed as mean±SEM of values for the indicated number of determinations. V_{max} and K_m values for the decarboxylation of L-DOPA, the *O*-methylation or deamination of dopamine were calculated from non-linear regression analysis by using the GraphPad Prism statistics software package (Motulsky, 1994). Geometric means are given with 95% confidence limits, and arithmetic means are given with SEM. Statistical analysis was performed by 1-way ANOVA followed by the Newman-Keuls post hoc test. A value of $P<0.05$ was assumed to denote a significant difference.

RESULTS

Na^+,K^+-ATPase Activity

Basal Na^+,K^+-ATPase activity (in nmol Pi/mg protein/min) in the jejunum of obese rats (111±6) was higher ($P<0.05$) than that observed in lean rats (88±8) under the same sodium regimen (NS intake). In both obese

and lean rats the increase in sodium intake was accompanied by significant increases in basal Na^+,K^+-ATPase activity (Fig. 1). As shown in Figure 2, SKF 38393 (10 nM), a selective D_1 dopamine receptor agonist, significantly reduced Na^+,K^+-ATPase activity in jejunal epithelial cells of obese rats on HS intake, but not in obese rats on NS intake and lean rats on either NS or HS intake. This concentration (10 nM) of SKF 38393 has been shown to exert maximal inhibition upon jejunal Na^+,K^+-ATPase activity (Vieira-Coelho and Soares-da-Silva, 2000).

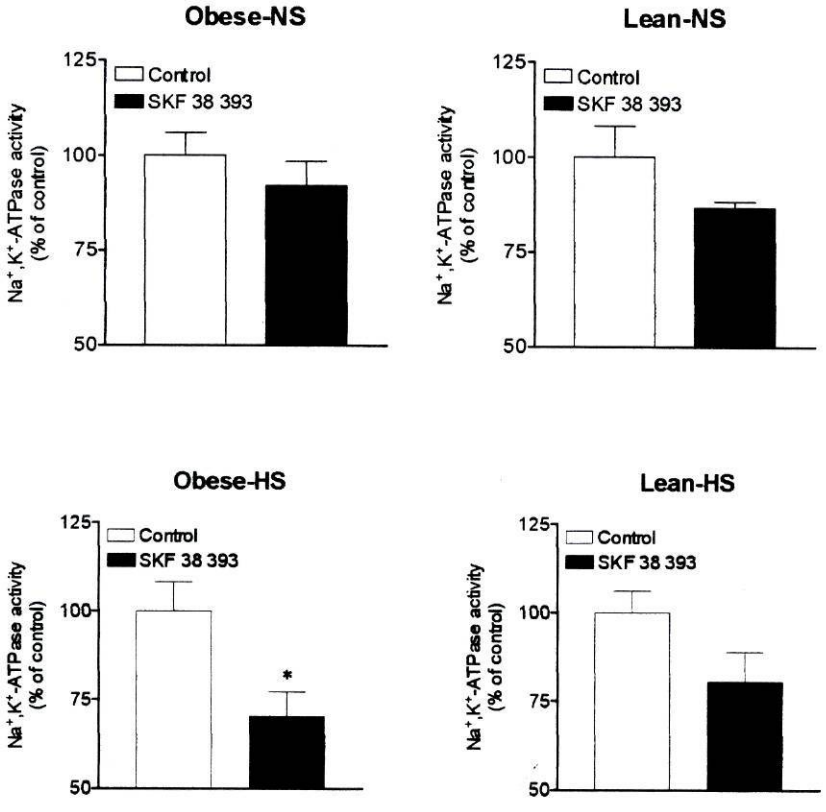


FIGURE. 2. Effect of SKF 38 393 (10 nM) on jejunal Na^+,K^+-ATPase activity from obese and lean rats on NS and HS intake. Columns represent means of four determinations per group and vertical lines show SEM. Significantly different from corresponding control values (* $P<0.05$).

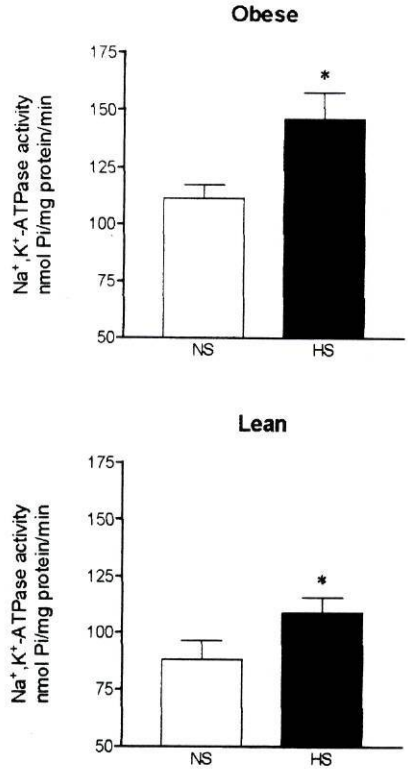


FIGURE. 1. Na^+,K^+-ATPase activity in isolated jejunal epithelial cells in (A) obese Zucker rats and (B) lean Zucker rats on NS and HS intake. Columns represent means of four determinations per group and vertical lines show SEM. Significantly different from corresponding values during NS intake (* $P<0.05$).

TABLE 1

Levels (pmol/g) of L-DOPA and dopamine in obese and lean rats on NS or HS intake. Values are means ±S.E.M. (n=4).

	Obese	
	NS	**HS**
L-DOPA	19.9±1.6	22.8±2.3
Dopamine	21.7±2.6	28.9±6.4
	Lean	
	NS	**HS**
L-DOPA	24.2±2.4	31.7±2.6
Dopamine	18.1±2.2	19.9±1.7

Tissue catecholamines

Data on tissue levels of catecholamines and metabolites in the jejunal mucosa are given in the Table 1. There were no significant changes in tissue levels of L-DOPA and DA between obese and lean rats on NS intake. Likewise changing from NS to HS intake failed to alter levels of L-DOPA and DA in both obese and lean rats.

AADC Activity

Incubation of homogenates of jejunal mucosa with L-DOPA (100 to 10,000 μM) resulted in a concentration-dependent but saturable formation of dopamine. V_{max} values for jejunal AADC in obese rats on NS intake were found to be significantly ($P<0.05$) lower than those observed in lean rats (Table 2). Changing from NS to HS intake failed to change AADC activity in lean rats, but resulted in marked in AADC activity in obese rats. In all experimental conditions, the decarboxylation reaction was a saturable process with K_m values of the same magnitude (Table 2).

MAO Activity

Incubation of homogenates of jejunal mucosa with dopamine (1 to 500 μM) resulted in a concentration-dependent formation of DOPAC. V_{max} values for jejunal MAO in obese rats on NS intake were similar to those in lean rats (Table 2). HS intake significantly ($P<0.05$) decreased the V_{max} values in both obese and lean rats (Table 2). In all experimental conditions, the deamination reaction was a saturable process, with K_m values of the same magnitude.

TABLE 2

Kinetic parameters (V_{max} and K_m) of AADC, COMT and MAO activity in homogenates of jejunal mucosa obtained from lean and obese rats on NS and HS intake for 24 h. **AADC** activity is expressed as the rate of formation of dopamine (μmol/mg protein/15 min). **MAO** activity is expressed as the rate of formation of DOPAC (pmol/mg protein/20 min). **COMT** activity is expressed as the rate of formation of 3-MT (pmol/mg protein/15 min). K_m values for AADC are expressed as mM and for MAO and COMT in μM. Symbols represent means of 4 experiments per group and vertical lines show SEM.

		Lean		Obese	
		NS	HS	NS	HS
AADC	V_{max}	132.7±3.3 #	169.5±6.1	60.5±5.5	265.2±32.9 *
	K_m	3.03±0.2	3.1±0.27	1.39±0.4	4.65±1.25
MAO	V_{max}	252.2±12.7	144.4±24.9 *	341.4±49.6	93.6±14.8 *
	K_m	12.7±9.2	51.7±26.2	126.4±40.4	78.2±30.4
COMT	V_{max}	845.0±61.7 #	729.0±35.3	1525.3±130.0	991.3±103.2 *
	K_m	510.3±136.2	408.4±77.3	607.6±179.1	824.2±269.6

* significantly different from corresponding values for NS rats ($P<0.05$)

\# significantly different from corresponding values for obese rats ($P<0.05$)

COMT Activity

Incubation of homogenates of jejunal mucosa with dopamine (50 to 5000 μM) resulted in a concentration-dependent formation of 3-MT. V_{max} values for jejunal COMT in obese rats on NS intake were significantly ($P<0.05$) higher than those observed for lean rats under the same experimental conditions (Table 2). HS intake decreased the V_{max} values in obese rats only (Table 2). In all experimental conditions, the O-methylation reaction was a saturable process, with K_m values of the same magnitude (Table 2).

DISCUSSION

Studies performed in other animal models have shown that dopamine of peripheral origin plays an important role in sodium homeostasis. At the intestinal level, the inhibitory effects of dopamine upon jejunal sodium absorption and Na^+,K^+-ATPase activity in rat jejunal epithelial cells are limited to animals under 20 days of age, adult animals being insensitive to the inhibitory effects of dopamine (Finkel et al., 1994; Lucas-Teixeira et al., 2000b; Vieira-Coelho et al., 1997; Vieira-Coelho and Soares-da-Silva, 2000; Vieira-Coelho et al., 1998). In response to HS intake, jejunal Na^+,K^+-ATPase in young rats remains sensitive to inhibition by dopamine, whereas in adult rats dopamine is devoid of inhibitory effect upon the sodium pump (Vieira-Coelho et al., 1998). This may assume particular importance, especially at a young age because the kidney has a limited capacity to regulate fluids and electrolyte homeostasis, though nephrogenesis is completed at birth (Robillard et al., 1992). The lack of effect of dopamine upon jejunal

Na^+,K^+-ATPase activity in adult animals coincides with the period in which the renal function reaches maturation (Finkel et al., 1994; Lucas-Teixeira et al., 2000a; Lucas-Teixeira et al., 2000b; Vieira-Coelho et al., 1998), suggesting that water and electrolyte homeostasis would be primarily maintained by the kidney and not the intestine.

The findings reported in the present study show that inhibition of jejunal Na^+,K^+-ATPase activity by dopamine is dependent on salt intake in obese Zucker rats while lean rats failed to respond to dopamine, regardless their salt intake. In obese rats, HS intake increased intestinal dopaminergic tonus, evidenced by an increase in AADC activity and enhanced sensitivity of jejunal Na^+,K^+-ATPase activity to inhibition by D_1–like dopamine receptor stimulation. This is in full agreement with the evidence obtained in young Wistar and young Sprague-Dawley rats, in which the inhibitory effect of dopamine upon jejunal Na^+,K^+-ATPase activity was found to be a D_1 receptor mediated event, but contrasts with that observed in adult animals either on NS or HS intake (Lucas-Teixeira et al., 2000b; Vieira-Coelho and Soares-da-Silva, 2000; Vieira-Coelho et al., 1998). Despite the increase in AADC activity in obese rats on HS intake, no significant changes in jejunal dopamine were observed. It is possible that the lack of correlation between the increase in AADC activity and dopamine tissue levels might be due to deficient availability of L-DOPA or reduced uptake into the jejunal epithelial cells. Reduced ability to take up L-DOPA into renal epithelial cells has been observed in other animal models (Armando et al., 1995).

The observation that HS intake increased AADC activity and the sodium pump became sensitive to inhibition by dopamine suggests that the system is somehow trying to respond to the increase in salt intake by decreasing the sodium absorption at the intestinal level. However, since no increase in dopamine levels were actually detected, these findings support the hypothesis that due to a deficiency in substrate utilization the jejunal dopaminergic system fails to exert effective control over sodium transporting mechanisms. This situation may, in conjunction with other factors, contribute to the increase in blood pressure observed when these animals are exposed to a continues HS intake (Carlson et al., 2000; Morgan et al., 1995; Reddy and Kotchen, 1992). In contrast to that observed in obese rats, HS intake in lean rats failed to enhance the sensitivity of Na^+,K^+-ATPase activity to inhibition by D_1–like receptor stimulation and AADC activity. This is in agreement with findings obtained in adult Wistar and Sprague-Dawley rats (Lucas-Teixeira et al., 2000b; Vieira-Coelho and Soares-da-Silva, 2000; Vieira-Coelho et al., 1998), in which all the electrolyte homeostasis is maintained by a fully functional renal system.

The findings described here also show that under NS intake obese Zucker rats have an increased jejunal Na^+,K^+-ATPase activity when compared to the lean controls, which may suggest that obese rats would be endowed with enhanced ability to take-up sodium. This is in agreement with previous studies reporting upregulation of the sodium pump in tissues involved in uptake and processing of nutrients (intestinal mucosa and liver) in the obese animals (Ferrer-Martinez et al., 1996). During NS intake, obese rats appear to have a decrease in dopaminergic tonus when compared to the lean rats. This is evidenced by the reduced AADC activity, the enzyme responsible for the synthesis of dopamine. Although dopamine in adulthood is known to have no inhibitory effect upon the jejunal Na^+,K^+-ATPase (Lucas-Teixeira et al., 2000b; Vieira-Coelho and Soares-da-Silva, 2000; Vieira-Coelho et al., 1998), it would interesting in future studies to address the

issue of whether inhibition of dopamine formation may affect the sodium pump activity. Despite differences in basal Na^+,K^+-ATPase activity, both obese and lean rats responded to HS intake with increases in sodium pump activity. This may suggest that the intestine in Zucker rats reacts to an increase in sodium delivery by increasing its capacities to absorb sodium. This increase in the sodium pump activity may actually occur in order to sustain the metabolic pressure associated with an induction of several Na^+- dependent transport system, as it was observed in the liver of obese rats (Ruiz-Montasell et al., 1994). Since, as mentioned previously, no change was observed in the dopamine levels despite the differences in AADC activity, this observation contrasts with that at the kidney level in which HS intake is usually accompanied by enhanced availability of dopamine and decreases in Na^+,K^+-ATPase activity (Bertorello et al., 1988; Seri et al., 1990; Vieira-Coelho et al., 1999). Though it is rather difficult to conceive a model in which HS intake is accompanied by enhanced ability to absorb sodium, it may make sense consider that sodium balance during adulthood is maintained in equilibrium through renal function. However, other studies have provided evidence that in conditions of reduced renal function the intestinal system reacts appropriately. In uninephrectomized rats there is a marked reduction in jejunal Na^+,K^+-ATPase activity accompanied by recovery of sensitivity to inhibition by dopamine (Vieira-Coelho et al., 2000). It is unlikely that recovered sensitivity to inhibition by dopamine results from changes in the basal activity of the enzyme. In fact, reductions in Na^+,K^+-ATPase activity in Wistar rats subjected to fasting periods of 48 h or prolonged (2 weeks) low salt intake was not accompanied by the recovery of dopamine inhibitory effect upon the sodium pump (Lucas-Teixeira et al., 2000a). It is likely that HS intake in obese Zucker rats could function as a stimulus for the jejunum to take an active part in maintaining the electrolyte homeostasis, since these animals have impaired renal function (Bray, 1977), jejunal Na^+,K^+-ATPase activity becoming sensitive to inhibition by dopamine. Actually, at the kidney level a recent study reported a decrease in D_1-like dopamine receptor binding sites and diminished activation of G-proteins in the proximal tubular basolateral membranes from obese Zucker (Hussain et al., 1999; Lokhandwala and Hussain, 2000). However, due to the reason mentioned earlier, this seems not to be the case at the intestinal level. The deficient coupling which leads to reduced inhibition by dopamine of Na^+,K^+-ATPase activity in proximal tubular cells and additionally the reduced capacity to synthesize dopamine in the jejunum, may contribute together to sodium retention and development of hypertension in obese Zucker rats.

In conclusion, it is suggested that inhibition of jejunal Na^+-K^+ ATPase activity through D_1–like dopamine receptors is dependent on salt intake in the obese Zucker rats and lean rats failed to respond to the activation of D_1 dopamine receptors irrespective of their salt intake.

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O assunto abordado nesta dissertação faz parte de uma tema mais geral que tem como objectivo estudar o mecanismo de regulação e acção dos sistemas monoaminérgicos periféricos. Os dados apresentados confirmam a existência de um sistema monoaminérgico intestinal altamente dinâmico, cujo efeito sobre o transporte epitelial pode ser modulado por variados factores intrínsecos e extrínsecos ao próprio organismo.

A nível renal sabe-se que a dopamina endógena ao ser libertada tem um efeito natriurético e diurético através da activação de receptores da dopamina do tipo D_1 (Jose et al, 1992; Lokhandwala and Amenta, 1991). O aumento da excreção de sódio pela dopamina é conseguido por inibição da ATPase-Na^+,K^+ basolateral (Seri et al, 1990) por activação da via de transdução de sinal que envolve a fosfolípase-C e a proteína cínase-C (Felder et al, 1989; Kansra et al, 1995), e ainda por inibição do trocador Na^+/H^+ apical por mecanismos de fosforilação mediados pela proteína cínase-A (Felder et al, 1990). Na fisiologia renal a disponibilidade em dopamina é, assim, de extrema importância e depende fundamentalmente da disponibilidade do percursor (L-DOPA), da sua rápida e eficiente descarboxilação em dopamina, da sua metabolização (Fernandes and Soares-da-Silva, 1990; Vieira-Coelho and Soares-da-Silva, 1996) e ainda dos mecanismos de libertação desta mesma amina (Soares-da-Silva, 1994). Como mencionado anteriormente a 5-HT tem também um papel muito importante a nível renal, uma vez que, tem um efeito anti-natriurético por estimulação da ATPase-Na^+,K^+ basolateral (Soares-da-Silva et al, 1996b; Soares-da-Silva e Pinto-do-Ó, 1996; Soares-da-Silva e Vieira-Coelho, 1998; Soares-da-Silva et al, 1996a). Todo este conhecimento sobre o sistema monoaminérgico renal, em particular o efeito da dopamina e 5-HT sobre a fisiologia renal, e da possibilidade que existe entre uma interacção entre este último e um sistema semelhante ao nível intestinal foi determinante para a tentativa de resposta das questões colocadas em cada um dos capítulos desta dissertação.

Existem muitas semelhanças entre ambos os sistemas monoaminérgicos, nomeadamente ao nível dos mecanismos de transporte de L-DOPA, das enzimas de síntese e metabolização, da presença de receptores específicos para estas aminas, e ainda das vias de transdução de sinal cujo alvo são inúmeros transportadores que regulam o fluxo transepitelial de iões (Na^+, Cl^-) e água e consequentemente a homeostasia hidroelectrolítica (Vieira-Coelho et al, 1997).

No capítulo I, os resultados apresentados permitem confirmar dados anteriores relativos à presença na mucosa jejunal de toda a maquinaria molecular necessária à existência de um sistema monoaminérgico intestinal, assim como confirmar a possibilidade de utilização de uma linha celular para o estudo *in vitro* das funções absorptiva e secretiva típicas do epitélio intestinal. A utilização de linhas celulares tem a vantagem de possibilitar a realização de inúmeros estudos numa população celular 100% homogénea, contrariamente ao que sucede com populações de células isoladas. Estudos de microscopia óptica e electrónica permitiram verificar, por exemplo, que apesar de a preparação de células isoladas de jejuno de rato ser constituída por mais de 99% de enterócitos, existe ainda uma pequeníssima percentagem de células neuroendócrinas. A linha celular utilizada neste primeiro trabalho e em paralelo com células isoladas de jejuno de rato foram as células Caco-2. Estas células têm origem num adenocarcinoma do cólon humano e têm a

capacidade de se diferenciarem em enterócitos quando em cultura (Pinto et al, 1983). Com base nestas características as células Caco-2 têm sido utilizadas como um modelo *in vitro* para estudos do transporte epitelial semelhantes aos que ocorrem ao nível do intestino delgado (Hidalgo et al, 1989; Grasset et al, 1984). Este trabalho permitiu confirmar nestas células a existência de processos celulares de síntese e metabolismo de monoaminas semelhante ao que se passa no próprio epitélio intestinal, de forma semelhante ao publicado anteriormente (Vieira-Coelho e Soares-da-Silva, 1998). Neste primeiro trabalho foi observado que estas células possuem um mecanismo de transporte de L-DOPA saturável, estereoselectivo e dependente da temperatura, e que a descarboxilação de L-DOPA em dopamina é também um processo dependente do tempo, da concentração e rapidamente saturável. Foi ainda observado que a dopamina recentemente formada também saí do compartimento celular por um processo saturável. Além da capacidade de captação do percursor de L-DOPA, também o percursor da 5-HT, a L-5-hidroxitriptofano (L-5-HTP), é captado por estas células, possibilitando assim síntese não apenas de dopamina, mas também de 5-HT. Os dados obtidos nas células isoladas do jejuno do rato de igual modo confirmam a existência dos mecanismos de transporte e formação de monoaminas, que englobam um sistema eficiente de captação de L-DOPA e L-5-HTP, e actividade considerável da enzima AADC. Relativamente aos parâmetros cinéticos foi possível observar que as enzimas AADC, COMT, MAO-A e MAO-B apresentaram afinidades (K_m) semelhantes para os respectivos substratos nos dois modelos celulares. No entanto, a velocidade máxima (V_{max}) para as enzimas AADC, MAO-A e MAO-B foi mais elevada nas células epiteliais isoladas do jejuno de rato do que nas Caco-2. No que diz respeito à COMT, nenhuma actividade foi detectada nas células Caco-2, contrariamente ao que se observa nas células isoladas de jejuno que possuem uma elevada actividade desta enzima. Apesar da semelhança entre a afinidade das enzimas para os seus substratos entre as células Caco-2 e as células da mucosa jejunal, é interessante verificar que existem diferenças em cada um destes modelos entre os valores de K_m da AADC para a L-DOPA e L-5-HTP. Esta enzima descarboxila preferencialmente a L-DOPA. Esta preferência deve-se muito provavelmente ao arranjo dos anéis aromáticos existentes na estrutura desta amina. Resultados semelhantes foram observados noutros tecidos (Shirota & Tomchick, 1988; Soares-da-Silva & Pinto-do-Ó, 1996). A razão pela qual existem diferenças nos parâmetros cinéticos entre os tipos celulares estudados deve depender de inúmeros factores que podem englobar: 1) alterações fenotípicas que as células sofrem em cultura, 2) alterações na actividade de variadas enzimas, nomeadamente das enzimas responsáveis pela síntese e metabolização da DA e 5-HT, ao longo de todo o processo de diferenciação, 3) origem tumoral das células Caco-2 que pode justificar, por exemplo, a perda de actividade de algumas enzimas, presentes em condições fisiológicas normais e 4) população celular mais homogénea nas células Caco-2.

Este estudo permitiu ainda identificar os mecanismos de transporte de fluídos presentes quer nas células Caco-2 quer nas células da mucosa jejunal, e que se confirmam pela possibilidade de medição de alguns parâmetros electrofisiológicos, tais como resistência transepitelial, diferença de potencial, corrente de curto-circuito basal (I_{sc}) que reflecte o movimento electrogénico de iões no epitélio, e ainda pela

sensibilidade destes parâmetros à presença de inibidores de alguns sistemas de transporte como a ubaína, que é um inibidor da ATPase-Na^+,K^+. De facto, em ambos os modelos celulares foi confirmada a presença da ATPase-Na^+,K^+ por este processo. É interessante verificar que as células Caco-2 apesar de possuírem uma actividade considerável desta bomba, os seus níveis são semelhantes aos medidos para as células da mucosa jejunal isoladas de ratos jovens e inferiores aos medidas nas células isoladas de ratos adultos, o que pode estar relacionado com um perfil funcional imaturo destas células com 7 a 10 dias de cultura, tal como foram utilizadas.

Este trabalho, além de confirmar a presença da maquinaria enzimática necessária para a formação e metabolismo de monoaminas e transporte de electrólitos ao nível da mucosa jejunal permitiu demonstrar a existência de um ambiente celular propício à actividade de um sistema monoaminérgico periférico semelhante ao encontrado ao nível renal e intestinal nas células Caco-2.

Estando comprovada a existência de um sistema monoaminérgico intestinal, outras questões foram colocadas no capítulo 2, nomeadamente ao nível da regulação e factores que modulam a actividade deste sistema. Tal como foi referido na introdução sabe-se que inúmeros factores intrínsecos e extrínsecos ao organismo modulam o transporte hidroelectrolítico. Como alvo desta regulação encontram-se os mecanismos celulares de que dependem todos os tipos de transporte transepitelial. Estudos anteriores demonstraram que factores como a ontogenia e o conteúdo em sal determinam a resposta da mucosa jejunal ao sistema monoaminérgico intestinal (Finkel et al, 1994). Neste trabalho foi observado que apenas ratos jovens respondem a uma sobrecarga salina com uma diminuição da absorção de sódio e aumento dos níveis de dopamina. Fenómeno este não observado nos animais adultos. Estes dados sugeriram de imediato o envolvimento da dopamina endógena na regulação do transporte de sódio ao nível intestinal. Estando o movimento vectorial de iões através do epitélio intestinal, em particular o transporte de sódio directamente dependente da actividade da ATPase-Na^+,K^+, uma das questões levantadas coloca a hipótese do efeito da dopamina ocorrer através da modulação da actividade bomba de sódio. Com o objectivo de estudar esta hipótese fomos, então, determinar a actividade da ATPase-Na^+,K^+ em células isoladas de jejuno de ratos da estirpe Sprague-Dawley adultos (40 dias) e jovens (20 dias) sujeitos a uma dieta normo ou hiperssalina durante, respectivamente, 7 e 4 dias. Foi verificado que numa situação controlo, ou seja, com uma dieta normossalina as células isoladas do jejuno de ratos adultos além de serem dotados de uma maior actividade da ATPase-Na^+,K^+ que as células isoladas de ratos jovens apenas nestes se observa uma diminuição da actividade basal acompanhado por um aumento dos níveis de dopamina e diminuição de 5-HT na mucosa jejunal após a ingestão de uma dieta hiperssalina. Este efeito resultante do aumento de aporte de sódio nos ratos de 20 dias relativamente à dopamina deixou de se observar quando estes animais foram tratados 1 h antes da experiência com benserazida, um inibidor da enzima AADC e consequentemente da síntese de dopamina.

A ATPase-Na^+,K^+, é formada por duas subunidades, α e β, sendo a subunidade α a que catalisa a hidrólise do ATP e possui os locais de ligação para os iões sódio e potássio. Cada subunidade possui ainda 3 isoformas, sendo o tipo de isoforma presente na membrana plasmática específica para

cada tipo celular. Utilizando técnicas de biologia molecular fomos verificar quais as isoformas da subunidade α típicas do epitélio jejunal e adicionalmente confirmar os resultados obtidos por métodos bioquímicos relativos à actividade da ATPase-Na^+,K^+. A análise dos "Western-blot" demonstram que apenas a isoforma α_1 é expressa ao nível da membrana plasmática. Foi interessante verificar ainda que existe uma diminuição de cerca de 30% na abundância desta isoforma nos animais jovens submetidos a uma dieta hiperssalina, o que está de acordo com os dados obtidos para a actividade da ATPase-Na^+,K^+ determinada através da hidrólise do [^{32}P]-ATP. Com a finalidade de associar o efeito da dopamina sobre o transporte intestinal com a bomba de sódio, foi determinado o efeito da dopamina exógena sobre a actividade da ATPase-Na^+,K^+ jejunal. A incubação das células isoladas da mucosa jejunal com dopamina induziu apenas nos ratos jovens uma diminuição da actividade dependente da concentração com um IC_{50} de aproximadamente 100 nM, sendo a ATPase-Na^+,K^+ das células isoladas de ratos adultos insensíveis a esta amina. Foi interessante ainda observar que a inibição induzida pela dopamina (1 μM) foi revertida pela presença de 5-HT (10 μM).

Estes dados apoiam a hipótese mencionada anteriormente que defende uma modulação do transporte epitelial pela dopamina e a 5-HT a nível celular através da regulação da actividade da ATPase-Na^+,K^+ em animais jovens submetidos a uma dieta hiperssalina. A falta de resposta da ATPase-Na^+,K^+ jejunal nos animais adultos ao aumento do aporte de sódio e mesmo durante uma dieta normossalina coincide com a altura em que os rins adquirem a total capacidade de manutenção da homeostasia hidroelectrolítica, uma vez que, atingem a maturidade (Robillard et al, 1992a; Robillard et al, 1992b). Relativamente à actividade da bomba de sódio parece que a diferença dos níveis basais entre os ratos jovens e adultos pode estar relacionada com diferenças no tónus dopaminérgico, já que os ratos adultos submetidos a uma dieta com um teor normal de sal apresentam níveis mais baixos quer de L-DOPA quer de DA. Em relação à 5-HT, a amina mais abundante do epitélio intestinal e um conhecido secretagogo, e uma vez que ao nível dos túbulos renais a 5-HT estimula a actividade da ATPase-Na^+,K^+ (Soares-da-Silva et al, 1995; Soares-da-Silva et al, 1996), foi colocada a hipótese de que a diminuição dos níveis desta amina após a ingestão de uma dieta rica em sódio fosse responsável pela diminuição da actividade da ATPase-Na^+,K^+ observada nos ratos jovens. O facto de contrariamente ao que aconteceu com a dopamina os níveis de 5-HT não terem sido modificados com o tratamento pela benserazida, não impede, no entanto, esta relação, mas apenas sugere que a 5-HT se poderá encontrar armazenada num compartimento de difícil manipulação insensível alterações por um tratamento agudo (Erspamer, 1954). É possível assim concluir que o efeito da dopamina sobre o transporte intestinal que tem como mecanismo celular alvo a ATPase-Na^+,K^+ está dependente de factores como a idade dos animais e o conteúdo em sal da dieta. Os dados sugerem ainda uma possível complementaridade funcional entre o rim e o intestino, uma vez que, a inibição da absorção de sódio durante a ingestão de uma dieta rica neste sal só está presente nos ratos numa idade em que os rins por serem imaturos não conseguem desempenhar com total eficiência todos os processos que contribuem para homeostasia hidroelectrolítica do organismo.

Tendo sido demonstrado que o desenvolvimento ontogénico tem um papel

preponderante na sensibilidade da ATPase-Na^+,K^+ à dopamina, nos níveis desta amina, bem como na actividade desta bomba durante uma curta exposição a uma dieta rica em sal, decidimos investigar se de facto é possível alterar, por um lado, o efeito da dopamina sobre a ATPase-Na^+,K^+ e, por outro, os mecanismos envolvidos no transporte de água e electrólitos em células isoladas da mucosa jejunal, modificando o período de exposição a diferentes dietas salinas. Para responder a esta questão fomos avaliar determinados parâmetros electrofisiológicos que traduzem o estado do transporte transepitelial (corrente de curto circuito, I_{sc}), os níveis de actividade da ATPase-Na^+,K^+ e de monoaminas em células isoladas da mucosa jejunal. Estas avaliações foram realizadas em ratos adultos (60 dias de idade) da estirpe Wistar os quais foram divididos em dois grupos. Num grupo foi testado o efeito da ingestão crónica de uma dieta hipo, normo ou hiperssalina, e no outro grupo o efeito da ingestão durante 24 h destas dietas após um período de jejum de 72 h que teve como finalidade minorar os efeitos da dieta normal a que os animais estavam sujeitos.

Os resultados obtidos demonstram a importância não apenas do conteúdo em sal, mas também do tempo de exposição às diferentes dietas salinas no transporte epitelial e nos níveis de dopamina e de L-DOPA na mucosa jejunal. Assim, a ingestão de um dieta hipossalina durante 2 semanas aumentou os níveis e a taxa de síntese de dopamina na mucosa jejunal (dada pela razão dopamina/L-DOPA). Este aumento de dopamina foi acompanhado por uma diminuição na actividade da ATPase-Na^+,K^+ e da corrente de curto circuito basal, indicando uma diminuição no transporte intestinal. Sendo, a ATPase-Na^+,K^+ de ratos adultos insensível à inibição pela dopamina, estes dados sugerem que a relação entre o aumento do tónus dopaminérgico e a diminuição da actividade da bomba de sódio deverá ocorrer por um outro mecanismo celular que surge a longo prazo, e que difere do observado nos ratos jovens, onde um aumento do aporte de sódio aumenta os níveis de dopamina com consequente diminuição da actividade da ATPase-Na^+,K^+. Este tipo de resposta é fundamental pois evita um excesso da absorção de sódio numa idade em que o rim não está completamente funcional. A análise dos mecanismos celulares de transporte envolvidos através da medição da corrente de curto-circuito na presença de ubaína (inibidor da ATPase-Na^+,K^+) ou furosemida (inibidor do co-transportador $Na^+,K^+,2Cl^-$) complementaram os resultados obtidos respeitantes à bomba de sódio, na medida em que, nos ratos sujeitos a 2 semanas de dieta hipossalina, a diminuição da actividade da ATPase-Na^+,K^+ não foi acompanhada, como se esperava, por uma alteração na corrente de curto-circuito na presença de ubaína. Um outro dado interessante refere-se à importância que a presença de alimentos no tracto gastrointestinal exerce sobre todo este sistema. De facto, apesar da actividade da ATPase-Na^+,K^+ nos 3 grupos de animais sujeitos a 72 h de jejum ser semelhante entre si, observou-se uma significativa diminuição desta actividade e nos níveis de dopamina e L-DOPA quando comparada com os animais mantidos durante 2 semanas nas diferentes dietas salinas, dados estes que estão de acordo com outros anteriormente publicados (Murray & Wild, 1980). Em resumo, podemos referir que em animais adultos apesar da exposição prolongada a dietas com diferentes teores em sal alterar os níveis de dopamina, a actividade da ATPase-Na^+,K^+ e o transporte de electrólitos, a bomba de sódio permanece insensível à inibição pela dopamina.

Na terceira parte deste capítulo fomos avaliar de seguida se, contrariamente ao que acontece com os ratos adultos cuja ATPase-Na^+,K^+ jejunal permanece insensível à dopamina, nos ratos jovens (20 dias) a sensibilidade a esta amina pode, de facto, ser alterada ou é uma adaptação característica de um determinado período do desenvolvimento ontogénico não sendo, por isso, modulável por alterações na dieta ou do regime alimentar. Para tal, fomos determinar a actividade da ATPase-Na^+,K^+ e os níveis de monoaminas na mucosa jejunal em dois grupos de ratos jovens da estirpe Wistar. Um grupo foi mantido com a progenitora alimentando-se de leite materno e o outro foi submetido a uma dieta sólida semelhante à ingerida por animais adultos durante 48 h. Os resultados demonstram, de facto, que o efeito inibitório da dopamina sobre a actividade da ATPase-Na^+,K^+ jejunal de ratos jovens é uma característica que depende da dieta e não exclusivamente da idade, na medida em que a mudança de uma dieta em leite materno para uma dieta sólida é acompanhado não apenas por uma perda de sensibilidade da ATPase-Na^+,K^+ à dopamina, mas também por um aumento dos níveis basais desta bomba iónica e por uma diminuição dos níveis de dopamina. O efeito inibitório da dopamina (1 μM) sobre a ATPase-Na^+,K^+ de células isoladas do jejuno de ratos jovens a amamentarem foi mediado via receptor dopaminérgico do tipo D_1. O envolvimento deste tipo de receptor foi apoiado pela observação de que pode ser completamente revertido na presença de um antagonista específico D_1 (SKF 83 566) e não D_2 (S-sulpiride) e mimetizado por um agonista específico D_1 (SKF 38 393). Foi interessante verificar que a perda de sensibilidade ocorre simultaneamente com um aumento dos níveis basais da bomba de sódio. Se considerarmos que os ratos adultos possuem comparativamente com os ratos jovens uma actividade basal mais elevada, poderíamos ser levados a concluir que a razão pela qual a ATPase-Na^+,K^+ isolada de ratos adultos não é sensível depende exclusivamente dos níveis basais de actividade. No entanto, se considerarmos resultados anteriormente publicados (Lucas-Teixeira et al, 1999) onde foi demonstrado que apesar das células isoladas da mucosa jejunal de ratos adultos sujeitos a um período de jejum de 48 h sofrerem uma diminuição da actividade basal da ATPase-Na^+,K^+, esta permanece insensível à dopamina.

Com a finalidade de determinar se a diferença observada entre os animais adultos e os animais jovens, assim como entre os animais jovens submetidos às diferentes dietas, poderia ser causada por uma diferença na densidade dos receptores da dopamina, foi determinada a abundância dos locais de ligação para o receptor D_1 ao nível da membrana plasmática das células de jejuno isoladas de rato nos vários protocolos experimentais. Nenhuma alteração foi, no entanto, detectada. De facto, existem inúmeros outros factores que podem estar na base das diferenças observadas entre os animais adultos e jovens e sujeitos a diferentes dietas, nomeadamente o conteúdo em sal e em proteínas das dietas que é bastante diferente entre o leite materno e a dieta sólida. Considerando os resultados apresentados na primeira parte deste capítulo referentes a uma diminuição da actividade da ATPase-Na^+,K^+ com uma dieta hiperssalina sem perda da sensibilidade à dopamina, é possível sugerir que a dieta sólida deverá conter alguma outra substância além do sódio responsável simultaneamente pela ausência de efeito da dopamina assim como pela alteração da disponibilidade da dopamina endógena. A sensibilidade à dopamina e a actividade

basal da ATPase-Na^+,K^+ está assim dependente da composição da dieta.

Resumindo os dados obtidos para os efeitos mediados pela dopamina ao nível do transporte intestinal neste capítulo, é possível concluir que de facto o sistema dopaminérgico é modulado por inúmeros factores externos ou inerentes ao próprio organismo nomeadamente a idade, a composição e tempo de exposição às dietas assim como o tipo de regime alimentar. Neste capítulo foi ainda possível confirmar que o mecanismo de transporte celular alvo da dopamina é a ATPase-Na^+,K^+.

Os efeitos da dopamina ao nível da mucosa intestinal podem ser de dois tipos: pró-absorptivo ou anti-secretivo mediado por adrenoceptores α e β e anti-absorptivo mediado por receptores da dopamina. Assim, por exemplo, em íleo de coelho e íleo e cólon de rato foi observado que a dopamina estimula a absorção de sódio e cloro (Donowitz et al, 1982) e a absorção de fluídos, respectivamente, através de receptores da dopamina e α-adrenoceptores. Efeitos anti-secretivos e pró-absorptivos, mediados pelos dois tipos de receptores referidos acima, foram também observados no jejuno de rato (Wahawisan et al, 1997). Um estudo mais recente veio ainda demonstrar que os efeitos anti-secretivos e pró-absorptivos da dopamina exógena resultam da activação de adrenoceptores do tipo α_2 (Vieira-Coelho and Soares-da-Silva, 1998). Neste trabalho foi demonstrado que a dopamina libertada pelas células da mucosa jejunal tem um efeito anti-secretivo ou pró-absorptivo que se manifesta por uma diminuição da I_{sc} em ratos adultos e jovens. Esta redução observada para elevadas concentrações de dopamina (acima do 1 μM) é dependente da activação de adrenoceptores α, uma vez que, a presença de fentolamina deslocou para a direita a curva concentração-resposta com um consequente aumento nos valores de EC_{50}. O envolvimento de adrenoceptores α_2 e não de adrenoceptores do tipo α_1 e β, foi apoiada pela observação de que nem o propanolol nem a prazosina, respectivamente, tiveram a capacidade de deslocar curva à dopamina, e adicionalmente pela observação de que apenas o agonista específico para receptores do tipo α_2, o UK 14,304, mimetizou o efeito da dopamina sobre a I_{sc}. Este trabalho permitiu ainda determinar que o mecanismo iónico envolvido após activação dos receptores α_2-adrenérgicos foi o NKCC, na medida em que dos vários inibidores utilizados para diferentes transportadores (amilorido - canais de Na^+, 5-N-etil-N-isopropil-amilorido - trocador Na^+/H^+, ubaína - ATPase-Na^+,K^+, furosemida - co-transportador Na^+,K^+,$2Cl^-$), apenas a furosemida (1 mM) foi eficaz a antagonizar o efeito induzido pela dopamina sobre a I_{sc}, resultados estes que estão de acordo com outros autores (Liu & Coupar,1997). Foi interessante verificar ainda que apenas nos ratos jovens a presença de fentolamina revelou um efeito bifásico da curva dose-resposta à dopamina. Para baixas concentrações desta amina (inferior a 1μM) o efeito foi anti-absorptivo e mediado pela activação de receptores dopaminérgicos do tipo D_1.

Tendo por base estes resultados fomos avaliar no capítulo 3 desta dissertação qual a influência de uma alteração do regime alimentar nos fluxos iónicos (I_{sc}) através do epitélio jejunal em ratos adultos e jovens da estirpe Wistar durante uma estimulação dos adrenoceptores α_2. Os resultados obtidos neste trabalho confirmam a observação anterior de que a activação de adrenoceptores α_2 pelo UK 14,304 exerce um efeito no transporte iónico ao nível do jejuno de rato através da modulação das correntes geradas pelo co-transportador

$Na^+,K^+,2Cl^-$ (NKCC). Foi observado também que a ausência de alimentos no tracto gastrointestinal altera a diminuição dependente da concentração induzida pelo UK 14,304 sobre a I_{sc}. Tal como acontece para o efeito da dopamina sobre a ATPase-Na^+,K^+, também o efeito do UK 14,304 sobre a I_{sc} é diferente entre os ratos adultos e jovens, e pode ser alterado nestes últimos pelo tipo de regime alimentar. De facto, os resultados demonstram que nos ratos de 60 dias a resposta (diminuição da I_{sc}) resultante da estimulação de adrenoceptores α_2 de localização basolateral pelo UK 14,304 não foi alterada após um período de 48 h de jejum, tendo as curvas de dose-resposta para o UK 14,304 nos ratos adultos controlo e submetidos a jejum valores semelhantes de EC_{50} e E_{max}. Pelo contrário, nos ratos de 20 dias verificou-se uma potenciação do efeito máximo (E_{max}) induzido pelo UK 14,304 após um período de 24 h de jejum quando comparados com os animais controlo. Este resultado poderia ser explicado ou por um aumento da afinidade do receptor para o agonista ou por um aumento do número receptores. A primeira hipótese foi de imediato excluída, uma vez que não ocorreu nenhuma alteração nos valores de EC_{50}. A segunda hipótese e de acordo com os dados obtidos neste trabalho é a mais viável, uma vez que, os resultados com radioligandos mostraram claramente um aumento do número de locais de ligação para os adrenoceptores α_2. Foi interessante verificar ainda que no jejuno de animais adultos, apesar das diferenças observadas na corrente de curto-circuito basal e na resistência do tecido entre os animais controlo e os submetidos a 48 h de jejum, estes responderam do mesmo modo à estimulação dos adrenoceptores α_2. Com o objectivo de determinar as vias iónicas envolvidas na resposta do UK 14,304, o seu efeito na I_{sc} foi avaliado na presença de furosemida (1 mM), um inibidor do NKCC. Nos ratos adultos em regime alimentar normal e em jejum a furosemida apenas parcialmente reverteu o efeito do UK 14,304 o que sugere a existência de um outro componente na resposta deste agonista α_2. Nos animais jovens observou-se, no entanto, uma completa reversão do efeito do UK 14,304 na presença da furosemida nos ratos controlo (regime alimentar sem restrições) e apenas parcial no jejuno de ratos submetidos a 24 h de restrição alimentar. Este resultado é consistente com a hipótese de que o estado de jejum leva a um aumento da actividade do co-transportador NKCC. De facto, esta hipótese é apoiada pelas observação de que nos ratos jovens a actividade da ATPase-Na^+,K^+ não é modificado por apenas 24 h de jejum nem modulável pelo UK 14,304.

Em resumo, é possível afirmar que tal como acontece para os efeitos da dopamina, o efeito que a activação de adrenoceptores, em particular do tipo α_2, exerce no transporte iónico depende não apenas da idade, mas de outros factores tal como o regime alimentar. No entanto, ao contrário do que acontece para a dopamina, o mecanismo celular na base desta modulação não é a ATPase-Na^+,K^+, mas o co-transportador NKCC.

Após avaliados os factores que modulam o efeito da activação de receptores da dopamina e adrenoceptores ao nível do transporte intestinal, fomos de seguida avaliar no capítulo 4 as acções da 5-HT. A 5-HT, tal como a dopamina, pode ser localmente sintetizada e libertada (Soares-da-Silva e Pinto-do-Ó, 1996; Sole et al, 1986; Stier et al, 1984), assim como actuar através de receptores específicos nas próprias células do epitélio. Resultados anteriores quer ao nível renal (Soares-da-Silva, 1996a, b), quer ao nível intestinal (ver primeira parte do capítulo 2) demonstram que a 5-HT tem a capacidade de modular a

actividade da ATPase-Na^+,K^+. De facto, foi verificado que a 5-HT tem um efeito contrário à dopamina relativamente ao transporte de sódio, uma vez que reverte o efeito inibitório da dopamina por estimulação da actividade da ATPase-Na^+,K^+. Tendo este sistema 5-hidroxitriptaminérgico características tão semelhantes às que se observam com a dopamina, decidimos neste capítulo averiguar o efeito da 5-HT na actividade da ATPase-Na^+,K^+ jejunal em ratos jovens e adultos da estirpe Wistar e determinar o efeito do jejum e de um regime normal após um período de restrição alimentar sobre o transporte de sódio, assim como os níveis de 5-HT e seus metabolitos nestas condições experimentais. Foi interessante observar que tal como acontece para a dopamina, apenas a ATPase-Na^+,K^+ jejunal de células isoladas de ratos jovens é sensível a uma estimulação pela 5-HT, estando este efeito dependente das condições experimentais, uma vez que, nos animais que foram submetidos primeiro a um período de jejum seguido por um regime alimentar normal este efeito desapareceu. Esta perda de sensibilidade à 5-HT foi acompanhada por uma aumento da actividade basal da ATPase-Na^+,K^+. Várias questões se colocam com estes dados. Quanto à questão sobre a sensibilidade à 5-HT ser um factor dependente da idade, pelo exposta acima podemos concluir que não existe nenhuma relação de dependência, uma vez, que o regime alimentar claramente alterou esta situação nos ratos de 20 dias. Quanto à questão da sensibilidade a esta amina depender apenas dos níveis de actividade da bomba de sódio, a resposta é semelhante à dada anteriormente para a dopamina, que se relaciona com o facto de nos animais adultos uma diminuição da actividade da ATPase-Na^+,K^+ após 48 h de jejum não ter sido acompanhada por um reaparecimento da sensibilidade nem à dopamina nem à 5-HT. Adicionalmente, se considerarmos que nos ratos jovens o aumento da actividade da bomba de sódio surge quando estes passam a consumir alimentos sólidos, tal como os ratos adultos, poderíamos especular que a perda da sensibilidade deverá estar relacionada com algum componente presente nesta dieta tal como foi sugerido para a dopamina. De facto, a composição do leite materno e da dieta sólida é completamente diferente. Vários destes componentes têm sido descritos como serem importante reguladores da actividade da bomba de sódio, nomeadamente o conteúdo em sal e proteínas. Se pensarmos nos resultados apresentados no capítulo 2 relativos a uma diminuição da actividade da ATPase-Na^+,K^+ em ratos jovens submetidos a uma dieta hiperssalina, somos levados a sugerir que outros componentes deverão ser assim os responsáveis por determinar a sensibilidade da bomba de sódio não apenas à dopamina, mas também à 5-HT, como mencionado no capítulo anterior. Em resumo, podemos referir que mais uma vez em ratos jovens os regime alimentar e o tipo de dieta são fundamentais na modulação da resposta de sistemas monoaminérgicos sobre o transporte epitelial.

De tudo o que tem sido descrito, verifica-se que a modulação do efeito de sistemas monoaminérgicos sobre o transporte intestinal e, em particular, sobre a ATPase-Na^+,K^+ dependem de factores relacionados com o tipo de dieta, o regime alimentar e a idade. Este último assume maior relevância em ratos mais jovens cuja função renal não se encontra completamente maturada. Nos ratos adultos, pelo contrário, a dopamina e a 5-HT deixam de modular o transporte de sódio através da ATPase-Na^+,K^+ intestinal, porque essa função passa a ser exclusivamente desempenhada pelos rins. Esta observação levanta uma questão

importante. Será que nos animais adultos o sistema monoaminérgico intestinal pode ser activado quando a função renal se encontra diminuída? De facto, experiências anteriores realizadas com modelos animais onde esta situação se verifica vieram demonstrar uma alteração do estado de activação do sistema dopaminérgico intestinal. O objectivo do capítulo 5 foi, assim, avaliar o estado do sistema dopaminérgico intestinal em modelos animais que apresentam alterações neste sistema ao nível renal.

Neste âmbito encontram-se os trabalhos realizados em modelos animais de hipertensão. A hipertensão é uma patologia que muitos estudos indicam poder ser causada por uma anomalia no sistema dopaminérgico renal. Uma alteração a este nível pode ter com consequência uma redução dos efeitos natriuréticos com retenção de sal e aumento da tensão arterial. Estudos realizados por vários investigadores têm vindo a demonstrar que estas anomalias podem estar relacionadas com uma diminuição da capacidade de síntese de dopamina pelas células epiteliais (Kuchel & Shigetomi, 1992; Soares-da-Silva et al, 1995; Yoshimura et al, 1987), com um deficiente acoplamento entre os receptores dopaminérgicos e as vias de transdução de sinal (Felder et al, 1993; Hussain & Lokhandwala, 1997; Kansra et al, 1995) ou ainda com a ausência de determinado tipo de receptores da dopamina. Foi demonstrado, por exemplo, que ratinhos aos quais foi inibida a expressão de receptores do tipo D_{1A} ou D_3 desenvolvem hipertensão (Albrecht et al, 1996; Asico et al, 1998). Existe assim, em modelos animais de hipertensão, uma estreita correlação entre as alterações acima referidas e a capacidade de manutenção da homeostasia hidroelectrolítica que se manifesta na falta de tónus inibitório da dopamina sobre a ATPase-Na^+,K^+ renal. O objectivo do trabalho descrito nesta primeira parte do capítulo 5 foi então determinar a actividade basal da ATPase-Na^+,K^+ jejunal e a sua sensibilidade à dopamina em 3 situações experimentais distintas: 1) dieta hipossalina, 2) dieta normossalina e 3) dieta hiperssalina, em ratos espontaneamente hipertensos (SHR) e respectivos animais controlo (ratos Wistar-Kyoto, WKY). Os resultados obtidos demonstram que, de facto, a ATPase-Na^+,K^+ jejunal dos animais hipertensos é insensível à dopamina independentemente do conteúdo em sal da dieta, contrariamente ao que acontece nos ratos normotensos. Nestes a ATPase-Na^+,K^+ é sensível ao efeito inibitório da dopamina mediado por receptores D_1, efeito este que se perde com o aumento da ingestão de sódio (dieta hiperssalina). Foi interessante verificar ainda que independentemente do conteúdo em sal das dietas, os ratos hipertensos têm uma maior actividade basal da ATPase-Na^+,K^+, o que sugere uma maior capacidade para absorver sódio. De facto, aumentos da actividade do trocador Na^+/H^+ na membrana apical de enterócitos isolados de animais SHR foram já reportados (Acra & Ghishan, 1991). Quanto aos animais normotensos (WKY) a sua ATPase-Na^+,K^+ jejunal é apenas sensível à dopamina durante uma dieta hipossalina. A passagem para uma dieta com maior teor em sal (normo e hiperssalina) é acompanhada por uma perda do efeito inibitório sobre a ATPase-Na^+,K^+, o que está de acordo com os resultados anteriores obtidos com a ATPase-Na^+,K^+ jejunal de ratos Wistar. Pode parecer contraditório, o modo de funcionar do sistema dopaminérgico intestinal. Seria lógico que, de modo a contribuir para a homeostasia hidroelectrolítica, o maior aporte de sódio fosse um estímulo para uma diminuição da absorção deste ião. No entanto, é interessante relembrar que nos animais adultos essa função parece ser

exclusivamente desempenhada pelos rins numa situação fisiológica normal, e que o sistema intestinal apenas é activado quando o sistema renal não está a funcionar apropriadamente. É o que acontece em estudos realizados em ratos uninefrectomizados (Vieira-Coelho et al, 1999). Nestes animais verificou-se o reaparecimento da sensibilidade à inibição pela dopamina a nível da ATPase-Na^+,K^+ intestinal, com a finalidade de diminuir a absorção do sódio contribuindo, deste modo, para a homeostasia do organismo. Quando este "cross-talk" não está presente por anomalia no sistema dopaminérgico periférico surge uma patologia, que é o caso do modelo animal de hipertensão. Parece assim, haver um factor sistémico responsável pela interacção entre os dois sistemas de modo que quando um está alterado o outro responda apropriadamente Este factor natriurético intestinal teria a capacidade de sentir o aumento de aporte de sódio e alterar a função intestinal de modo a que o intestino absorve-se mais sódio. Neste modelo animal de hipertensão a razão para a falha deste "cross-talk" e a ausência da inibição ATPase-Na^+,K^+ jejunal pela dopamina, resulta de uma anomalia ao nível da sistema dopaminérgico intestinal, em particular de um defeito do acoplamento do receptor com as vias de transdução de sinal, cujo alvo é a ATPase-Na^+,K^+ jejunal. Esta falha leva a uma alteração da homeostasia para o sódio e consequente desenvolvimento de hipertensão. Em resumo, a inibição da actividade da ATPase-Na^+,K^+ pela dopamina nos animais normotensos é dependente do aporte de sódio, enquanto que os animais hipertensos não respondem com essa inibição seja qual for o tipo de dieta salina.

Além do modelo animal de hipertensão, outros modelos existem onde também se verificam alterações no funcionamento do sistema dopaminérgico renal. É o que se observa no modelo animal de obesidade em ratos da estirpe Zucker, que têm sido extensivamente utilizados no estudo da relação entre a obesidade e a hipertensão (Boese et al, 1985; Kurtz et al, 1989). Estes animais têm uma função renal diminuída (Fiske et al, 1986) que deverá ter como consequência um aumento da retenção de sódio e consequente desenvolvimento de hipertensão (Bray, 1977). Tal como acontece para o modelo animal de hipertensão, a dopamina não exerce qualquer efeito inibitório sobre a bomba de sódio renal, como resultado de um deficiente acoplamento entre os receptores dopaminérgicos e as vias de transdução de sinal (Hussain et al, 1999). Se considerarmos a existência de uma complementaridade funcional entre o intestino e o rim, uma das dúvidas que se levanta e à qual tentámos responder na segunda parte deste capítulo, diz respeito ao estado de activação do sistema dopaminérgico intestinal, numa situação onde declaradamente o sistema renal se encontra com uma falha na capacidade de manter o equilíbrio iónico e hídrico. O objectivo foi assim avaliar a actividade da ATPase-Na^+,K^+ em animais obesos e respectivos controlo, bem como a sua sensibilidade à dopamina na presença de uma dieta normo e hiperssalina. Os resultados obtidos demonstram que relativamente à actividade basal da ATPase-Na^+,K^+ durante uma dieta normossalina, os ratos obesos quando comparados com os animais controlo possuem uma maior actividade basal, compatível com uma maior capacidade absorptiva. De facto, estudos anteriores demonstraram que neste modelo ocorre uma hiperactividade da bomba de sódio nos tecidos e orgãos associados com a absorção de nutrientes e hiperfagia (Ferrer-Martinez, 1996). De acordo com esta noção,

verificámos que a quantidade de comida ingerida pelos animais obesos é maior do que a ingerida pelos animais controlo. O aumento da actividade da ATPase-Na^+,K^+ parece estar relacionada com uma diminuição do tónus dopaminérgico resultante da diminuição da actividade da enzima AADC responsável pela síntese de dopamina. Foi também interessante verificar que uma dieta hiperssalina durante 24 h é acompanhada apenas nos animais obesos por um aparecimento da sensibilidade da ATPase-Na^+,K^+ jejunal à inibição pela dopamina. Esta característica é importante se considerarmos que estes animais possuem uma função renal diminuída. Sendo assim, a dopamina ao inibir a bomba de sódio intestinal estará a contribuir para a homeostasia evitado deste modo a absorção excessiva de sal resultante da ingestão de uma dieta hiperssalina.

Após a análise dos dados descritos em todos os capítulos, é possível concluir sobre a existência de um sistema monoaminérgico intestinal de natureza parácrina e autócrina, e este reveste-se de uma importância crucial para a homeostasia hidroelectrolítica do organismo e se encontra regulado por inúmeros factores. Factores estes que podem ser extrínsecos ou intrínsecos ao próprio organismo, e que incluem: 1) idade, 2) tipo de regime alimentar, 3) conteúdo em sal das dietas, 4) tipo de dieta e 5) estádio fisiológico do sistema monoaminérgico ao nível renal. É também possível concluir que a modulação do transporte intestinal, pela dopamina e 5-HT, ocorre por regulação dos mecanismos celulares de transporte iónico, em particular a ATPase-Na^+,K^+ e o co-transportador Na^+,K^+,$2Cl^-$. Todo este tipo de regulação e, em particular, a possibilidade de interacção que existe entre o sistema intestinal e o sistema renal, assume particular relevância nas situações em que este último não se encontra completamente funcional, como seja durante o desenvolvimento ontogénico e em patologias que apresentem alterações na função renal.

Apesar do vasto conhecimento adquirido nestes últimos anos sobre estes dois sistemas há ainda muito para investigar quer sobre o a fisiologia e o modo de acção de cada um individualmente, quer sobre e modo como interagem e transmitem a informação entre ambos.

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A realização deste trabalho permitiu não apenas confirmar a presença de um sistema monoaminérgico, em particular dopaminérgico e 5-hidroxitriptaminérgico, a nível renal e jejunal, mas também caracterizá-lo segundo algumas novas vertentes, de modo a complementar trabalhos anteriormente publicados na área da fisiologia renal e intestinal.

O sistema monoaminérgico periférico exerce um importante papel na regulação da actividade do aparelho digestivo, nomeadamente na regulação da absorção e secreção intestinal. Na base dos mecanismos de transporte de cuja actividade dependem estes processos absorptivos e secretivos, encontram-se respectivamente a ATPase-Na^+,K^+ e o co-transportador Na^+,K^+,$2Cl^-$. A regulação exercida pelo sistema monoaminérgico sobre estes mecanismos e, consequentemente sobre o transporte hidroelectrolítico, é do tipo autócrino/ parácrino. Esta característica resulta, por um lado, da capacidade que as células da mucosa têm para sintetizar e metabolizar a dopamina e a 5-hidroxitriptamina, e por outro, da existência de receptores específicos sobre os quais actuam as aminas sintetizadas e libertadas pela própria mucosa.

Este trabalho permitiu assim demonstrar que a influência do sistema monoaminérgico periférico sobre os mecanismos de transporte depende não só de factores inerentes ao próprio organismo, mas também de factores e estímulos externos, tais como a presença ou ausência de alimentos no intestino, o conteúdo em sal e proteínas da dieta, idade e estádio de desenvolvimento.

De facto, factores como o desenvolvimento ontogénico, a ingestão de uma dieta com diferentes concentrações de sódio e períodos de jejum modificam de modo significativo o transporte epitelial por modulação, não apenas da actividade basal da bomba de sódio e outros transportadores, mas também por alteração da sensibilidade destes mecanismos de transporte aos efeitos da dopamina, da 5-hidroxitriptamina (5-HT) e de agonistas dos adrenoceptores α.

Assim, apoiando a importância destes factores sobre o transporte transepitelial, foi observado que, a ingestão de uma dieta rica em sódio conduz, apenas nos animais jovens, a uma diminuição da absorção intestinal deste ião por inibição da ATPase-Na^+,K^+ mediada pela dopamina. De igual modo, nos animais jovens a mudança de uma dieta com base em leite materno para uma dieta sólida, com diferente conteúdo em sal e proteínas, modificou o transporte epitelial, conduzindo a um aumento dos níveis basais de actividade da bomba de sódio e perda da sua sensibilidade à dopamina.

Relativamente à activação dos adrenoceptores α, apenas nos ratos jovens se verificou uma potenciação do efeito máximo induzido pelo UK 14,304 (agonista selectivo dos adrenoceptores α_2) sobre o transporte transepitelial, após um período de jejum. Também o efeito da 5-HT foi claramente influenciado pelo tipo de dieta e idade, já que só nos ratos de 20 dias em amamentação se observou efeito estimulatório para esta amina sobre a bomba de sódio.

De acordo com o mencionado anteriormente, foi interessante verificar que, de um modo geral, os mecanismos de transporte nos animais adultos permanecem insensíveis ao efeito das aminas estudadas, independentemente da dieta e do regime alimentar. Estes resultados estão relacionados com o facto de a regulação dos mecanismos de transporte hidroelectrolítico a nível intestinal ser fundamental para a homeostasia do organismo nos animais jovens, uma vez que nestes a função renal não se encontra completamente maturada.

Esta observação levantou ainda uma questão interessante relacionada com o estado de activação do sistema monoaminérgico intestinal, em situações em que a função renal se

encontra alterada como consequência de determinadas patologias. Os dados obtidos com os estudos realizados em dois modelos animais (hipertensão e obesidade), vieram demonstrar que de facto, se verifica a activação do sistema dopaminérgico intestinal nas situações em que o rim não responde aos efeitos desta amina.

Em conclusão, este trabalho permitiu-nos demonstrar que a existência e a regulação por inúmeros factores de um sistema monoaminérgico intestinal, de natureza parácrina/ autócrina, é crucial para a homeostasia hidroelectrolítica do organismo.

The data presented in this thesis, apart from confirming previous results regarding the presence of a peripheral monoaminergic system, in particular dopaminergic and 5-hidroxytryptaminergic, contributes with additional information to complete the present knowledge about renal and intestinal physiology.

The peripheral monoaminergic system performs a very important role in the regulation of the gastrointestinal physiology, namely in the regulation of the intestinal absorption and secretion. The transport mechanisms that are the basis for these absorptive and secretive processes are, respectively, the Na^+,K^+- ATPase and the $Na^+,K^+,2Cl^-$- cotransporter. The activity of these transporters, and consequently the water and electrolyte transport, is modulated by the monoaminergic system through an autocrine/ paracrine fashion. This results from the fact that the mucosal epithelial cells are able, not only, to synthesise and degrade dopamine and 5-hidroxytryptamine, but also are endowed with specific receptors for the amines.

The present work demonstrates that the modulation of the transport mechanisms present in the jejunal mucosa are influenced by a variety of factors, some of which depend on characteristics to the organism, while others are environmental and include the presence or absence of food in the gastrointestinal tract, salt and proteins in the diet, age and developmental stage.

In fact, factors like the ontogenic development, salt content in the diet and fasting periods were shown to significantly change the epithelial transport by modulating both the basal activity of the transporters and their sensitivity to dopamine, 5-hidroxytryptamine and α_2-adrenoceptors agonists.

In support of this view, it was observed that the intake of a high salt diet induced, only in young rats, a decrease in the intestinal sodium absorption through an inhibition of Na^+,K^+-ATPase activity mediated by dopamine. Likewise, the change from a milk to a solid diet, which has a different salt and protein content, changed epithelial transport, seem by an increase in Na^+,K^+-ATPase activity and loss of sodium pump sensitivity the inhibitory effects of dopamine.

In regard to the activation of α_2-adrenoceptors, it was observed an increase in the maximal effect induced by the UK 14,304 upon the transepithelial transport after a fasting period, but only in young rats. Similarly, the effect of 5-HT was clearly influenced by the type of diet and age, since only in young rats submitted to a milk diet, this amine significantly stimulated Na^+,K^+-ATPase activity.

In agreement with that mentioned previously, it is interesting to verify that in general transport mechanisms in adult animals remain insensitive to the effects of dopamine, 5-HT and adrenoceptor activation, regardless the diet and food regimen. These results are related to the fact that in young animals the regulation of water and electrolyte transport at the intestinal level is essential for body homeostasis, since at this age the kidneys are not fully developed.

This observation raised an interesting question related to the activation status of the monoaminergic peripheral system in conditions of decreased renal function as a consequence of certain pathologies. The data obtain with the study of two animals models (hypertension and obesity) demonstrated that, in fact, the intestinal dopaminergic system is activated in situations of renal insensitivity to this amine.

In conclusion, this work demonstrates that the existence and regulation by a variety of factors of an autocrine/paracrine intestinal monoaminergic system, is crucial for body water and electrolyte homeostasis.