João Tiago de Sousa Pinto Guimarães

Contribuição para o estudo das Monoaminoxídases A e B

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e para o João, o Tomás, o António, o Rodrigo e o Francisco

Aos meus Pais e Irmãos

Ao Excelentíssimo Senhor
Professor Doutor Patrício Soares da Silva

Prefácio

Este volume que apresento como dissertação de doutoramento, não é aquilo que desejava que fosse. E não o é por várias razões. Pela modéstia dos trabalhos que o compõem, pelo tempo que demorou a ser apresentado e, sobretudo, por ser fraco espelho da excelência daqueles com quem tive a sorte de aprender nos últimos dez anos.

Como em tudo na vida, também aqui houve um início. No meu caso, primeiro passo do percurso que hoje sigo, foi o convite feito pelo Professor Doutor Hipólito Reis para ser parte do grupo docente da disciplina de Bioquímica. Se a atracção pela vida académica era algo de "endógeno" e portanto fácil de "ser activado", já a possibilidade de estar na área das ciências básicas me levou a encarar de outro modo a minha vida clínica. Em consequência desta primeira opção decidi, em boa hora, enveredar pela actividade clínica numa especialidade laboratorial. Não posso deixar de agradecer publicamente, aqui e agora, ao Professor Doutor Hipólito Reis, para além dos seus conselhos e censuras, a possibilidade deste percurso. Tenho plena consciência da heterodoxia do mesmo, mas as vicissitudes não me impedem de considerar que em cada momento ajuizei correctamente perante as circunstâncias, o que me leva a partilhar o pensamento de Raúl Brandão quando escreveu nas suas Memórias que «*Se tivesse de recomeçar a vida, recomeçava-a com os mesmos erros e paixões. Não me arrependo, nunca me arrependi*». Tenho na actividade pedagógica que exerço na Faculdade de Medicina do Porto uma das funções mais gratificantes, exigentes e estimulantes da minha vida profissional.

Mas, o eu que fez este percurso, não é o mesmo eu que, hoje, lança um olhar por estes últimos anos. E, assim como tenho como certas as opções que, a seu tempo, tomei, também tenho como certo, que esse percurso poderia e deveria ter sido melhor. Sobretudo a nível científico o

modelo que vivi, e que muitos outros viveram, não é o mais ajustado ou desejável. A conciliação de actividade clínica, pedagógica e científica é difícil, sujeita a múltiplas oscilações de vontades (triplas!) e com fortes probabilidades frustrante. Não será assim de espantar que, com frequência, se afirme que a actividade científica feita por médicos é escassa. Mas também, quem é que a estima? A chamada pós-graduação, não é feita a pensar neles, nem os recursos têm sido aplicados em favor dos seus destinatários naturais. Há quem evoque as chamadas "leis do mercado"! Mas essas não podem ser aqui aplicadas. Pode, isso sim, é ser lembrado o velho postulado de que "a oferta gera a sua própria procura". Tenho como certa, que uma vez criadas as condições que permitam atrair os alunos e jovens licenciados para as actividades científicas, não faltarão vocações e interessados. Porque, paradoxalmente, muitos sabem afirmar que o futuro exigirá médicos que conciliem as três vertentes – a da educação, a da saúde e a da ciência. A minha opção académica procura essa síntese.

No meu percurso científico, tive a sorte de ter como Mestre o Professor Doutor Patrício Soares da Silva. Pessoa exigente, dotada de grande capacidade de trabalho, com grande domínio dos assuntos e uma notável capacidade de crítica científica. Não podia ter encontrado melhor orientador dos meus trabalhos. Além destas qualidades, que outros poderão também conhecer, tenho ainda de realçar a sua paciência para com o andamento da "minha ciência". Sendo o Professor Patrício Soares da Silva um dos mais produtivos investigadores do nosso país (e seguramente dos maiores do mundo por metro quadrado de espaço disponível...), nem por isso, deixa de ter tempo para discutir qualquer resultado, esgrimir interpretações ou ouvir um desabafo ou espírito mais desalentado. Se já tomava parte na transmissão e na aplicação do saber, foi com o Professor Patrício Soares da Silva que apreciei o que é tomar parte, mesmo que incipientemente, na criação desse saber. Facilmente se compreende que, uma quota parte do que sou, devo ao Professor Patrício Soares da Silva. Mas aquilo que mais lhe quero agradecer é a amizade que me dedicou nestes anos.

Neste meu caminho sou devedor de muitas outras pessoas. Ao Professor Doutor Walter Osswald, quero agradecer a forma como desde sempre me distinguiu, fosse nas palavras, fosse na amizade atenta que me manifestava. Ao Professor Doutor Daniel Moura, devo a paciência com que me escutava, o entusiasmo que sempre põe em tudo, a cultura que sempre manifesta, e a amizade que faz transparecer. À Professora Doutora Isabel Azevedo, devo um agradecimento peculiar! Para além da disponibilidade para nos ouvir, devo-lhe um agradecimento sobretudo "prospectivo" e menos "retrospectivo". A clareza com que vê os assuntos, o pragmatismo do seu raciocínio, a facilidade com que agrega as pessoas, associados aos valores que tão bem preza, fazem prever um futuro para o Serviço de Bioquímica em que vou, caso possa, ter gosto de tomar parte. Quero ainda recordar, o grande efeito que em mim sempre tiveram as visitas, sobretudo no universo familiar, dos Professores Doutores Ullrich Trendelenburg e Klaus Starke, como geradores do gosto pela vida académica.

Viver no Instituto de Farmacologia e Terapêutica durante este período, uma espécie de "ir para fora cá dentro", permitiu-me o contacto diário com pessoas de excepção. Não posso deixar de referir a consideração pelos Srs. Professores Doutores Eduardo Rodrigues Pereira, Jorge Polónia, António Albino Teixeira, Fernando Brandão e Rosa Begonha, bem como o companheirismo e provas de amizade do António Sarmento, do José Pedro Nunes, do Manuel Vaz da Silva, do Nuno Borges e da Sofia Magina. À Doutora Maria Quitéria Paiva tenho que agradecer o rigor e exigência, mas sobretudo a amizade sólida.

No grupo de investigação do Professor Patrício Soares da Silva encontrei um grupo pequeno, mas coeso. Não posso deixar de agradecer os ensinamentos da Professora Doutora Maria Helena Fernandes, a camaradagem, boa disposição e disponibilidade da Maria Augusta Vieira Coelho, a amizade da Paula Serrão, da Perpétua Pinto do Ó e do Pedro Gomes.

Durante três meses pude viver uma actividade diária e exclusivamente de investigação na Unidade 388 do INSERM, em Toulouse. Aí sob a orientação do Doutor Angelo Parini e com a ajuda da Cécile Vindis, da Nanou Pizzinat, da Anne Remaury e da Claudie Cambon aprendi algumas técnicas de foro molecular aplicadas ao estudo das MAOs. Para além dos ensinamentos que muito agradeço, não posso deixar de testemunhar o bom ambiente e a facilidade com que fui recebido por todos.

Mas tenho muitas outras dívidas de gratidão.... Ao aceitar o desafio dos Drs. Manuel Strecht Monteiro e Fernando Moreira, a quem desde já agradeço toda a confiança e amizade que por mim sempre demonstraram, lancei-me numa das boas aventuras que uma vida profissional na medicina pode proporcionar- a abertura de raiz de uma instituição hospitalar com um modelo de gestão inovador. Aquilo que começou como uma aposta arriscada, veio a revelar-se como uma oportunidade "once in a lifetime"! Compreensívelmente, a preparação não só do Serviço de Patologia Clínica do Hospital de São Sebastião, como também todas as outras funções desempenhadas nesse hospital, acabaram por ser o principal factor para o atraso na elaboração desta tese. Permitiram-me, contudo, uma vivência única e muito enriquecedora. Mas o "excesso" de vida activa também pode ser prejudicial! Sentia que estava a diminuir o já pouco espaço que me sobrava para a reflexão, algo tão essencial à vida académica. Daí que, o retorno ao Hospital de São João, instituição que pelas suas características (desejavelmente!) específicas permite conciliar uma vida activa e uma vida mais de reflexão, tenha sido a opção certa no momento certo. Dos tempos de Santa Maria da Feira, tenho gratas recordações e muita aprendizagem a agradecer. Ao Dr. Hugo Meireles tenho que agradecer a grande amizade, estima e confiança que me dispensou, mas, acima disso, o modo esclarecido e superior como tem conduzido os destinos do Hospital. À Dra. Belina Nunes tenho que agradecer, para além da grande amizade que perdura, a possibilidade de viver diariamente em contacto com a excelência da sua

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À Mariana e aos nossos João Maria, Tomás Maria, António Maria, Rodrigo Maria e Francisco Maria. Palavras? Quais?

Glossário

a.a. – ácido aminado
AAAD – descarboxílase dos ácidos aminados aromáticos
AD – adrenalina
Ala – alanina
Asn – asparagina
cDNA – ácido desoxirribonucleico complementar
COMT – catecol-o-metil-transférase
Cys – cisteína
Da – dalton, unidade de massa molecular
DA – dopamina
DNA – ácido desoxirribonucleico
DOMA – ácido di-hidroximandélico
L-DOPA – di-hidroxifenilalanina (enantiómero L)
DOPAC – ácido di-hidroxifenilacético
DOPEG – di-hidroxifeniletilglicol
E.C. – "Enzyme Comission "- Comissão de nomenclatura enzímica da IUBMB
FAD – Dinucleotídeo da flavina e da adenina
Gly – glicina
5-HT – 5-hidroxitriptamina ou serotonina
Ile – isoleucina
I.U.B.M.B. – International Union of Biochemistry and Molecular Biology
Kb – quilobase
KO – "knock out"
MAO – oxídase das monoaminas ou monoaminoxídase
Met – metionina
MPTP – 1-metil-4-fenil-1,2,3,6-tetrahidropiridina
MPP+ – 1-metil-4-fenilpiridinio
mRNA – ácido ribonucleico mensageiro
mtDNA – ácido desoxirribonucleico mitocôndrico
NA – noradrenalina
PEA – beta-feniletilamina
Phe – fenilalanina
PST – fenolsulfotransférase
SSAO – oxídase de aminas sensível à semi-carbazida
("semicarbazide-sensitive amine oxidase")
sDA – sulfato de dopamina
Thr – treonina
Tyr – tirosina
VAP-1 – peptideo de adesão vascular ("vascular adhesion peptide")

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Capítulo I

Introdução e objectivos

Considerações gerais

A descrição por Mary Hare, em 1928, de um novo mecanismo enzímico de oxidação da tiramina pode ser considerado o ponto de partida da investigação nesta área (Hare, 1928). Os trabalhos subsequentes que indicaram que as catecolaminas (Blaschko et al., 1937) e a histamina (Zeller, 1938) eram, também, substratos dessa mesma enzima, vieram consolidar a importância da actividade então descrita por Mary Hare. Nos mais de 70 anos que se seguiram, o interesse pela caracterização dessa actividade enzímica foi-se alargando e aprofundando, acompanhando a natural evolução das metodologias e dos conhecimentos de que a ciência foi dispondo.

A Oxídase das Monoaminas (Monoaminaoxídase – MAO – ver glossário para as abreviaturas) [classificada pela Comissão de Enzimas da "International Union for Biochemistry and Molecular Biology" (IUBMB) como - amine: oxygen oxidoreductase (deaminating) (flavin-containing), E.C. 1.4.3.4] (I.U.B.M.B., 2001) é uma flavoproteína, presente nos seres eucariontes, que existe em duas formas, A e B, ubiquamente distribuídas pelo organismo, e responsáveis pela desaminação oxidativa de numerosas aminas, neuro- e vasoactivas, bem como de numerosos produtos xenobióticos, incluindo protoxinas. Genericamente, estas duas formas da enzima distinguem-se pelos seus substratos, especificidade de inibição, distribuição tecidular e ainda por propriedades e características moleculares e imunológicas. A sua actividade oxidativa faz-se sentir essencialmente sobre as aminas aromáticas primárias, muito embora as aminas alifáticas primárias e as aminas secundárias ou terciárias também possam ser seus substratos (Youdim et al., 1988; Hsu et al., 1989; Weyler et al., 1990; Singer et al., 1995; Boulton et al., 1998; Shih et al., 1999; Abell et al., 2000).

Caracterização molecular – do gene à proteína

A distinção de duas formas de MAO foi inicialmente proposta com base nos resultados obtidos mediante o uso dos inibidores clorgilina (Johnston, 1968) e selegilina (deprenil) (Knoll et al., 1972). Desde logo se colocou a questão de se saber se se estava perante duas proteínas diferentes ou se, como alguns autores pretendiam, a distinção nas formas A e B era apenas um conceito resultante das múltiplas características de uma mesma enzima. Entre estes últimos, uns explicavam as diferenças encontradas pelos ambientes de membrana (Houslay et al., 1973) e outros sugeriam que se estava perante um fenómeno de modificação pós-translacional de um precursor enzímico comum, codificado por um único gene (Cawthon et al., 1981). Contudo, e por outro lado, já em 1979, os grupos de Breakefield e de Callingham com estudos electroforéticos feitos em gel de poliacrilamida, em que usaram um inibidor de ambas as formas de MAO, a pargilina (marcada radioactivamente), mostraram duas bandas proteicas perfeitamente separadas, correspondentes à forma A e à forma B (Callingham et al., 1979; Cawthon et al., 1979). Nestes estudos os pesos moleculares determinados foram, respectivamente, 63 000 Dalton (Da) para a MAO-A e 60 000 Da para a MAO-B. Pouco tempo depois, Denney e Abell (Denney et al., 1982) fazendo uso da técnica do hibridoma desenvolvida por Kohler e Milstein (Kohler et al., 1975) – que permitiu pela primeira vez a obtenção de grandes quantidades de anticorpos monoclonais – conseguiram obter um anticorpo mono-clonal específico para a MAO-B capaz de discriminar as duas formas em cromatografia. Nesse mesmo sentido, e poucos anos mais tarde, também a diferente expressão por essas mesmas formas nas experiências de inactivação foto-dependente, veio reforçar essa hipótese de diferenças conformacionais ou estruturais dos respectivos locais activos (Chen et al., 1984; 1985). Estes dados, reveladores de propriedades electroforéticas e imunológicas distintas, vieram confirmar as diferenças cinéticas encontradas, dando corpo à existência de um suporte proteico próprio de cada uma das formas de MAO que a se-

quenciação, em 1987, veio confirmar em definitivo (Bach et al., 1988). Estes autores, usaram sondas de oligonucleótidos derivadas de sequências peptidícas de MAO A e de MAO B de fígado humano, para isolarem e sequenciarem clones do ácido desoxiribonucleico complementar (cDNA) respectivo, o que, para além de ter demonstrado a existência de genes distintos, permitiu relacionar as características descritas para as enzimas e o suporte proteico e genético responsável pelas mesmas. Sabe-se então, que na espécie humana, a MAO-A, com 527 ácidos aminados (a.a.) e a MAO-B com 520 a.a., têm uma identidade de a.a. de 73%, e possuem pesos moleculares de 59 700 Da a forma A e de 58 800 Da a forma B, em concordância com os valores anteriormente referidos nas separações electroforéticas. Os genes que codificam cada uma das formas de MAO estão situados, muito próximos um do outro, no braço curto (p) do cromossoma X (Pintar et al., 1981; Kochersperger et al., 1986), em áreas adjacentes a Xp11.23. A precisão dessa localização varia apenas em função da metodologia utilizada (Ozelius et al., 1988; Levy et al., 1989; Lan et al., 1989b). A análise dos cDNAs e das sequências genómicas demonstrou que ambas as regiões codificadoras dos genes da MAO-A e da MAO-B compreendem 15 exões e 14 intrões, sendo que todos os intrões interrompem as sequências codificadoras nos mesmos pontos apresentando, assim, uma organização exão-intrão idêntica (Grimsby et al., 1991). O estudo das sequências de ácido desoxirribonucleico (DNA) que flanqueiam a extremidade 5' dos genes para a MAO-A e para a MAO-B permitiu caracterizar os genes promotores, responsáveis pela activação da transcrição (Shih et al., 1994; Denney, 1995). Trata-se de dois fragmentos situados a cerca de 40 resíduos do codão de iniciação, ambos ricos em guanina (G) e em citosina (C), que contêm locais potenciais de ligação para factores de regulação da transcrição (como para o Sp1) e que têm cerca de 60% de identidade das suas sequências. É de referir, ainda, o facto de os genes constitucionais, isto é os genes que são continuamente expressos, tenderem a ter sequências ricas em G e em C nos seus promotores ("GC boxes") (Stryer, 1994b). Todas estas semelhanças descritas, em particular na relação exão-intrão, sugerem

que as duas formas A e B da enzima resultam da duplicação de um gene ancestral comum (Hsu et al., 1989; Weyler et al., 1990; Stryer, 1994a).

Caracterização topográfica – localização celular e distribuição tecidular

As MAOs são enzimas integrais da membrana externa da mitocôndria (Schnaitman et al., 1967; Greenawalt et al., 1970) e a MAO-A foi a primeira proteína da membrana mitocôndrica externa de seres eucarióticos a ser clonada (Hsu et al., 1989). O seu processo de inserção na membrana é aparentemente semelhante ao de outras enzimas com idêntica localização, não contendo a proteína qualquer sequência sinal que a conduza até à sua posição final (Zhuang et al., 1988). A fixação na membrana externa da mitocôndria é determinada por sequências próximas da extremidade carboxílica da proteína (Bach et al., 1988; Grimsby et al., 1991).

Sendo enzimas mitocôndricas, as MAOs existem na quase totalidade dos tecidos em que foram pesquisadas. A distribuição relativa da MAO-A e da MAO-B tem sido estudada por numerosos métodos e todos eles demonstram que ela varia muito. Essa variação existe quer inter-espécies, pois é diferente o predomínio de cada uma das formas nos diferentes tecidos, quer intra-espécie, pois é diferente a sua distribuição relativa nos diferentes tecidos. No sistema nervoso central de primatas (incluindo o humano) e do Rato, a MAO-A está essencialmente contida nas áreas onde existem neurónios adrenérgicos e noradrenérgicos, enquanto a MAO-B ocorre nas áreas onde existem neurónios serotoninérgicos (Levitt et al., 1982; Westlund et al., 1985; Fowler et al., 1987). Nos tecidos periféricos, a MAO-A e a MAO-B co-exprimem-se em quase todos, havendo algumas excepções. A placenta humana e os fibroblastos apresentam exclusivamente o tipo A, enquanto que os linfócitos e as plaquetas apresentam apenas a forma B (Thorpe et al., 1987; Youdim et al., 1988). No que respeita aos outros tecidos periféricos a

expressão das duas formas faz-se em proporções variáveis. Quanto à actividade total ela varia desde um mínimo para o músculo esquelético (MAO-A e MAO-B) até um máximo no gânglio cervical superior (MAO-A) ou no fígado (MAO-B) (Youdim et al., 1988). A distribuição do ácido ribonucleico mensageiro (mRNA) das MAOs é, de um modo geral, consistente com a sua actividade catalítica e com os estudos morfológicos (Grimsby et al., 1990; Saura et al., 1996). Existe ainda actividade das MAOs em numerosas linhas celulares utilizadas experimentalmente.

Caracterização estrutural – a enzima

A localização das MAOs na membrana externa da mitocôndria torna a sua cristalização extremamente difícil. Simultaneamente, as MAOs não são semelhantes a qualquer outra proteína cuja estrutura esteja já estabelecida. Como consequência destes dois factos a sua estrutura tri-dimensional não é, ainda, conhecida, sendo apenas possível dispôr de modelos parciais para a estrutura da MAO-A (Wouters et al., 1998). A sequenciação dos genes das MAOs, a mutagénese directa e a construção de quimeras, têm permitido esclarecer a sua estrutura primária e antecipar "tendências" na sua estrutura secundária e terciária.

Assim, há uma homologia de 88% entre a estrutura primária da MAO-B hepática humana e a do Rato, com uma distribuição das regiões de identidade ao longo de toda a proteína (Bach et al., 1988; Ito et al., 1988). De igual modo o cDNA para a MAO-A hepática do Rato gera uma sequência de a.a. com uma homologia de 88% relativamente à mesma forma hepática humana (Kuwahara et al., 1990). Estudos feitos no Boi mostraram que o cDNA para a MAO-A codifica uma proteína com 527 a.a., a qual possui 87% de semelhança com a humana, enquanto que a semelhança com a MAO-B da mesma espécie é de apenas 67% (Powell et al., 1989). Também se encontra uma total identidade na sequência deduzida dos a.a. da MAO-B nas plaquetas

e no cortex frontal humanos (Chen et al., 1993). Há várias regiões bastante conservadas entre as duas formas de MAO (Bach et al., 1988; Hsu et al., 1988).

Cada uma das MAOs está covalentemente ligada a uma molécula de dinucleotídeo da flavina e da adenina (FAD), sem a qual são inactivas (Weyler, 1989). O local de fixação do FAD, em posições equivalentes em ambas as proteínas, é um pentapeptídeo de serina-glicina(Gly)-(Gly)-cisteína(Cys)--tirosina(Tyr) (Kearney et al., 1971; Walker et al., 1971; Nagy et al., 1981). Essa ligação covalente faz-se entre um grupo metilo situado na posição 8α do FAD e uma Cys situada na posição 406 na MAO-A e na posição 397 na MAO-B, respectivamente (Bach et al., 1988; Gottowik et al., 1993). Importa referir que os 35 a.a. onde se encontra este pentapeptídeo são idênticos, nas duas formas de MAO e nas diferentes espécies (Hsu et al., 1988; Powell et al., 1989; Kuwahara et al., 1990; Chen et al., 1993), sendo codificados pelo exão 12, que é o mais conservado de todos os exões entre as MAOs humanas (Grimsby et al., 1991). A molécula de FAD para se poder fixar covalentemente tem de se ligar, previamente, a outras regiões das MAOs que funcionam como locais de ancoração. Estas regiões incluem vários resíduos na extremidade amina da MAO-A e da MAO-B, bem como alguns a.a. dispersos (Kwan et al., 1995; Zhou et al., 1995; Kirksey et al., 1998; Zhou et al., 1998).

O local activo está situado num segmento intermédio entre as extremidades amina e carboxílica, correspondendo no Homem, para a MAO-B, às porções 62-103 e 146-220 e para a MAO-A, ao segmento 112-395 (Gottowik et al., 1995). Na MAO-B, a sequência 146-220 parece ser a responsável pela ligação do inibidor lazabemida (Gottowik et al., 1995). No Rato é o segmento 120-220 o responsável pela determinação da especificidade de substrato para ambas as MAOs, enquanto que a região 220-400 está relacionada com a actividade catalítica relativa em relação a esses mesmos substratos (Tsugeno et al., 1995; Tsugeno et al., 1997). Estão descritos outros segmentos que são essenciais para a actividade de cada uma das MAOs estendendo-se, no Homem, entre os resíduos 215-375, para a MAO-A e entre os resíduos 206-366, para a MAO-B (Grimsby et al., 1996; Geha et al., 2000). A extremidade

carboxílica é importante para a actividade da MAO-B, não por estar envolvida no local activo, mas por ser essencial para a interacção com a membrana (Chen et al., 1996; Rebrin et al., 2001). Uma outra região descrita diz respeito aos locais de ligação imidazolínicos do tipo I_2, os quais foram identificados como correspondendo às formas A e B da MAO (Raddatz et al., 1995; Tesson et al., 1995). Verifica-se que o local de ligação imidazolínico na MAO-B está localizado entre os aminoácidos lisina 149 e metionina (Met) 222 da MAO-B humana (Raddatz et al., 1997). Este local de ligação imidazolínico compreende uma região envolvida na metabolização e selectividade dos substratos (Gottowik et al., 1995; Tsugeno et al., 1997).

A estrutura secundária mostra a existência de cadeias alfa helicoidais (α) e folhas beta pregueadas (β) em número muito semelhantes em ambas as MAOs. A extremidade amina de ambas as formas apresenta um pregueamento β-α-β que corresponde ao local de fixação do ADP e está envolvido na ligação não-covalente do FAD às MAOs (Wouters et al., 1995). Quanto à estrutura terciária, apenas existe uma proposta parcial baseada nos conhecimentos provenientes do estudo das estruturas primária e secundária e nas analogias com outras enzimas flavoproteicas. É possível delimitar quatro regiões na MAO-A que correspondem a um primeiro domínio α/β (resíduos 15-120 e 280-368), um segundo domínio α/β (resíduos 120-280), um terceiro domínio α+β (resíduos 394-504) e um domínio correspondente à extremidade carboxílica (Wouters et al., 1998). Muito embora os estudos com expressão dos cDNAs da MAO-A e da MAO-B em linhas celulares mostrem que a actividade destas enzimas está contida num único polipeptídeo (Lan et al., 1989a), este existe na forma de complexos oligoméricos de grandes dimensões. Esta estrutura quaternária das MAOs foi já demonstrada para a MAO-B bovina, que existe na forma de um hexâmero formado por um trímero de homodímeros da enzima (Shiloff et al., 1996).

Caracterização funcional – posicionamento metabólico, substratos e inibidores

As MAOs catalisam a desaminação oxidativa dos seus substratos, desaminação que se acompanha da redução simultânea do FAD, co-factor obrigatório desta reacção. Os produtos dessa reacção são a imina do substrato e a amónia. A primeira, hidroliza-se espontaneamente no aldeído correspondente, enquanto que a reoxidação simultânea do FAD, pelo oxigénio, leva à produção de peróxido de oxigénio (H_2O_2) (Youdim et al., 1988; Weyler et al., 1990). De entre os produtos da reacção e para além dos aldeídos resultantes, assumem especial relevo, quer a amónia produzida, pelo efeito que pode ter na regulação osmótica, quer o H_2O_2 libertado, por estar envolvido em numerosos processos intra e extracelulares.

A separação dos substratos, em função da sua especificidade, em substratos da MAO-A e em substratos da MAO-B, não pode ser considerada como tendo um valor absoluto. Contudo é possível considerar que há substratos preferencialmente metabolizados por cada uma das formas de MAO, enquanto outros são simultaneamente metabolizados pelos dois tipos. A MAO-A oxida preferencialmente as aminas biogénicas como a serotonina ou 5-hidroxitriptamina (5-HT), a noradrenalina (NA) e a adrenalina (AD), enquanto que a MAO-B oxida preferencialmente os compostos não catecolamínicos como a β-feniletilamina (PEA) e a benzilamina. A dopamina (DA), a tiramina e a triptamina são considerados substratos de ambas as formas (Youdim et al., 1988; Weyler et al., 1990). Não deveremos ignorar a possibilidade de os substratos poderem originar produtos com efeitos fisiológicos diferentes daqueles que eram os dos produtos originais. Neste caso é de realçar a capacidade que a MAO-B tem de transformar profármacos ou toxinas como seja o 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP). O MPTP, é oxidado por acção da MAO-B em 1-metil-4-fenilpiridinio+ (MPP+), neurotoxina dopaminérgica que induz um sindroma semelhante ao de Parkinson no Homem e nos primatas (Langston et al., 1983; Chiba et al., 1984; Langston

et al., 1984). O papel da MAO-B nesta situação é confirmado pelo facto de os ratinhos "knock out" (KO) para o gene da MAO-B (KO MAO-B) não sofrerem a toxicidade pelo MPTP (Grimsby et al., 1997).

São conhecidos vários tipos de inibição das MAOs. A importância desta inibição ultrapassa o mero fenómeno inibitório, pois serviu de base à distinção entre MAO-A e MAO-B, a qual só existe como consequência das MAOs apresentarem diferente sensibilidade à inibição pela clorgilina e pela selegilina (Johnston, 1968; Knoll et al., 1972). A MAO-A é inactivada de modo irreversível pela clorgilina, enquanto a MAO-B é inactivada também irreversivelmente pela selegilina. Ambos estes inibidores actuam por ligação ao local de fixação do co-factor FAD. Um outro grupo importante de inibidores inclui os derivados N-(2-aminoetil)-aril-carboxamida, como a lazabemida e o Ro 41-1049, os quais partilham o mesmo mecanismo de acção do substrato e podem ser considerados como inibidores reversíveis activados pela enzima (Cesura et al., 1989; Da Prada et al., 1990). Também os fumadores apresentam inibição de ambas as formas de MAO, por mecanismos ainda não totalmente esclarecidos (Fowler et al., 1996a; Fowler et al., 1996b). Um outro grupo de inibidores é formado pelos detergentes utilizados nos processos de extracção (como por exemplo, triton X-100 e sais biliares), os quais têm actividade inibidora inespecífica, embora por vezes selectiva de uma das formas (Achee et al., 1981; Bancells et al., 1987). Compostos imidazolínicos e guanídicos inibem também a MAO-B, sendo contudo necessárias concentrações destes 10 a 50 vezes superiores às utilizadas para saturar os locais de fixação imidazolínicos (Palaty et al., 1989; Carpene et al., 1995; Tesson et al., 1995). Poderá ainda acontecer que, pela própria natureza da via catalítica, o inibidor acabe por funcionar como um substrato, dando origem à formação de um produto. Está nestas circunstâncias a selegilina que é metabolizada em compostos anfetamínicos, os quais poderão ser responsáveis por alguns dos seus efeitos terapêuticos. Interessa ainda referir que os inibidores, pelo facto de terem afinidades e selectividades elevadas, em geral superiores às dos substratos, poderem

ser boas ferramentas para o estudo do centro activo destas enzimas (Dostert et al., 1991).

O papel metabólico desempenhado pelas MAOs na inactivação das catecolaminas vai ser de dois tipos. Por um lado transformam essas catecolaminas, por desaminação oxidativa, num aldeído. Por outro lado actuam sobre o produto da acção da catecol-O-metiltransférase (COMT). Outra das enzimas envolvidas nesta metabolização é a fenolsulfotransférase (PST), cujo produto, o sulfato de dopamina (sDA) é, não só um produto final, como também é uma forma de reserva de DA, uma vez que pode ser "dessulfatado" originando a forma activa (Kuchel, 1994). Finalmente, será ainda de considerar o papel que a glicurono-conjugação, pela glicuronídase, tem na metabolização destas aminas.

Caracterização patológica – relevância clínica e modelos animais

A Doença de Parkinson, doença neurodegenerativa progressiva é a situação clínica mais relevante envolvendo as MAOs. Foi em 1983 que se estabeleceu pela primeira vez uma relação entre as MAOs e a doença de Parkinson (Langston et al., 1983). Desde então esta relação entre MAOs e doença de Parkinson tem vindo a ser cada vez melhor estudada, seja no esclarecimento do modo como estas enzimas estão envolvidas na patogénese da doença, seja pelo papel terapêutico dos inibidores das MAOs (Tetrud et al., 1989; Hauptmann et al., 1996; Cohen et al., 1997; Lozano et al., 1998). As MAOs poderão ainda estar associadas a outras doenças neurodegenerativas como a doença de Alzheimer (Sano et al., 1997; Burke et al., 2001).

A ausência da MAO-A tem sido associada a alterações comportamentais, nomeadamente agressividade, como no caso de uma família holandesa com deficiência de MAO-A por substituição de um único nucleotídeo no exão 8 do gene da MAO-A (Brunner et al., 1993a; Brunner et

al., 1993b). Mesmo a ausência total de MAO-A e de MAO-B, em doentes com doença de Norrie é compatível com a vida, embora causando um fenótipo neurológico muito grave (Sims et al., 1989; Lan et al., 1989b; Collins et al., 1992). Contudo, as mutações dos genes da MAO-A e da MAO-B não são fenómenos frequentes (Tivol et al., 1996; Schuback et al., 1999). As MAOs estão ainda associadas a outras alterações comportamentais relacionadas com o tabagismo e o álcool (Devor et al., 1993; Fowler et al., 1996a; Fowler et al., 1996b). Os inibidores da MAO-A são também utilizados no tratamento de certas formas de depressão.

Estes achados clínicos têm correspondência nos modelos animais. O KO do gene para a MAO-A em ratinhos resulta em comportamento agressivo e elevação dos níveis de 5-HT e NA (Cases et al., 1995) bem como em alterações da aprendizagem (Kim et al., 1997). Quando se faz o mesmo ao gene da MAO-B as consequências são, quer uma alteração do comportamento perante o stress, mas sem se observar agressividade, quer um aumento dos níveis de PEA (Grimsby et al., 1997).

Objectivo do trabalho

O objectivo deste trabalho consiste na caracterização do papel das MAOs em alguns tecidos periféricos, nomeadamente o rim, o jejuno e o coração. Pretende-se, através da sua caracterização, por estudos cinéticos de metabolização, da sua inibição e de estudos moleculares, definir melhor o papel que lhe cabe na biotransformação das aminas pelos tecidos referidos. Dá-se, ainda, especial relevo à procura de novas características que possam distinguir as duas formas de MAO.

No Capítulo II estudou-se a actividade de ambas as formas de MAO em células e túbulos renais, procurando distinguir o papel de cada uma dessas formas no destino metabólico da DA. Para tal recorreu-se ao isolamento de células do epitélio tubular e ainda de túbulos contornados

proximais, procurando isolar as unidades funcionais individuais. Além disso utilizaram-se vários substratos e inibidores de forma a caracterizar melhor essas mesmas actividades (**publicação 1**). Ainda no âmbito desta linha de estudo fez-se a caracterização comparativa com o rim de Rato de uma linha celular epitelial classicamente utilizada para estudar a função renal, as células OK (Opossum kidney) – derivadas do rim da fêmea do marsupial americano *Didelfis virginiana* – e cuja caracterização do ponto de vista de actividade desaminativa não estava ainda feita (**publicação 2**).

Seguidamente, no Capítulo III, e para caracterizar a actividade das MAOs em tecidos com actividade dopaminérgica relevante, estudou-se no jejuno o efeito de inibidores de cada uma das formas de MAO sobre a sua actividade (**publicação 3**). Procurou-se ainda caracterizar o modo como se comportavam células isoladas do jejuno, e uma outra linha celular – as células Caco-2 – linha celular epitelial derivada de adenocarcinoma de cólon humano, quanto ao padrão de síntese de DA e de 5-HT e quanto à capacidade desaminativa. Nesta linha celular, o estudo das enzimas que tomam parte na síntese e degradação de monoaminas estava também por fazer (**publicação 4**).

O Capítulo IV diz respeito ao estudo no coração, um dos tecidos metabolicamente mais activos e onde o papel das duas formas de MAO está pior caracterizado no que respeita à metabolização de monoaminas. O estudo das MAOs a nível cardíaco demonstrou a existência de um padrão particular de metabolização da PEA pelo coração de Rato (**publicação 5**). Este facto fez progredir o estudo de modo a procurar estabelecer uma correspondência entre as propriedades cinéticas encontradas e o suporte molecular correspondente, na procura de diferenças eventualmente existentes a esse nível (**publicação 6**). Como referência comparativa para o conjunto dos estudos realizados com o coração de Rato, utilizou-se o cortex renal da mesma espécie.

Finalmente, em trabalhos cujos resultados são apresentados no Capítulo V, avaliou-se a influência do envelhecimento na capacidade de metabolização de NA por alguns tecidos periféricos (**publicação 7**).

Capítulo II) *no rim:*

1) *"The activity of MAO A and B in rat renal cells and tubules";*
Life Sci. (1998), 62:727-737
2) *"Opossum kidney (OK) cells in culture synthesize and degrade the natriuretic hormone dopamine: a comparison with rat tubular cells";*
Int. J. Biochem. Cell Biol. (1997), 29:681-688

Capítulo III) *no intestino:*

3) *"Inhibitory effects of Ro 41-1049, lazabemide and tolcapone on rat jejunal MAO-A, MAO-B and COMT activities";*
Pharmacol. Comm. (1995), 5:213-219
4) *"Caco-2 cells in culture synthesize and degrade dopamine and 5-hydroxytryptamine: a comparison with rat jejunal epithelial cells";*
Life Sci. (1999), 64:69-81

Capítulo IV) *no coração:*

5) *"Unusual pattern of β-phenylethylamine deamination in the rat heart";*
Neurobiology (2000), 8:109-118
6) *"Differential substrate specificity of monoamine oxidase in the rat heart and renal cortex";*
(Enviado para publicação)

Capítulo V) *com a idade:*

7) *"Influence of maturation and ageing on the biotransformation of noradrenaline in the rat";*
J. Neural Transm. [Suppl.] (1998), 52:225-232

Capítulo II

Actividade da MAO-A e da MAO-B em tecidos renais. Estudos realizados em células e túbulos renais de rato e numa linha celular renal.

PUBLICAÇÃO 1

"The activity of MAO-A and B in rat renal cells and tubules"

Life Sci. (1998), 62:727-737

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THE ACTIVITY OF MAO A AND B IN RAT RENAL CELLS AND TUBULES

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Summary

The present study reports on the presence of type A and B monoamine oxidase (MAO) activity and their sensitivity to selective MAO-A and MAO-B inhibition by Ro 41-1049 and lazabemide, respectively, in homogenates of isolated rat renal tubules. Non-linear analysis of the saturation curve of ^{3}H-5-hydroxytryptamine (^{3}H-5-HT) deamination revealed a K_m of 351±71 μM (n=4) and a V_{max} of 25±2 nmol mg protein^{-1} h^{-1}. Deamination of ^{14}C-ß-phenylethylamine (^{14}C-ß-PEA) was also a saturable process yielding K_m values of 58±12 μM and V_{max} values of 24±2 nmol mg protein^{-1} h^{-1}. Ro 41-1049 produced a concentration-dependent inhibition of ^{3}H-5-HT deamination with a K_i of 24 nM. Deamination of ^{14}C-ß-PEA was found to be reduced by lazabemide in a concentration-dependent manner with a K_i value of 17 nM. The effect of these selective MAO inhibitors on dopamine fate and DOPAC formation in isolated tubular epithelial cells was also studied. In these studies a clear inhibition of DOPAC formation was observed with Ro 41-1049 (250 nM), while 250 nM lazabemide was found not to increase the accumulation of newly-formed DA in those tubular epithelial cells loaded with 50 μM L-DOPA. In conclusion, the results presented here confirm the presence of both MAO-A and MAO-B activity in renal tubular epithelial cells, that MAO-A is the predominant enzyme involved in the deamination of the natriuretic hormone dopamine and that the deamination of newly-formed dopamine is a time-dependent process which occurs early after the decarboxylation of L-DOPA.

Key Words: **monoamine oxidase, kidney, dopamine, lazabemide, Ro 41-1049**

Monoamine oxidase (MAO, amine-oxygen oxidoreductase, E.C 1.4.3.4) is a mitochondrial membrane bound flavin-containing enzyme which catalyses the oxidative deamination of biogenic and xenobiotic amines to their corresponding aldehydes (1, 2). It inactivates such biogenic amines as adrenaline, noradrenaline, serotonin, dopamine and various trace amines, thus regulating their levels. There are two molecular forms of the enzyme MAO-A and MAO-B, encoded by separate genes (3), classically differentiated on the basis of substrate specificity (4), inhibitor sensitivity (5), electrophoretic motility (6) and also immunochemical properties (7).

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Epithelial cells of renal proximal tubules synthesise dopamine from L-3,4-dihydroxyphenylalanine (L-DOPA) present in the tubular filtrate (8), and a considerable amount of the amine has been found to be deaminated to 3,4-dihydroxyphenylacetic acid (DOPAC) in the kidney (9). The relevance of this is even more re-enforced by evidence suggesting that DA formed in tubular epithelial cells may act as an autocrine and/or paracrine substance in the regulation of sodium handling through the activation of specific tubular receptors (10). The inhibitory effect of DA on the reabsorption of sodium in renal tubules has been ascribed to represent the most prominent effect of the amine on the homeostasis of sodium and extracellular fluid (11). As the renal tissues are endowed with one of the highest MAO activities in the body (12), the availability of DA may be more dependent on its own degradation then on its synthesis from L-DOPA. Although both type A and B MAOs were demonstrated autoradiographically to be present in the kidney (12) and to be involved in the deamination of renal DA (13), it has been suggested that MAO-A might be located in the compartment where the synthesis of the amine occurs, whereas MAO-B would be located outside this cellular compartment (13, 14).

Since previous studies on the presence of types A and B MAO in renal tissues have been performed in kidney homogenates it was felt worthwhile to perform the assay of MAO-A and MAO-B in isolated proximal convoluted tubules (PCT of the rat kidney) using preferential substrates (5-hydroxytryptamine for MAO-A and ß-phenylethylamine for MAO-B) and determine the effect of selective inhibitors on the deamination of the two specific substrates; compounds Ro 41-1049 and lazabemide are both members of a class of highly selective, mechanism-based and reversible inhibitors for MAO-A and MAO B, respectively (15). The present study also reports on the formation and deamination of DA in isolated tubular epithelial cells and the effect of MAO-A and MAO-B selective inhibitors Ro 41-1049 and lazabemide on DA fate and DOPAC formation.

Materials and methods

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal), 45 to 60 days old and weighing 200 to 280 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12 hours light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum*. The experiments were all carried out during daylight hours.

Isolation of rat renal tubules

The isolation of renal tubules was based on the techniques previously described and the tubules obtained were found to be predominantly proximal nephron segments (16). In brief, rats were killed by decapitation under ether anaesthesia and the kidneys removed through a midline abdominal incision, after which they were decapsulated, cut in half and placed in ice-cold Collins solution [containing (in mM): KH_2PO_4, 15; K_2HPO_4, 50; KCl, 15; $NaHCO_3$, 15; $MgSO_4$, 60; and Glucose, 140; pH 7.4]. The outer cortex was cut out with fine scissors and minced with a scalpel into a fine paste. The cortical paste was filtered sequentially through a series of Nybolt nylon sieves, first 180 µm and then 75 µm. Unseparated cortex remained on the upper (180 µm) sieve, while the lower one (75 µm) retained predominantly proximal nephron segments. The sieves were rinsed continuously with cold Collins solution throughout. Thereafter the retained tubules where washed off with cold Collins solution and collected into a pellet by centrifugation at 200 *g*, for 5 min, at 4°C; renal tubules used for the deamination studies were suspended in 67 mM phosphate buffer, pH 7.2, before being homogenised.

Isolation of rat tubular epithelial cells

Tubular epithelial cells were obtained from rat renal cortex using a methodology similar to that described above. In brief, the outer cortex was cut out with fine scissors and minced with a scalpel into a fine paste like consistency, incubated with collagenase (0.065%) for 60 minutes at 37°C in an atmosphere of 95% O_2 and 5% CO_2, and thereafter poured through graded sieves (180, 75, 53,and 23 µm) to obtain a single cell suspension, as observed under light microscope.

Determination of MAO-A and B activities

The renal tubules were homogenised in 67 mM phosphate buffer, pH 7.2, at 4°C with a Thomas teflon homogeniser kept continuously on ice. MAO-A and MAO-B activities were determined using, respectively, [^{3}H]-5-hydroxytrytpamine (^{3}H-5-HT) (50 to 2000 µM) and [^{14}C]-ß-phenylethylamine (^{14}C-ß-PEA) (5 to 500 µM) as preferential substrates (17). The reaction mixture contained 50 µL of homogenate and 50 µL of 67 mM phosphate buffer and increasing concentrations of each substrate (^{3}H-5-HT for MAO-A and ^{14}C-ß-PEA for MAO-B). After 20 minutes of incubation at 37°C with continuous oxygenation and shaking, the tubes were transferred to an ice-water bath and the reaction stopped by the addition of 10 µL of 3 M HCl. The deaminated products were then extracted with ethyl acetate (500 µL) and measured by liquid scintillation counting. The MAO activity is expressed in nanomoles of substrate metabolised per mg of protein per hour of incubation (nmol mg protein^{-1} h^{-1}).

For each substrate, deamination was determined in the absence and in the presence of a MAO inhibitor. Homogenates were pre-incubated for 30 minutes at 37°C either with phosphate buffer or the inhibitor, Ro 41-1049 (250 nM) when using ^{14}C-ß-PEA (5 to 500 µM) as substrate and lazabemide (250 nM) when using ^{3}H-5-HT (50 to 2000 µM).

In another set of experiments selective MAO-A and MAO-B inhibitors, respectively Ro 41-1049 and lazabemide, were used to study the deamination of ^{3}H-5-HT and ^{14}C-ß-PEA. Homogenates were prepared the same way and pre-incubated for 30 min at 37°C in the presence of increasing concentrations of each inhibitor, ranging from 0.5 to 500 nM.

Synthesis of dopamine by tubular cells

Isolated tubular epithelial cells were either handled in a modified Hank's medium buffered with Tris HCl and supplemented with 0.5 mM $CaCl_2$, or in Dulbeco's Modified Eagle Medium (DMEM). The experiments on the synthesis of dopamine were carried out at 30° C in an atmosphere of 95% O_2 and 5% CO_2. The cell suspension was preincubated for 15 min, in a water shaking bath, and thereafter incubated with increasing concentrations of L-DOPA (1 to 100 µM) for further 15 min. In another set of experiments, the cell suspension was incubated with 50 µM L-DOPA for increasing periods of time (1 to 30 min). In both situations reactions were stopped by the addition of 2.0 mL of 0.2 M perchloric acid.

Experiments with tubular cells and MAO-A and B inhibitors

The experiments performed in order to test the effect of selective type A and B MAO inhibitors on the production of DOPAC consisted in the incubation of a suspension of isolated tubular epithelial cells in the modified Hank's medium for increasing incubation periods (1 to 30 min) with 50 µM L-DOPA; Ro 41-1049 (250 nM) and lazabemide (250 nM) were present during both the preincubation and incubation periods. Tolcapone (1 µM) was added to the incubation medium in order to inhibit catechol-O-methyltransferase (COMT) activity.

Assay of catecholamines

The assay of dopamine and DOPAC were performed by means of high-pressure liquid chromatography, as previously described (16). The mobile phase was a degassed solution of citric acid (0.1 mM), sodium octylsulphate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM) and methanol (8% v/v), adjusted to pH 3.5 with perchloric acid (2 M) and pumped at a rate of 1.0 ml/min. 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.

Protein assay

The protein content in cell and in tubule suspensions was determined according to the method of Bradford (18) with human albumin as a standard.

Viability of cells and tubules

Cells and tubules were incubated at 37°C for 2 minutes with trypan blue (0.2%w/v) in phosphate buffer and observed under light microscope. More than 95 % of cells and tubules excluded the dye, a condition for viability.

Data analysis

The V_{max} and K_m values for the deamination of ^{3}H-5-HT and ^{14}C-β-PEA, as determined in saturation experiments, were calculated by non-linear regression analysis for one site binding, using the GraphPad Prism statistics software package (19). For the calculation of the IC_{50} 's for lazabemide and Ro 41-1049 the parameters of the equation for one site inhibition were fitted to the experimental data (19). K_i 's were calculated as defined by Cheng and Prusoff (20) for competitive inhibition. Arithmetic means are given with S.E.M. or geometric means with 95% confidence values. Statistical analysis was done with a one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference.

Drugs

Drugs used were: collagenase type I (Sigma), ß-phenylethylamine hydrochloride (Sigma), 5-hydroxytryptamine hydrochloride (Sigma), [^{14}C]-ß-phenylethylamine hydrochloride (50 Ci/mmol) (NEN Chemicals), [^{3}H]-5-hydroxytryptamine creatinine sulphate (23.6 Ci/mmol) (NEN Chemicals), Ro 41-1049 [*N*-(2-aminoethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide hydrochloride] (RBI) and Ro 19-6327 (generic name lazabemide) [N-(2-aminoethyl)-5-chloro-2-pyridine carboxamide hydrochloride] (F.Hoffmann-La Roche Ltd), L-ß-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma).

Results

MAO-A and MAO B kinetics in tubular homogenates

Deamination of ^{3}H-5-HT in homogenates of isolated rat renal tubules was found to be dependent on the concentration used and to be saturable at nearly 500 μM (Fig. 1); non-linear analysis of the

saturation curve revealed a K_m of 351±71 μM (n=4) and a V_{max} of 25±2 nmol mg protein^{-1} h^{-1}. Deamination of ^{3}H-5-HT in the absence of lazabemide was of the same magnitude of that observed in the presence of the MAO-B inhibitor (Fig. 1 and Table 1). Deamination of ^{14}C-ß-PEA was also a saturable process yielding K_m values of 58±12 μM and V_{max} values of 24±2 nmol mg protein^{-1} h^{-1}. When deamination was studied in the presence of the MAO-A inhibitor Ro 41-1049 no changes in V_{max} were observed, though there was a slight rightward shift of saturation curve accompanied by a slight increase in K_m values (see Table 1).

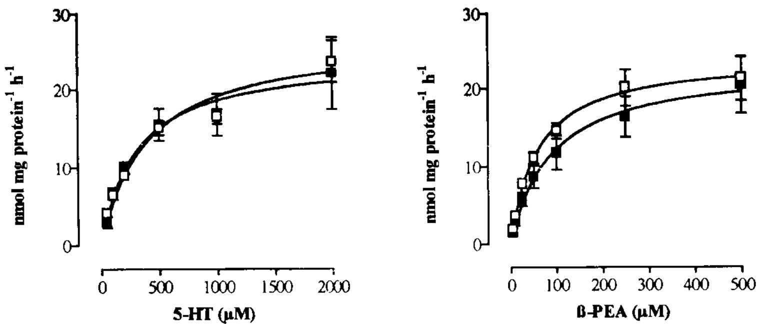

Fig. 1

MAO-A (using 5-HT) and MAO-B (using ß-PEA) activities in rat renal tubules. Control (open symbols) versus inhibitor (filled symbols; Ro 41-1049 for MAO B, and lazabemide for MAO A). Symbols represent means of four experiments per group and vertical lines show SEM.

Table 1. Kinetic parameters (K_m and V_{max}) of MAO-A and MAO-B activities in homogenates of isolated rat renal tubules. Values are means ±SEM of four duplicate determinations.

		V_{max} (nmol/mg protein/h)	K_m (μM)
ß-PEA	control	24±2	58±12
	Ro 41-1049	23±3	89±28
5-HT	control	25±2	351±71
	Lazabemide	24±3	296±112

Inhibitory constants for MAO inhibitors

In this series of experiments the effects of increasing concentrations of selective MAO-A and MAO-B inhibitors on the deamination of ^{3}H-5-HT and ^{14}C-ß-PEA were studied. Ro 41-1049 produced a concentration-dependent inhibition of ^{3}H-5-HT deamination with a K_i of 24 nM (Fig. 2 and Table 2). Deamination of ^{14}C-ß-PEA was found to be reduced by lazabemide in a concentration-dependent manner with a K_i value of 17 nM (Fig. 2 and Table 2).

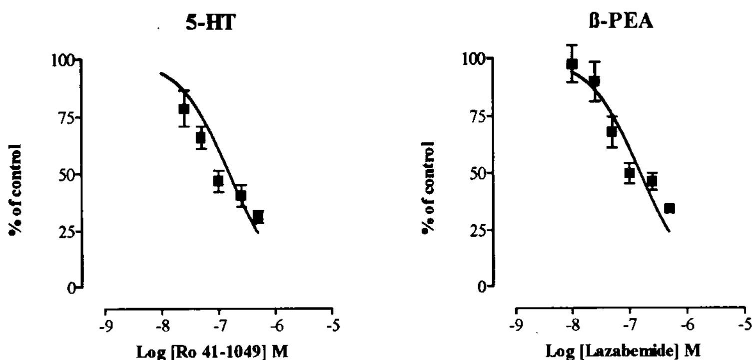

Fig. 2

Effect of increasing concentrations of Ro 41-1049 and lazabemide on rat renal tubular MAO-A and MAO-B activities. Symbols represent means of four experiments per group and vertical lines show SEM.

Table 2. IC_{50} and K_i values of Ro 41-1049 and lazabemide for inhibition of deamination of ^{3}H-5-HT (MAO-A) and ^{14}C-ß-PEA (MAO-B), as determined with virtually saturating concentrations of the substrates, in homogenates of isolated rat renal tubules. Values are geometric means with 95% confidence intervals (n=4).

	Lazabemide	Ro 41-1049
IC_{50} (nM)	142 (87;231)	91 (54;153)
K_i (nM)	17 (10;28)	24 (14;40)

Synthesis and deamination of dopamine by tubular cells

The formation of dopamine from L-DOPA and its deamination to DOPAC by renal tubular cells was found to be dependent on the concentration of the substrate, the duration of incubation and also on the type of medium used. For the same substrate concentration and incubation time the levels of dopamine and DOPAC were always higher when using Hanks' medium than with DMEM (Figs 3 and 4). The amounts of dopamine and DOPAC (in nmol mg protein^{-1}) formed in the presence of 50 µM L-DOPA were, respectively, 3.3±0.2 and 2.0±0.8 at 1 min incubation and increased to 55.1±4.0 and 17.7±0.7 at 30 min incubation for Hanks' medium. In experiments performed with DMEM these values were for dopamine and DOPAC (in nmol mg protein^{-1}), respectively, 1.86±0.6 and 0.2±0.1 at 1 min incubation, and increased to 37.6±3.0 and 6.6±0.8 at 30 min. When incubated for 15 min in the presence of 100 µM L-DOPA the amounts of dopamine and DOPAC formed (in nmol mg protein^{-1}) were, respectively, 38.8±0.5 and 9.6±0.3 for Hanks' medium and 24.1±1.4 and 4.8±0.6 for DMEM. The ratio DOPAC/dopamine was 0.3±0.02 (range 0.2-0.38) in experiments performed with Hanks' medium, compared to 0.13±0.01 (range 0.09-0.18) when using DMEM.

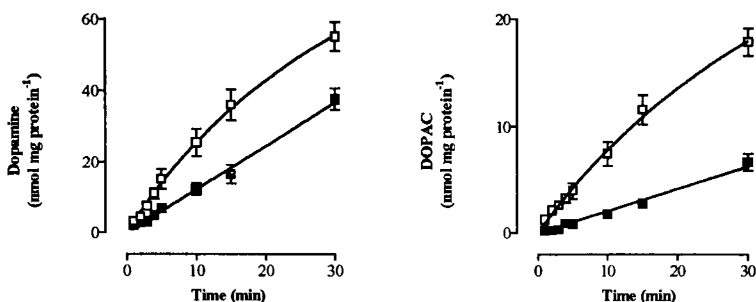

Fig. 3

Time dependence of dopamine and DOPAC formation (in nmol mg protein^{-1}) in the presence of 50 µM of L-DOPA (open symbols, Hanks' medium n=4; filled symbols, DMEM n=4-8). Symbols represent means of four to eight experiments per group and vertical lines show SEM.

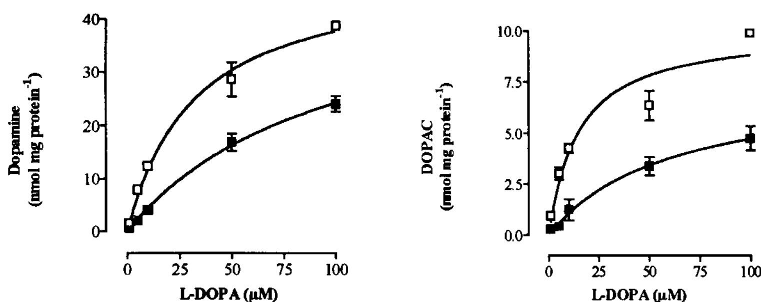

Fig. 4

Dopamine and DOPAC formed (in nmol mg protein^{-1}) in the presence of increasing concentrations of L-DOPA; incubation time was 15 minutes (open symbols, Hank's medium; filled symbols, DMEM). Symbols represent means of four experiments per group and vertical lines show SEM.

Effects of MAO inhibitors on dopamine and DOPAC formation in tubular cells

As shown in table 3, the effect of Ro 41-1049 was a reduction in the time-dependent production of DOPAC with no significant changes in the formation of dopamine. The time-dependent formation of dopamine and DOPAC in the presence of lazabemide was found to be similar to that occurring in control conditions. In experiments performed in the presence of both Ro 41-1049 (250 nM) and lazabemide (250 nM) the reduction in the formation of DOPAC was found to be similar to that obtained in the presence of the selective MAO-A inhibitor alone (Table 3). Levels of DOPAC at 30 min incubation in the presence of 250 nM Ro 41-1049 or Ro 41-1049 +

lazabemide were almost 50% those in control conditions (Table 3). The DOPAC/dopamine ratios (Fig. 5) increased with time for, both, control and lazabemide (250 nM), while remaining almost constant after Ro 41-1049 (250 nM).

Table 3. Effects of Ro 41-1049 (250 nM) and Lazabemide (250 nM) on dopamine and DOPAC formation for different incubation periods in the presence of 50 μM L-DOPA. Values are means±SEM of 6 experiments per group.

Dopamine (nmol mg protein^{-1})

Time(min)	Control	Ro 41-1049	Lazabemide	Ro 41-1049 + Lazabemide
1	1.0±0.1	0.9±0.1	0.9±0.1	1.1±0.2
3	2.9±0.5	3.3±0.3	3.0±0.2	3.0±0.3
5	5.8±0.7	5.5±0.6	5.1±0.5	5.3±0.6
10	9.1±0.8	9.9±0.9	8.9±1.2	9.7±1.2
15	12.5±1.2	12.9±1.4	12.8±2.4	12.6±1.6
30	18.9±1.9	22.4±3.0	18.6±2.2	20.7±3.2

DOPAC (nmol mg protein^{-1})

Time(min)	Control	Ro 41-1049	Lazabemide	Ro 41-1049 + Lazabemide
1	0.2±0.1	0.2±0.1	0.3±0.1	0.2±0.1
3	0.3±0.1	0.3±0.0	0.4±0.1	0.3±0.1
5	0.8±0.1	0.5±0.1 *	0.8±0.1	0.5±0.0 *
10	2.0±0.1	1.0±0.1 *	2.0±0.4	1.2±0.1 *
15	3.3±0.2	1.6±0.2 *	3.5±0.7	1.9±0.2 *
30	7.7±0.6	4.1±0.5 *	6.9±0.7	4.3±0.6 *

* ($P<0.05$) significantly different from corresponding control values by Newman-Keuls test.

Discussion

The metabolism of dopamine is mainly dictated by the presence of enzymes like catechol-O-methyltransferase (COMT) and MAO (21). The role of COMT in the metabolism of renal DA has been shown to be quite reduced (22). This information added to the knowledge that the kidney has one of the highest MAO activities in the body (12), makes deamination the leading inactivating pathway for dopamine (22). Dopamine of renal origin is deaminated to DOPAC by both forms of MAO (13). However, it has been suggested that MAO-A is predominantly active in the compartment where the synthesis of dopamine takes place, whereas MAO-B would be located outside this compartment (13, 23). Functional studies in rat kidney slices have shown that MAO-A activity in the cortex is twice as high as MAO-B (24), and that inhibition of MAO-A rather than MAO-B inhibition, results in increased accumulation of newly formed dopamine (13, 23). This would agree with the view that MAO-B might be responsible for the deamination of renal dopamine leaving the compartment where the synthesis of dopamine has occurred (23). *In vivo* studies in the rat have also shown a reduction in DOPAC tissue levels when using Ro 41-1049, but no effects were found when lazabemide was administered (25). Analysis of the outflow of dopamine and DOPAC from kidney slices also supports the suggestion that MAO-A is the main form of MAO involved in the deamination of newly-formed dopamine (14). Furthermore, the released dopamine appears to be taken up into a MAO-A rich compartment,

whereas the deamination of dopamine by MAO-B is a minor process as dictated by a slow diffusion of the amine towards the enzyme (14). The authors have even considered the possibility of a multi-compartmental system, MAO-B being located in non-tubular cells (14). Favouring this hypothesis are the data from autoradiographic studies by Saura et al. (12) who showed that MAO-A is homogeneously distributed, while MAO-B has a heterogeneous distribution throughout the renal cortex. However, denervation studies have shown not to affect MAO-A and MAO-B activities (26).

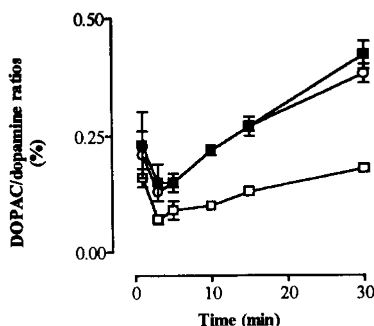

Fig. 5

DOPAC/dopamine ratios in experiments performed in isolated epithelial cells incubated with L-DOPA in the absence of inhibitor (closed squares) and the presence of Ro 41-1049 (open squares; 250 nM) or Lazabemide (open circles; 250 nM). Symbols represent means of six experiments per group and vertical lines show SEM.

The present study was intended to isolate as far as possible the functional units of the renal dopaminergic system in order to obviate the interference of surrounding cellular elements. The experiments reported here in isolated tubular epithelial cells show that these cells appear to constitute a suitable system for studying dopamine formation and amine deamination. However, the extent of amine synthesis and its deamination to DOPAC were both found to be greater when using Hanks' medium rather than DMEM, for the same concentration of L-DOPA and length of incubation. In addition, DOPAC/dopamine ratios while using Hanks' medium were greater than with DMEM. Therefore, Hanks' medium appears to be a more suitable medium. The reason for the less suitability of DMEM in experiments aimed to study the formation of dopamine from L-DOPA is not apparent, but could be related to the fact that DMEM contains large quantities of various neutral amino acids which might compete with L-DOPA for uptake (27).

In studies in which isolated tubular epithelial cells have been incubated in the presence of the MAO-A inhibitor, Ro 41-1049 (250 nM), a clear inhibition of DOPAC formation was observed. It is interesting to note that DOPAC formation was a time-dependent process, as evidenced by the progressive increase in DOPAC/dopamine ratios, and the effect of Ro 41-1049 was a suppression of this time-dependent dopamine deamination process, though it did not abolish amine deamination. As reported here, 250 nM lazabemide was found not to increase the accumulation of newly-formed dopamine in kidney slices loaded with 50 μM L-DOPA; only at higher concentrations of L-DOPA (100 and 250 μM) was Ro 41-1049 found to increase the accumulation of newly-formed dopamine (13, 23). By contrast, inhibition of MAO-B with lazabemide (250 nM) had no effect both on dopamine and DOPAC levels. Co-incubation of

lazabemide with the MAO-A inhibitor was found not to potentiate the effects of Ro 41-1049. These results re-enforce the "multi-compartment" theory, in that MAO-A appears to be mainly located in the compartment where dopamine synthesis takes place, the tubular epithelial cell. In agreement with previous data (13, 14, 23, 25), the results presented here also support the view that MAO-B is not a major entity for deamination of newly formed dopamine. However, as discussed below, epithelial cells were shown to be endowed with considerable MAO-B activity. Therefore, it is possible that the lack of involvement of MAO-B in the deamination of newly formed dopamine might be related to the reduced accessibility of the amine to MAO-B (14) as a result of its heterogeneous distribution (12).

MAO-A and MAO B differ in their amino acid sequence, substrate specificity and biological function, but they both have the same location in the mitochondrial outer membrane (2). One possible explanation for the apparent lack of involvement of MAO-B in deamination of dopamine could be the presence of different populations of mitochondria, the "A" and the "B" types, in different places within the cell. In studies performed on rat brain MAO, it was possible to separate three distinct populations of mitochondria using discontinuous Ficoll gradients (28). These were metabolically active and exhibited distinctly different MAO activities when incubated with 5-HT and β-PEA. Another aspect worth considering when understanding the apparent discrepancy between the results obtained when using cell suspensions (where lazabemide produced no effect on DOPAC formation) and the results obtained in experiments performed in cell homogenates (where lazabemide inhibited ^{14}C-ß-PEA deamination with a K_i of 17 nM) may lay in the use of different substrates. At least in the rat brain it has been shown that dopamine is largely a preferential substrate for MAO-A (29,30), while β-PEA is the traditional preferential MAO B substrate (1).

Data obtained using specific substrates for MAO-A and MAO-B clearly showed that both enzymes were present in tubular epithelial cells. The K_m values obtained for deamination of ^{3}H-5-HT and ^{14}C-ß-PEA in homogenates of renal tubules were quite similar to those reported before while using kidney homogenates (24) and the values reported for other tissues (see for a review 1). It is interesting, however, to consider the finding that V_{max} values for both MAO-A and MAO-B in homogenates of renal tubules were 5- to 6-times lower than those obtained in kidney homogenates; this may indicate that other cellular elements in the rat renal cortex rather than the tubules are endowed with MAO activities. Another argument favouring the view that both MAO-A and MAO-B are present in tubular epithelial cells concerns the inhibition produced by compounds Ro 41-1049 and lazabemide.

In conclusion, the results presented here confirm the presence of both MAO-A and MAO-B activity in renal tubular epithelial cells, that MAO-A is the predominant enzyme involved in the deamination of the natriuretic hormone dopamine and that the deamination of newly formed dopamine is a time-dependent process which occurs early after the decarboxylation of L-DOPA.

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PUBLICAÇÃO 2

"Opossum kidney (OK) cells in culture synthesize and degrade the natriuretic hormone dopamine: a comparison with rat tubular cells"

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Opossum Kidney (OK) Cells in Culture Synthesize and Degrade the Natriuretic Hormone Dopamine: A Comparison with Rat Renal Tubular Cells

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To explore further the usefulness of opossum kidney (OK) cells in the study of renal dopaminergic physiology, we have undertaken the study of aromatic L-amino acid decarboxylase (AAAD), catechol-*O*-methyltransferase (COMT) and type A and B monoamine oxidase (MAO-A and MAO-B), the main enzymes involved in the synthesis and degradation of dopamine. The V_{max} values for AAAD, using L-DOPA as the substrate, in rat renal tubular cells were found to be significantly ($P < 0.01$) higher (120-fold) than in OK cells. However, K_m values in OK cells (1.1 mM [0.3, 1.9]) were similar to those observed in rat renal tubular cells ($K_m = 1.0$ mM [0.8, 1.2]). The V_{max} values for COMT (in nmol/mg protein/30 min) in OK cells (2.1 ± 0.2) were similar to those in the rat renal tubular cells (1.6 ± 0.1), whereas K_m values in OK cells (2.3 μM [0.1, 4.5]) differ considerably (4.8-fold, $P < 0.01$) from those in rat renal tubular cells (11.2 μM [9.2, 13.1]). The V_{max} values (in nmol/mg protein/20 min) for deamination of [^{3}H]-5-hydroxytryptamine, the specific MAO-A substrate, was similar in rat renal tubular cells (12.4 ± 1.0) and OK cells (12.9 ± 1.1); K_m values also did not differ between these two preparations. In contrast to rat renal tubular cells, deamination of [^{14}C]-β-phenylethylamine, the substrate for MAO-B, in OK cells was found to be non-saturable and to represent less than 10% of that observed in homogenates of rat tubular cells. In conclusion, OK cells in culture are endowed with the synthetic and metabolic machinery needed to form and degrade dopamine. The amounts of the enzymes AAAD, COMT and MAO-A found in this cell line are likely to be sufficient to reproduce, under *in vitro* conditions, the environment in which the renal dopaminergic system normally operates.
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INTRODUCTION

Dopamine of renal origin is believed to play a considerable role in the regulation of tubular sodium handling, as a result of activation of specific tubular dopamine receptors (Lee, 1993). It is assumed that these effects on tubular sodium absorption are the consequence of inhibition of Na^+–K^+-ATPase and Na^+–H^+ exchanger activities at the level of the proximal nephron (Aperia *et al.*, 1987; Bertorello and Aperia, 1990; Felder *et al.*, 1990). The source of dopamine responsible for the natriuretic effects of the amine is believed to reside in epithelial cells of the proximal convoluted tubules. These cells are rich in aromatic L-amino acid decarboxylase (AAAD) activity, use circulating or filtered L-DOPA as a source for dopamine, and have been suggested to be the basic entities of a local non-neuronal dopaminergic system (Soares-da-Silva, 1994; Soares-da-Silva and Fernandes, 1990a). An interesting particularity of this system concerns the fact that these AAAD-rich epithelial cells also are endowed with dopamine receptors, leading to the possibility that dopamine may act as an

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autocrine/paracrine substance (Siragy *et al.*, 1989).

Several renal cell lines are often used as model physiological systems of renal proximal tubule function, namely because, in most cases, their utilization enables free access to apical and basolateral cell surfaces. The OK cells are an established epithelial cell line, derived from the kidney of a female American opossum, which have been used for the purpose of studying dopamine receptors and the renal actions of the amine (Bates *et al.*, 1993; Glahn *et al.*, 1993; Nash *et al.*, 1993; Perrichot *et al.*, 1995). The OK cells can synthesize dopamine from L-DOPA in sufficient quantities to elicit both the maximum dopamine-stimulated cAMP accumulation and inhibition of Na^+–Pi co-transport (Glahn *et al.*, 1993). The OK cells were first shown to express exclusively the D_{1A} receptor, though the expression of both dopamine D_{1A} and D_{1B} receptor subtypes was detected in the opossum brain and kidneys (Nash *et al.*, 1993). More recently, Perrichot *et al.* (1995), while studying the involvement of dopamine receptor subtypes in the regulation of renal P_i transport by dopamine, either from exogenous sources or synthesized locally from L-DOPA, came to the conclusion that OK cells also synthesize dopamine and the newly formed amine modulates phosphate transport through the activation of both D_1 and D_2 receptors, positively and negatively coupled to adenylate cyclase.

The amount of dopamine that is available for the activation of dopamine receptors may depend not only on the delivery of L-DOPA to the kidney and on the activity of AAAD, but also on the extent of dopamine degradation. In fact, renal tissues are endowed with one of the highest monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT) activities in the body (Kopin, 1985), and previous work has shown a relevant role of both type A and B MAO and COMT in the deamination and *O*-methylation, respectively, of newly formed dopamine in kidney slices incubated with L-DOPA (Fernandes *et al.*, 1991; Fernandes and Soares-da-Silva, 1990, 1993; Pestana and Soares-da-Silva, 1994a; Soares-da-Silva and Fernandes, 1990b; Vieira-Coelho *et al.*, 1994). In contrast to the process described in the kidney 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 dopamine in OK cells. To explore further the usefulness of OK cells for the study of renal dopaminergic physiology, we have undertaken the study of AAAD, COMT, MAO-A and MAO-B activities in these cells using specific substrates. Since most of information on the renal dopaminergic system has been obtained using rat renal proximal convoluted tubules, we decided also to use this preparation for the sake of comparison.

METHODS

Cell culture

The OK cells (ATCC CRL 1840) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and maintained in a humidified atmosphere of 5% CO_2–95% air at 37°C. The OK cells (passages 36–43) were grown in Minimum Essential Medium (Sigma Chemical Co., MO, U.S.A.) supplemented with 10% foetal bovine serum (Sigma), 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin (Sigma) and 25 mM *N*-2-hydroxyethylpiperazine-*N′*-2-ethanosulfonic acid (HEPES; Sigma). The cell medium was changed every 2 days, and the cells reached confluence after 3–5 days of seeding. For subculturing, the cells were dissociated with 0.05% trypsin–EDTA (Sigma), split 1:4 and subcultured in flasks with 75 or 162 cm^2 growth areas (Costar, Badhoevedorp, The Netherlands). For 24 hr prior to each experiment, the cell medium was free of foetal bovine serum. Experiments generally were performed 2–3 days after cells reached confluence and 6 days after the initial seeding and each cm^2 contained about 100 μg of cell protein.

Isolation of rat renal tubules

The isolation of renal tubules was based on the techniques previously described and the tubules obtained were found to be predominantly proximal nephron segments (Soares-da-Silva *et al.*, 1994). In brief, rats were killed by decapitation under ether anaesthesia and the kidneys removed through a midline abdominal incision, after which they were decapsulated, cut in half and placed in ice-cold Collins solution [containing (in mM): KH_2PO_4, 15; K_2HPO_4, 50; KCl, 15; $NaHCO_3$, 15; $MgSO_4$, 60; and glucose, 140; pH 7.4]. The outer cortex was cut out with fine scissors and minced with a scalpel into a fine paste. The cortical paste was filtered sequen-

tially through a series of Nybolt nylon sieves, first 180 μm and then 75 μm. Unseparated cortex remained on the upper (180 μm) sieve, while the lower one (75 μm) retained predominantly proximal nephron segments. The sieves were rinsed continuously with cold Collins solution throughout. The retained tubules then were washed off with cold Collins solution and collected into a pellet by centrifugation at 200 *g*, 5 min, 4°C; renal tubules used in incubation experiments were suspended in Hanks' medium. The Hanks' medium had the following composition (mM): NaCl, 137; KCl, 5; $MgSO_4$, 0.8; Na_2HPO_4, 0.33; KH_2PO_4, 0.44; $CaCl_4$, 0.25; $MgCl_4$, 1.0; Tris–HCl, 0.15; and sodium butyrate, 1.0; pH 7.4.

AAAD preparation and decarboxylation studies

The OK cells and rat renal tubular cells were homogenized in 0.5 M phosphate buffer (pH 7.0) with a Thomas teflon homogenizer 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 (50–5000 μM) was added to the medium for a further 15 min; the final reaction volume was 1 ml. The composition of the incubation medium was as follows (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) also were 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 (Shirota and Fujisawa, 1988). During incubation, homogenates of renal tubules were continuously shaken and gassed (95% O_2 and 5% CO_2) and maintained at 37°C. The reaction was stopped by the addition of 500 μl of 2 M perchloric acid and the preparations kept at 4°C for 60 min. The samples were then centrifuged (200 *g*, 2 min, 4°C) and 500 μl aliquots of the supernatant filtered on Spin-X filter tubes (Costar) and injected directly into the column of a high pressure liquid chromatograph for the quantification of dopamine.

COMT preparation and O-methylation studies

The COMT activity was evaluated by the ability of cell homogenates to methylate adrenaline to metanephrine, as previously described (Vieira-Coelho and Soares-da-Silva, 1996). 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 adrenaline (1–100 μM; 50 μl) in the presence of a saturating concentration of the methyl donor (*S*-adenosyl-L-methionine, 100 μM; Axelrod and Tomchick, 1958); the incubation medium also contained pargyline (100 μM), $MgCl_2$ (100 μM) and EGTA (1 mM). The preincubation and incubation were carried out at 37°C, in the dark, with continuous shaking and without oxygenation. At the end of the incubation period, the tubes were transferred to ice and the reaction was stopped by the addition of 25 μl of perchloric acid (2 M) and the final volume made up to 575 μl with 0.2 M perchloric acid. The samples then were centrifuged (200 *g*, 4 min, 4°C) and 300 μl aliquots of the supernatant filtered on Spin-X filter tubes (Costar) were used for the assay of metanephrine.

Assay of catecholamines

The assays for dopamine and metanephrine were performed by means of high pressure liquid chromatography, as previously described (Soares-da-Silva *et al.*, 1994). The mobile phase was a degassed solution of citric acid (0.1 mM), sodium octylsulphate (0.5 mM), sodium acetate (0.1 M), EDTA (0.17 mM), dibutylamine (1 mM) and methanol (8% v/v), adjusted to pH 3.5 with perchloric acid (2 M) and pumped at a rate of 1.0 ml/min. 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, dopamine, adrenaline and metanephrine ranged between 350 and 500 fmol.

MAO preparation and deamination studies

The MAO activity was determined in cell homogenates, as previously described (Fernandes and Soares-da-Silva, 1992). The OK cells and rat renal tubular cells were homogenized in 67 mM phosphate buffer, pH 7.2, at 4°C with a Thomas teflon homogenizer kept continuously on ice. The MAO activity was determined with [^{3}H]-5-hydroxytryptamine ([^{3}H]-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. The reaction mixture contained 50 μl of cell homogenate and 50 μl of 67 mM phosphate buffer and increasing concentrations of [^{3}H]-5-HT (50–2000 μM) and [^{14}C]-β-PEA (5–500 μM). 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 10 μl of 3 M HCl. The deaminated products were then extracted with ethyl acetate (500 μl) and measured by liquid scintillation counting. The MAO activity is expressed in nanomoles of substrate metabolized per mg of protein per 20 min of incubation (nmol/mg protein/20 min).

Protein assay

The protein content in cell homogenates (approx. 2 mg/ml), as determined by the method of Bradford (1976) with human serum albumin as a standard, was similar in all samples.

Alkaline phosphatase and γ-glutamyl transferase

Alkaline phosphatase (ALKP) and γ-glutamyl transferase (γ-GT) activities in homogenates of OK cells and renal tubules were measured using standard enzymic techniques (p-nitrophenyl phosphate and L-γ-glutamyl-p-nitroanilide as substrates for ALKP and γ-GT, respectively) on a Kodak Ektachem 250 analyser (Eastman Kodak Company, Clinical Diagnostics Division, Rochester, NY, U.S.A.).

Cell viability

The OK cells and rat renal tubular cells were preincubated for 15 min at 37°C and then incubated in the absence or the presence of L-DOPA, [^{3}H]-5-HT and [^{14}C]-β-PEA or adrenaline for a 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 95% of the cells excluded the dye.

Data analysis

The V_{max} and K_m values for the decarboxylation of L-DOPA, O-methylation of adrenaline or deamination of [^{3}H]-5-HT and [^{14}C]-β-PEA were calculated from non-linear regression analysis using the GraphPad Prism statistics software package (Motulsky *et al.*, 1994). Geometric means are given with 95% confidence limits and arithmetic means are given with SEM. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Student's t-tests for unpaired comparisons. A P value less than 0.05 was assumed to denote a significant difference.

Drugs

Drugs used were: adrenaline bitartrate (Sigma Chemical Company, St Louis, MO, U.S.A.), [^{3}H]-5-hydroxytryptamine creatinine sulphate (23.6 Ci/mmol) (NEN Chemicals, Dreieichenhain, Germany), 5-hydroxytryptamine hydrochloride (Sigma), β-phenylethylamine hydrochloride (Sigma), [^{14}C]-β-phenylethylamine hydrochloride (50 Ci/mmol) (NEN Chemical), pargyline hydrochloride (Sigma) and tolcapone (kindly donated by the late Professor Mosé Da Prada, Hoffman La Roche, Basle, Switzerland.

RESULTS

AAAD activity

Incubation of homogenates of renal tubular cells with L-DOPA (50–5000 μM) resulted in a concentration-dependent formation of dopamine (Fig. 1). The V_{max} values for AAAD using L-DOPA as the substrate in rat renal tubular cells were found to be significantly ($P < 0.01$) higher than those observed in OK cells (see Table 1). In fact, AAAD in rat renal tubular cells was approx. 120-fold that observed in OK cells. However, the decarboxylation reaction in OK cells was a saturable process with a K_m (1.1 mM [0.3, 1.9]) similar to that observed in rat renal tubular cells (K_m = 1.0 mM [0.8, 1.2]).

COMT activity

Incubation of homogenates of OK cells and rat renal tubular cells in the presence of increasing concentrations of adrenaline resulted in a concentration-dependent formation of metanephrine (Fig. 2). The kinetics, (V_{max} and K_m values) for COMT are given in Table 1. As shown in this table, the highest V_{max} values for COMT are those in OK cells closely followed by those in the rat renal tubular cells; however, differences in V_{max} values for COMT in these two preparations attained statistical significance ($P < 0.05$). The K_m values in OK cells (2.3 μM [0.1, 4.5]) differ considerably (4.8-fold, $P < 0.01$) from those in rat renal tubular cells.

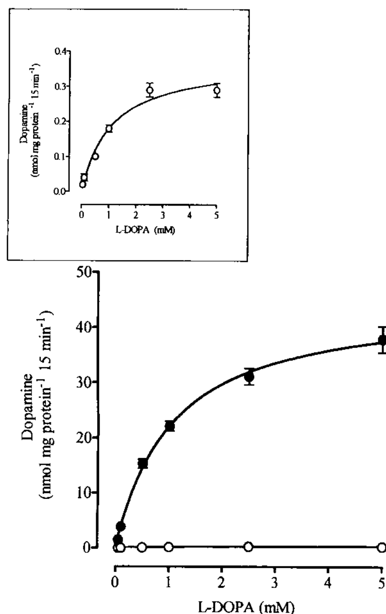

Fig. 1. Decarboxylation of L-DOPA (50–5000 μM) in homogenates of OK cells (open circles) and rat renal tubular cells (closed circles); inset shows non-linear decarboxylation of L-DOPA in OK cell homogenates. The results are levels (in nmol/mg protein/15 min) of dopamine formed during a 15 min incubation period. Each point represents the mean of five experiments per group; vertical lines show SEM.

MAO activity

Rat renal tubular cells were found to deaminate quite actively both [^{3}H]-5-HT and [^{14}C]-β-PEA. Figure 3(A) and (B) show the saturation curves obtained when homogenates of rat tubular cells were incubated in the presence of increasing concentrations of [^{3}H]-5-HT and [^{14}C]-β-PEA, respectively. Deamination of ^{3}H-5-HT by OK cells also was found to be dependent on the concentration of the substrate and was similar to that observed in rat renal tubular cells [Fig. 3(A)]; as shown in Table 1, K_m values for MAO-A did not differ significantly between the two preparations. By contrast, when OK cells were incubated in the presence of the substrate for MAO-B, deamination of [^{14}C]-β-PEA was found to be non-saturable and representing less than 10% of that observed in homogenates of rat tubular cells [Fig. 3(B)]. The kinetics (V_{max} and K_m) of MAO-A and MAO-B are shown in Table 1.

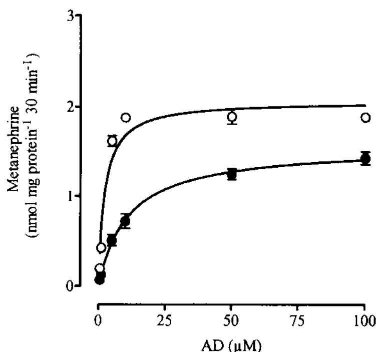

Fig. 2. The O-methylation of increasing concentrations (1–100 μM) of adrenaline in homogenates of OK cells (open circles) and rat renal tubular cells (closed circles). The results are levels (in nmol/mg protein/30 min) of metanephrine formed from added adrenaline. Each point represents the mean of five experiments per group; vertical lines show SEM.

Table 1. Kinetic parameters (V_{max} and K_m) of AAAD, COMT, MAO-A and MAO-B activities in homogenates of OK cells and homogenates of rat renal tubular cells

	OK cells	Rat renal tubular cells
AAD		
V_{max} (nmol/mg protein/15 min)	0.37 ± 0.03	45.1 ± 1.1**
K_m (mM)	1.1 (0.3, 1.9)	1.0 (0.8, 1.2)
COMT		
V_{max} (nmol/mg protein/30 min)	2.1 ± 0.2	1.6 ± 0.1*
K_m (μM)	2.3 (0.1, 4.5)	11.2 (9.2, 13.1)**
MAO-A		
V_{max} (nmol/mg protein/20 min)	12.9 ± 1.1	12.4 ± 1.0
K_m (μM)	407.9 (150.5, 665.3)	191.6 (84.4, 298.8)
MAO-B		
V_{max} (nmol/mg protein/20 min)	n.s.	9.0 ± 1.2
K_m (μM)	n.s.	104.3 (27.8, 180.8)

Values are arithmetic means ± SEM and geometric means with 95% confidence intervals ($n = 5$).
n.s., non-saturable reaction [see Fig. 3(B)].
Significantly different from corresponding control values (*$P < 0.05$; **$P < 0.01$) using Student's t-test.

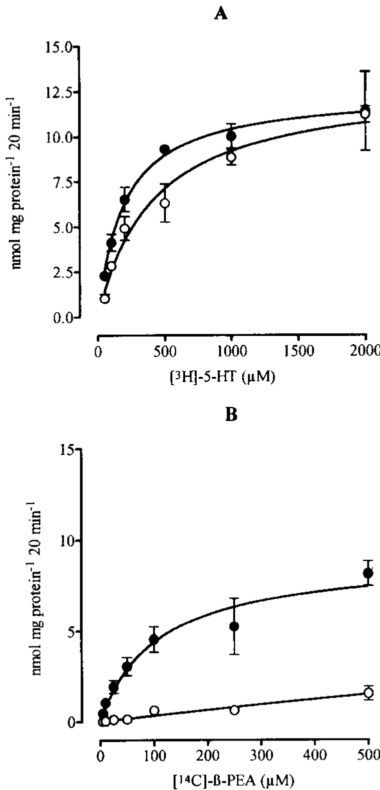

Fig. 3. (A) Type A monoamine oxidase activity (deamination of [^{3}H]-5-HT; 50–2000 μM); (B) type B monoamine oxidase activity (deamination of [^{14}C]-β-PEA; 5 500 μM) in homogenates of OK cells (open circles) and rat renal tubular cells (closed circles). Each point is the mean of five experiments per group; vertical lines indicate SEM.

ALKP and γ-GT activities

As shown in Table 2, both ALKP and γ-GT activities in homogenates of rat renal tubules were found to be significantly higher than in homogenates of OK cells. This difference was evident particularly for γ-GT activity, the enzyme activity in the rat tubular preparation being 2800-fold that in OK cell homogenates. The OK cells also are endowed with a low ALKP activity (330-fold difference when compared with the rat renal preparation).

Table 2. Alkaline phosphatase (ALKP) and γ-glutamyl transferase (γ-GT) activities in homogenates of OK cells and homogenates of rat renal tubular cells

	OK cells	Rat renal tubular cells
ALKP	0.01 ± 0.003	3.3 ± 0.1*
γ-GT	0.005 ± 0.001	14.0 ± 1.2*

Values (in U/mg protein) are means ± SEM ($n = 5$). Significantly different from corresponding values in OK cells (*$P < 0.01$) using Student's t-test.

DISCUSSION

The results presented here show that OK cells are endowed with all the necessary synthetic and metabolic enzyme machinery to form and degrade dopamine, though significant differences do exist when data on OK cells are compared with results obtained in rat renal tubular cells.

Previous studies have shown that OK cells were endowed with the ability to decarboxylate L-DOPA to dopamine and the newly formed amine was responsible for the activation of specific dopamine receptors, as an autocrine/paracrine substance (Glahn *et al.*, 1993; Perrichot *et al.*, 1995). The present study is the first to demonstrate that the efficiency of this decarboxylation process is similar to that in rat renal proximal tubular cells. In fact, K_m values for AAAD in OK cells were similar to those in the rat renal preparation. However, the activity of an enzyme is reflected by the rate constant:

$$K_{enzyme} = V_{max}/K_m,$$

and this equation indicates that the higher the V_{max} and the lower K_m, the higher will be the activity of the enzyme (Trendelenburg, 1988). Accordingly, K_{enzyme} for rat renal tubular cells ($K_{enzyme} = 45.08$) is approx. 130-fold that in OK cell ($K_{enzyme} = 0.34$). Obviously, this is an important aspect to have in mind when considering OK cells as a model of the renal dopaminergic system; per mg of protein, decarboxylation of L-DOPA in rat renal tubular cells is much more than in OK cells. It is possible, however, that this difference might be even greater, since there is no evidence that all tubular cells can decarboxylate L-DOPA to dopamine and OK cells in culture constitute a homogeneous cell population.

The *O*-methylation catalysed by COMT proceeds by a pathway in which *S*-adenosyl-L-methionine serves as a methyl donor, is dependent upon Mg^{2+} ions, occurs preferentially at the *m*-hydroxyl group and adrenaline is the preferred substrate among the three endogenous catecholamines (dopamine, noradrenaline and adrenaline) (Roth, 1992; Trendelenburg, 1988). The results obtained here on the

O-methylation of adrenaline show that the reaction is slightly greater, but statistically significantly, in OK cells than in rat renal tubular cells. The main difference, however, between these two cell preparations concerns the affinity for the substrate in rat renal COMT and OK cell COMT. In fact, K_m values for rat renal COMT were 4.8-fold those for OK cell COMT. The possibility that this difference might be related to the heterogeneous origin of the cell population in these preparations is rather unlikely. The most likely explanation might be in considering the presence in OK cells of a greater proportion of membrane-bound COMT (MB-COMT). In fact, there are two major classes of COMT based on their subcellular location a soluble cytosolic form (S-COMT) and a membrane-bound form (Roth, 1992; Vidgren *et al.*, 1994). The S-COMT generally is assumed to be the predominant form of the enzyme, as indicated by greater V_{max} values in comparison with those observed for MB-COMT. In contrast, MB-COMT is a biochemically distinct entity and is endowed with a higher affinity for the catechol substrates (Roth, 1992).

Dopamine is a substrate for both MAO-A and MAO-B, but deamination of dopamine in the kidney proceeds through a pathway in which MAO-A is the predominant form of MAO involved in deamination of the amine (Fernandes and Soares-da-Silva, 1990; Pestana and Soares-da-Silva, 1994a; Vieira-Coelho *et al.*, 1994). The rat kidney is endowed with both enzymes, as well as the human kidney (Fernandes and Soares-da-Silva, 1992). However, OK cells appear to be endowed only with MAO-A, since almost no deamination of $[^{14}C]$-β-PEA was found to take place in this preparation. The form of MAO-A and the activity of the enzyme in OK cells is quite similar to that occurring in rat renal tubular cells; in fact, K_m and V_{max} values are not statistically different between the two cell preparations.

In the present study, we have examined also the ALKP and γ-GT activities in homogenates of rat renal tubules and OK cells and found in the former cell preparation an almost complete absence of these enzymes. This agrees with results reported by other authors (Netzer and Gstraunthaler, 1993). The consequences of this deficiency are difficult to predict, but with respect to the renal dopaminergic system it should be pointed out that the lack of γ-GT will definitively compromise the synthesis of dopamine from γ-glutamyl-L-DOPA, a dopamine prodrug (Lee, 1993; Pestana and Soares-da-Silva, 1994b).

In conclusion, OK cells in culture are endowed with the synthetic and metabolic machinery needed to form and degrade dopamine. The amounts of the enzymes AAAD, COMT and MAO-A found to occur in this cell line are most probably quite sufficient to reproduce, in *in vitro* conditions, the environment in which the renal dopaminergic system normally operates.

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Capítulo III

Actividade da MAO-A e da MAO-B no jejuno. Estudos realizados em jejuno de rato e numa linha celular intestinal.

PUBLICAÇÃO 3

"Inhibitory effects of Ro 41-1049, lazabemide and tolcapone on rat jejunal MAO-A, MAO-B and COMT activities"

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INHIBITORY EFFECTS OF RO 41-1049, LAZABEMIDE AND TOLCAPONE ON RAT JEJUNAL MAO-A, MAO-B AND COMT ACTIVITIES

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The kinetics (V_{max} in nmol mg protein^{-1} h^{-1}; K_m in μM) of jejunal MAO-A (V_{max} = 33 ± 7 and K_m = 318 ± 32) and MAO-B (V_{max} = 38 ± 5 and K_m = 49 ± 7) were not affected by the addition of 250 nM Ro 19-6327 (MAO-B inhibitor) or 250 nM Ro 41-1049 (MAO-A inhibitor). Preincubation of homogenates with increasing concentrations of Ro 41-1049 (10 to 500 nM) produced a concentration decrease of ^{3}H-5-hydroxytryptamine deamination [IC_{50} = 222 nM (176, 281; 95% confidence limits)] (n = 4), whereas preincubation of homogenates with increasing concentrations of Ro 19-6327 (10 to 500 nM) produced a concentration decrease of ^{14}C-β-phenylethylamine deamination [IC_{50} = 274 nM (213, 353)] (n = 4). The kinetics (V_{max} in nmol mg protein^{-1} h^{-1}; K_m in μM) of COMT were as follows: V_{max} = 0.8 ± 0.1 and K_m = 81 ± 22. Tolcapone produced a concentration dependent decrease in the methylation of adrenaline in the intestinal mucosa [IC_{50} = 52 nM (49, 55)]. In conclusion, the results presented here show that the jejunal mucosa is endowed with MAO-A, MAO-B and COMT activities, which might be of physiological relevance in the metabolism of locally produce monoamines (dopamine and 5-hydroxytryptamine). Furthermore, Ro 41-1049, lazabemide and tolcapone are potent inhibitors of jejunal MAO-A, MAO-B and COMT, respectively.

KEY WORDS: MAO-A, MAO-B, COMT, jejunal mucosa, Ro 41-1049, lazabemide, tolcapone

INTRODUCTION

Increasing evidence in the past few years has suggested that dopamine may modulate a variety of intestinal functions, namely secretion of fluids, absorption of electrolytes and fluids, motility and control of blood flow (for review see Lefebvre *et al.*, 1988). The jejunal mucosa is endowed with a high aromatic L-amino acid decarboxylase activity (Vieira-Coelho and Soares-da-Silva, 1993) and a considerable amount of jejunal dopamine has been shown to derive from the decarboxylation of L-DOPA in epithelial cells of the jejunal mucosa (Bertorello *et al.*, 1993). There is evidence to suggest that jejunal dopamine has a role in the jejunal handling of water and electrolytes, this being particularly important in conditions of high sodium intake (Fynkel *et al.*, 1994). The physiological relevance of jejunal dopamine depends, however, on the amount and availability of the amine to activate its specific receptors, but this may be limited by the fact that the endogenous amine is subjected

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to extensive metabolism, namely deamination by monoamine oxidase (MAO) (Vieira-Coelho and Soares-da-Silva, 1993; Fynkel *et al.*, 1994).

The aim of the present work is to determine type A and B MAO activities in the jejunal mucosa of the rat and evaluate the sensitivity of the enzymes to inhibition by Ro 41-1049 and lazabemide (Ro 19-6327), two selective and reversible MAO-A and MAO-B inhibitors, respectively (Da Prada *et al.*, 1990). Because dopamine can also undergo methylation to 3-methoxytyramine and homovanillic acid, the activity of jejunal catechol-O-methyltransferase (COMT) was also determined, as well its sensitivity to inhibition by tolcapone (Ro 40-7592) (Borgulya *et al.*, 1989).

MATERIALS AND METHODS

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal) aged 45–60 days and weighting 200–300 g were used. Animals were kept two per cage under controlled environmental conditions (12 h light/dark cycle and room temperature 24°C). Food and tap water were allowed ad libitum. The rats were sacrified by decapitation under ether anaesthesia and the jejunum removed, cut in segments, opened longitudinally with fine scissors and rinsed free from blood and the jejunal content with saline (0.9% NaCl).

MAO activity was determined in homogenates of jejunal mucosa, as previously described (Fernandes and Soares-da-Silva, 1992). The jejunal mucosa was removed with a scalpel and homogenized in 67 mM phosphate buffer, pH = 7.2, at 4°C with a Duall-Kontes homogeneizer to a protein concentration of approximately 4 mg/ml of homogenate. MAO activity was determined with [^{3}H]-5-hidroxytryptamine ([^{3}H]-5-HT) as a preferential substrate for MAO-A and [^{14}C]-β-phenyletylamine ([^{14}C]-β-PEA) as a preferential substrate for MAO-B. The reaction mixture contained 50 μl of tissue homogenate and 50 μl of 67 mM phosphate buffer and increasing concentrations of [^{3}H]-5-HT (50 to 2000 μM) and [^{14}C]-β-PEA (5 to 500 μM). After 20 min of incubation at 37°C with oxygenation and continuous shaking, the tubes were transfered to an ice water bath and the reaction was stopped by the addition of 10 μl of 3 M HCl. The deaminated products were then extracted with ethyl acetate (500 μl) and measured by liquid scintillation counting. MAO activity is expressed in nanomoles of substrate metabolized per mg of protein per h of incubation (nmol mg protein^{-1} h^{-1}). The protein content in homogenates was similar in all samples. K_m and V_{max} values for the metabolization of [^{3}H]-5-HT and [^{14}C]-β-PEA in the jejunal mucosa were calculated by Eadie-Hofstee transformation of the data (V against V/S). For the calculation of the IC_{50} 's the parameters of the Hill-equation for multisite inhibition were fitted to the experimental data (Segel, 1975).

COMT activity was determined by evaluating the ability of jejunal homogenates to methylate adrenaline into metanephrine. Aliquots of 0.5 ml of the homogenate were preincubated for 20 min with 0.5 ml of phosphate buffer (0.5 M); thereafter, the reaction mixture was incubated for 30 min with increasing concentrations of adrenaline (5 to 500 μM) in the presence of a methyl donor (S-adenosylmethionine; 100 μM); the incubation medium contained also pargyline (100 μM), $MgCl_2$ (100 μM) and EGTA (1 mM). The preincubation and incubation were carried out at 37°C, in conditions of light protection, with continuous shaking and without oxygenation. At the end of the incubation period the tubes were transfered to ice and the reaction was stopped by the addition of 50 μl of perchloric acid (2 M). The

samples were then centrifuged (200 g, 4 min, 4°C) and 500 μl aliquots of the supernatant filtered on Millipore microfilters (MF1) were used for the assay of metanephrine by means of high pressure liquid chromatography with electrochemical detection (Soares-da-Silva *et al.*, 1994). K_m and V_{max} values for the metabolism of adrenaline in the jejunal mucosa were calculated by Eadie-Hofstee transformation of the data (V against V/S).

The protein content in jejunal homogenates was determined by the method of Bradford (1976) with human serum albumin as standard. The protein content was similar in all samples (approximately 0.10 mg 50 μl^{-1} homogenate).

DRUGS

Drugs used were: adrenaline bitartrate (Sigma Chemical Company, St. Louis, Mo, USA), [^{3}H]-5-hydroxytryptamine creatinine sulphate (23.6 Ci $mmol^{-1}$) (NEN Chemicals, Dreieichenhain, Germany), 5-hydroxytryptamine hydrochloride (Sigma), β-phenylethylamine hydrochloride (Sigma), [^{14}C]-β-phenylethylamine hydrochloride (50 Ci $mmol^{-1}$) (NEN Chemical), pargyline hydrochloride (Sigma), RO 41-1049 [N-(2-aminoethyl)-5-(3-fluorophenyl)-4-thiazolecarboxamide hydrochloride] (Research Biochemicals International, Natick, MA, USA). Lazabemide and tolcapone were kindly donated by Professor M. Da Prada (Hoffmann La Roche, Basle, Switzerland).

RESULTS

The kinetics (V_{max} in nmol mg $protein^{-1}$ h^{-1}; K_m in μM) of jejunal MAO-A and MAO-B were as follow (n = 7): MAO-A, V_{max} = 33 ± 7 and K_m = 318 ± 32; MAO-B, V_{max} = 38 ± 5 and K_m = 49 ± 7. Deamination of ^{3}H-5-HT in the presence of 250 nM Ro 19-6327 (V_{max} = 34 ± 6 and K_m = 343 ± 40) was similar to that observed in the absence of the MAO-B inhibitor (Fig. 1A). Deamination of ^{14}C-β-PEA in the presence of 250 nM Ro 41-1049 (V_{max} = 35 ± 9 and K_m = 53 ± 7) was also similar to that observed in the absence of the MAO-A inhibitor (Fig. 1B). Preincubation of homogenates with increasing concentrations of Ro 41-1049 (10 to 500 nM) produced a concentration dependent decrease of ^{3}H-5-HT deamination [IC_{50} = 222 nM (176, 281; 95% confidence limits)] (n = 4) (Fig. 2). Preincubation of homogenates with increasing concentrations of Ro 19-6327 (10 to 500 nM) produced a concentration dependent decrease of ^{14}C-β-PEA deamination [IC_{50} = 274 nM (353, 213)] (n = 4).

The kinetics (V_{max} in nmol mg $protein^{-1}$ h^{-1}; K_m in μM) of COMT were as follows: V_{max} = 0.8 ± 0.1 and K_m = 100 ± 14 (Fig. 3). Tolcapone produced a marked decrease in the methylation of adrenaline in the intestinal mucosa; the lowest concentration of tolcapone used (0.1 μM) reduced the methylation of adrenaline by 96% (Figure 4).

DISCUSSION

The results presented here show that the isolated jejunal mucosa is endowed with considerable MAO-A, MAO-B and COMT activities. Renal tubular epithelial cells have also been shown to produce dopamine from L-DOPA, similar to that described in the jejunal mucosa, and most of the newly-formed amine is deaminated to DOPAC, whereas only a minor

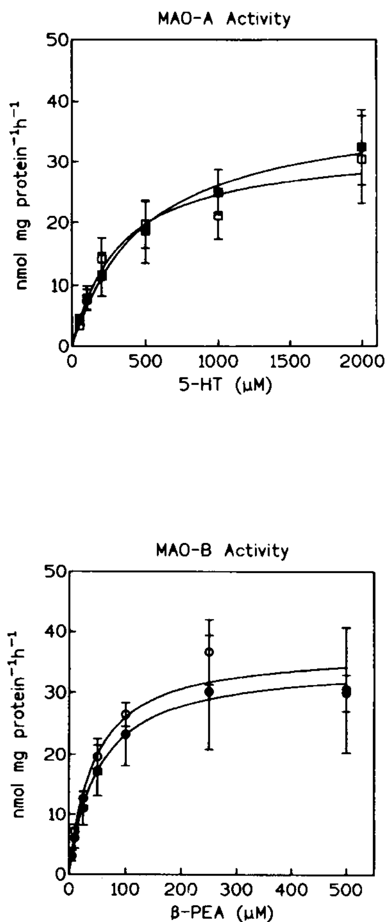

FIGURE 1 Part A shows type A monoamine oxidase activity in homogenates of rat jejunal mucosa in the absence (open squares) and the presence of 250 nM lazabemide (closed squares). Part B shows type B monoamine oxidase activity in homogenates of rat jejunal mucosa in the absence (open circles) and the presence of 250 nM Ro 41-1049 (closed circles). Each point is the mean of 4 to 6 experiments per group; vertical lines indicate S.E.M.

amount is methylated to 3-methoxytyramine (Fernandes and Soares-da-Silva, 1993). In renal tubular epithelial cells, newly-formed dopamine is deaminated to DOPAC predominantly by MAO-A. The kinetic characteristics of renal MAO-A (V_{max} = 62 ± 2 nmol mg protein^{-1} h^{-1} and K_m = 221 ± 13 μM) and MAO-B (V_{max} = 31 ± 1 nmol mg protein^{-1} h^{-1} and K_m = 39 ± 5 μM) (Fernandes and Soares-da-Silva, 1992) are similar to those described here for the jejunal mucosa; the main difference is that the rate of maximal activity of MAO-A in the kidney is twice that observed in the jejunal mucosa. It is possible, therefore, that in the jejunum MAO-B may assume a more important role in the deamination of endogenous

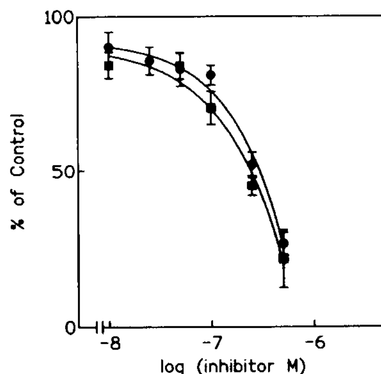

FIGURE 2 Effect of increasing concentrations of Ro 41-1049 (squares) and lazabemide (circles) on type A and B monoamine oxidase activities in the rat jejunal mucosa, respectively. Each point is the mean of 4 to 6 experiments per group; vertical lines indicate S.E.M.

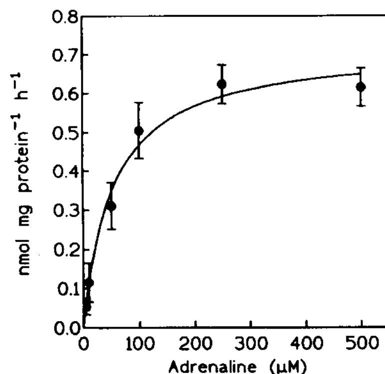

FIGURE 3 Catechol-O-methyltransferase activity in homogenates of rat jejunal mucosa. Each point represents the mean of four experiments per group; vertical lines indicate S.E.M.

dopamine, in contrast to that described in the kidney. By contrast, it might be suggested that methylation would be less important in the jejunum than in the kidney, considering the greater specific of COMT activity occurring in the renal tissues. In fact, renal COMT activity ($V_{max} = 3.5 \pm 0.1$ in nmol mg protein^{-1} h^{-1}; Vieira-Coelho and Soares-da-Silva, 1994) was found to be 4-fold that occuring in the jejunal mucosa. Another difference between the renal tubular epithelium and the jejunal mucosa for which we have no explanation for the momment, concerns the K_m values which are considerably greater in the jejunum than in renal tissues (kidney, $K_m = 20 \pm 2$ µM; jejunum; $K_m = 100 \pm 14$ µM).

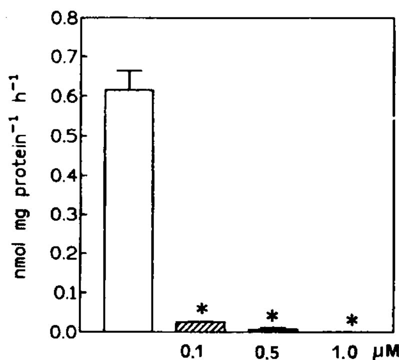

FIGURE 4 Inhibitory effect of increasing concentrations tolcapone (0.1, 0.5 and 1 μM) on the formation of metanephrine in homogenates of rat jejunal mucosa incubated with 500 μM adrenaline (control, open bar). Each bar represents the mean of four experiments per group; vertical lines indicate S.E.M. Significantly different from control values (* $P < 0.01$).

Ro 41-1049 and lazabemide were found to be potent inhibitors of MAO-A and MAO-B, respectively. On the other hand, for both MAO inhibitors no significant changes in MAO-A or MAO-B activities were found to occur when 250 nM lazabemide or Ro 41-1049 were used, respectively. This suggests that the compounds appart from being potent inhibitors are very selective as well. Tolcapone was also found in the intestinal mucosa a potent inhibitor of COMT. In fact, the lowest concentration used (0.1 μM) almost abolished the methylation of adrenaline. This contrast with the results obtained in the liver and kidney of the rat, where this concentration of tolcapone produced a less potent inhibitory effect (Vieira-Coelho and Soares-da-Silva, 1994). This fit well the evidence showing a reduced affinity of jejunal COMT for adrenaline in comparison with that reported for the liver and kidney (Vieira-Coelho and Soares-da-Silva, 1994).

In conclusion, the results presented here show that the jejunal mucosa is endowed with MAO-A, MAO-B and COMT activities, which might be of physiological relevance in the metabolism of locally produce monoamines (dopamine and 5-hydroxytryptamine). Furthermore, Ro 41-1049, lazabemide and tolcapone are potent inhibitors of jejunal MAO-A, MAO-B and COMT, respectively.

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PUBLICAÇÃO 4

"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.

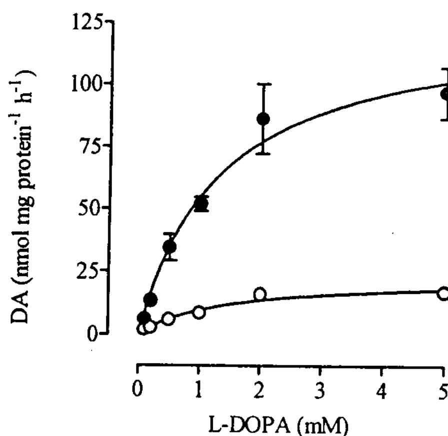

Fig. 1

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.

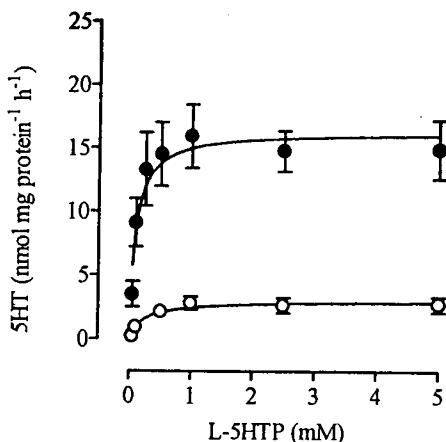

Fig. 2

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).

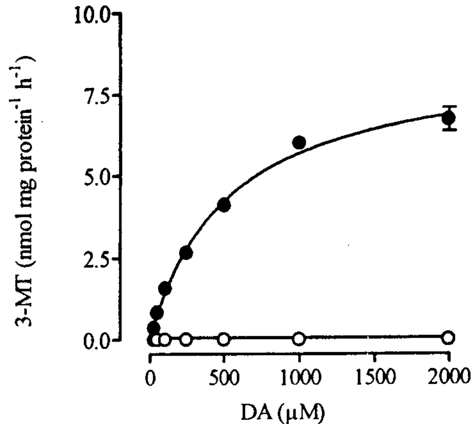

Fig. 3

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

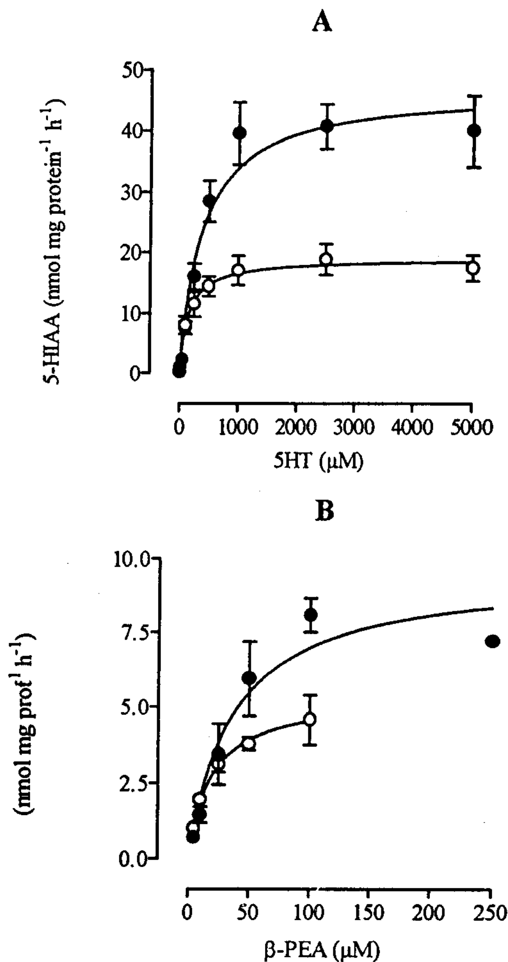

Fig. 4

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..

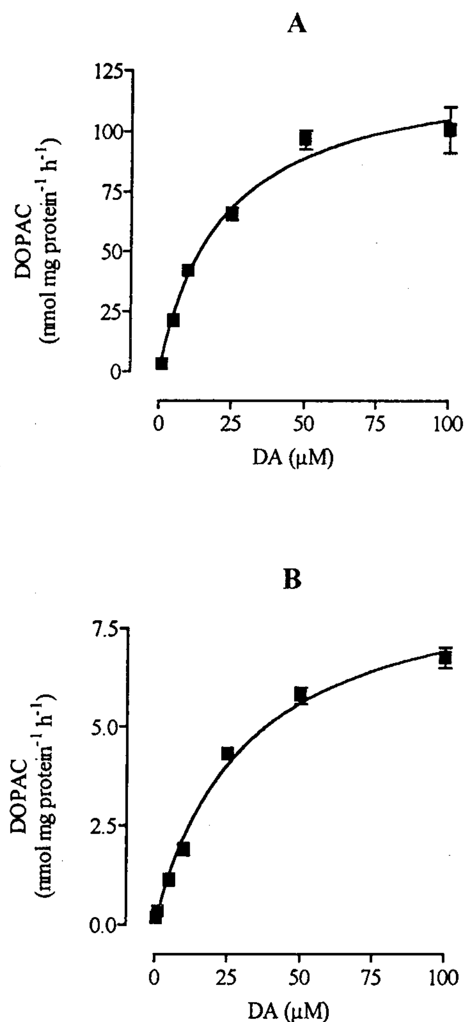

Fig. 5

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..

explanation for differences in enzyme kinetics. However, it remains to be explained why V_{max} values for L-DOPA are higher than those obtained with L-5-HTP, in spite of differences in affinity for both substrates. Another point worthwhile mentioning is that Caco-2 cells and rat jejunal epithelial cells do differ in the rate for maximal AADC activity. In fact, the activity of an enzyme is reflected by the rate constant $K_{enzyme}=V_{max}/K_m$, which indicates that the higher the V_{max} and the lower K_m, the higher will be the activity of the enzyme (29). Accordingly, K_{enzyme} for rat jejunal epithelial cells (K_{enzyme}=97.9) is 5 fold that in Caco-2 cells (K_{enzyme}=18.8). It is not possible to conclude that this difference is cell specific or inherent to species differences, because to our knowledge human intestinal AADC has not been studied.

O-methylation of monoamines by COMT proceeds through a pathway in which S-adenosyl-L-methionine serves as a methyl donor, is dependent upon the presence Mg^{2+} ions, occurs preferentially at the *m*-hydroxyl group and adrenaline is the preferred substrate among the three endogenous catecholamines (dopamine, noradrenaline and adrenaline) (29,30). The results obtained here show that CACO-2 cells are not able to *O*-methylate dopamine. In contrast, rat jejunal epithelial cells are endowed with considerable COMT activity. Kinetics values obtained in rat jejunal epithelial cells [V_{max}= 8.6±0.4 nmol mg protein^{-1} h^{-1}and K_m =515.6 (368.1, 663.0) μM] differ from those reported for the rat whole jejunal mucosa [V_{max}= 0.8±0.1 nmol mg protein^{-1} h^{-1}and K_m = 81 ±22 μM] (31). These differences may result from the fact that in experiments performed in the whole jejunal mucosa adrenaline was the substrate used and COMT has higher affinity adrenaline. On the other hand, differences in enzyme activity may be due to the fact that isolated cells constitute a more homogeneous cell preparation.

DA is a substrate for both MAO-A and MAO-B and 5-HT is a substrate only for MAO-A (32). The results presented here show that Caco-2 cells are endowed with both MAO-A and MAO-B, and the similar affinity of the two enzymes for the specific substrates, respectively 5-HT and ß-PEA, is similar to that observed in rat jejunal epithelial cells. Furthermore, K_m values for both type A and B MAO are also similar to those reported for the rat whole jejunal mucosa (31), and from guinea pig and cow isolated intestinal cells (33). Because epithelial cells are able to synthesise DA, and the amine is a common substrate for MAO-A and MAO-B, it was felt worthwhile to study its deamination in both preparations of epithelial cells. It is interesting to observe that K_m values for deamination of DA is in the low μM range, suggesting that the amine may be predominantly deaminated by MAO-B. However, the finding that V_{max} values in jejunal epithelial cells are 11-fold those in Caco-2 would suggest that some of MAO-A is participating in the deamination of the amine, as well. This reasoning is mainly derived from the fact that V_{max} values for 5-HT in jejunal epithelial cells are also considerably higher than those in Caco-2 cells. However, this may not be taken into great consideration since studies on the deamination of DA were not performed in the presence of selective MAO inhibitors, and only this could bring light to this particular aspect.

Caco-2 cells have been shown (34) to exist in three different states in culture: homogeneously undifferentiated (at subconfluence), heterogeneously polarized and differentiated (between 0 and 20 days after confluence), and homogeneously polarized and differentiated (after 30 days). Thus, it is quite possible that activities of enzymes involved in the synthesis and degradation of DA and 5-HT might change during differentiation.

Initial electrical measurements by Grasset et al. (13) on Caco-2 cells made it possible to define the epithelial properties of Caco-2 cells, which may resemble those of colonic crypt or foetal cells. These measurements also confirmed that functional differentiation is homogeneous in Caco-2 cells. It is interesting to note that our measurements are similar to used described by Grasset et al. (13) in their initial work (transepithelial electrical resistance = 154±6.5 $\Omega.cm^2$; potential difference = 0.29±0.02 mV; Isc = 1.9±0.1 μA cm^2). This indicates that cells have developed fluid transport mechanisms, which is line with data on Na^+-K^+ATPase activity and

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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Capítulo IV

Avaliação da metabolização da β-feniletilamina pelo coração de rato. Estudos cinéticos e moleculares comparativos com o cortex renal.

PUBLICAÇÃO 5

"Unusual pattern of β-phenylethylamine desamination in the rat heart"

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RESEARCH REPORT

UNUSUAL PATTERN OF ß-PHENYLETHYLAMINE DEAMINATION IN THE RAT HEART

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The present study was aimed to determine type A and B MAO activities in rat heart and renal cortex homogenates and evaluate the sensitivity of deamination of ^{3}H-5-HT and ^{14}C-ß-PEA to selective MAO-A and MAO-B inhibitors, respectively Ro 41-1049 and lazabemide. Deamination of ß-PEA in the rat heart was not affected (V_{max} = 53±10 vs 42±6 nmol mg protein^{-1} h^{-1}) by lazabemide (250 nM), but was significantly reduced (V_{max} = 10±1 nmol mg protein^{-1} h^{-1}) by Ro 41-1049 (250 nM). Deamination of ß-PEA in the rat heart is a low affinity process (when compared with that in the kidney) with high K_m values (244±98 vs 18.6±5.8 μM). On the other hand, deamination of 5-HT in the rat heart and renal cortex revealed high K_m values, which were similar to those for ß-PEA in the heart. Deamination of ß-PEA (1000 μM) in the rat heart was inhibited in a concentration-dependent manner by Ro 41-1049 with a K_i value of 32 nM (22, 48; 95% confidence limits), but not by the selective MAO-B inhibitor lazabemide (up to 500 nM). Inhibition of 5-HT (1000 μM) deamination in the rat heart by Ro 41-1049 was also a concentration-dependent process with a K_i value of 21 (16, 26) nM. Deamination of 5-HT (1000 μM) in the rat renal cortex, was inhibited in a concentration-dependent manner by Ro 41-1049 with a K_i value of 12 (8, 17) nM. Deamination of ß-PEA in the renal cortex was inhibited by lazabemide with a K_i of 5 (3, 7) nM. In the rat heart, in contrast to that in the renal cortex, the specific MAO-B substrate ß-PEA is deaminated by a form of MAO which most probably corresponds to MAO-A.

Key words: MAO-A, MAO-B, rat, heart, renal cortex

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INTRODUCTION

Monoamine oxidase (MAO, amine-oxygen oxidoreductase, E.C. 1.4.3.4) is a flavin-containing enzyme bound to the mitochondrial outer-membrane which catalyses the oxidative deamination of a variety of monoamines to their corresponding aldehydes (1, 2). MAO exists in two forms, A and B, encoded by separate genes (3, 4) with the same exon-intron organization (5). They have also been separated on a biochemical basis according to their substrate specificities and sensitivity to inhibitors (6, 7). Classically, MAO-A preferentially oxidizes biogenic amines such as 5-hydroxytryptamine (5-HT), adrenaline and noradrenaline, and is inactivated by the acetylenic inhibitor clorgyline. On the other hand, MAO-B preferentially oxidizes β-phenylethylamine (ß-PEA) and benzylamine and is inactivated by the irreversible inhibitor selegiline. Dopamine, tyramine and tryptamine are considered common substrates for both forms of MAO. However substrate specificities depend on the species being considered and also on the tissue studied (2, 8).

In the rat heart there is almost exclusively MAO-A with little MAO-B present (9), while in the rat renal cortex both forms of MAO are present (10-12). As already stated, ß-PEA is generally considered as MAO-B preferential substrate (2), but in the heart it has been described also as a substrate of MAO-A (13). Whether this substrate preference just reflects differences in tissue distribution of MAOs (14) or reflects structural differences between the two isozymes is not clear. As recently shown for both MAO-A and MAO-B, a single amino acid has a vital role in determining the substrate selectivity (15).

In the present study we determined type A and B MAO activities in rat heart and renal cortex homogenates and tested the sensitivity of deamination of ^{3}H-5-HT and ^{14}C-ß-PEA to selective MAO-A and MAO-B inhibition by, respectively, Ro 41-1049 and lazabemide (Ro 19-6327). These are highly selective, mechanism-based and reversible inhibitors of MAO activities (16), and lazabemide bears remarkable structural resemblance to the MAO-B preferential substrate ß-PEA (17, 18). These mechanism-based inhibitors, being nothing more than substrates that are converted by the normal catalytic pathway in products that inactivate the enzyme, are quite useful to study the mechanisms of action of the enzyme itself (19). Their interaction with MAOs appears to be similar to that occurring with substrates, being oxidized to form tightly, but reversible, bound adducts with the active sites. Taking advantage of the type of interaction of these MAO-A and MAO-B inhibitors it was decided to examine their effects upon deamination of 5-HT and ß-PEA in the rat heart.

METHODS

Male Wistar rats (Biotério do Instituto Gulbenkian de Ciência, Oeiras, Portugal), 45- to 60-day-old and weighing 200 to 280 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12-hour light/dark cycles and room temperature 24 °C). Food and tap water were allowed *ad libitum*. The experiments were all carried out during daylight hours.

Preparation of homogenates
In brief, rats were killed by decapitation under ether anaesthesia, the heart and kidneys removed through a midline thoraco-abdominal incision and immediately washed in ice-cold saline. The kidneys were decapsulated, cut in half and placed in ice-cold saline. The outer cortex was cut out with fine scissors and homogenised in 67 mM sodium phosphate buffer, pH 7.2, at 4 °C with a Thomas Teflon homogeneiser kept continuously on ice. As for the heart, after being rinsed with ice-cold saline, a portion of ventricle was used for the homogenisation in the same buffer conditions described for the kidney.

Assay of MAO-A and B Activities
MAO-A and MAO-B activities were determined using, respectively, [^{3}H]-5-hydroxytryptamine (^{3}H -5-HT) and [^{14}C]-ß-phenylethylamine (^{14}C-ß-PEA) as preferential substrates. The reaction mixture contained 50 μL of homogenate and 50 μL of 67 mM phosphate buffer and increasing concentrations of each substrate (^{3}H-5-HT for MAO-A and ^{14}C-ß-PEA for MAO-B). After 20 minutes of incubation at 37 °C with continuous oxygenation and shaking, the tubes were transferred to an ice-water bath and the reaction stopped by the addition of 10 μL of 3 M HCl. The deaminated products were then extracted with ethyl acetate (500 μL) and measured by liquid scintillation counting (20, 21). The MAO activity is expressed in nanomoles of substrate metabolized per mg of protein per hour of incubation (nmol mg protein^{-1} h^{-1}).

Saturation curves for deamination of ^{3}H-5-HT (50 to 2000 μM) were determined in the absence and in the presence of lazabemide (250 nM), whereas deamination of ^{14}C-ß-PEA (5 to 500 μM) was performed in the absence and the presence of both Ro 41-1049 (250 nM) and lazabemide (250 nM). Homogenates were pre-incubated for 30 min at 37 °C either with phosphate buffer or phosphate buffer plus the inhibitor, lazabemide or Ro 41-1049, and then incubated with the substrates, ^{14}C-ß-PEA or ^{3}H-5-HT, for further 20 min, in the same conditions as described above.

In another set of experiments, selective MAO-A and MAO-B inhibitors, respectively Ro 41-1049 and lazabemide were used to study the deamination of ^{3}H-5-HT (1000 μM) and ^{14}C-ß-PEA (1000 μM). Homogenates were prepared as described above and pre-incubated for 30 min at 37 °C in the presence of increasing concentrations of each inhibitor, ranging from 0.5 to 500 nM. This was also followed by an incubation with the substrates for further 20 min, in the same conditions as mentioned above.

Protein assay

The protein content in the homogenates were determined according to the method of Bradford (22), using human albumin as a standard.

Data analysis

V_{max} and K_m values for the deamination of ^{3}H-5-HT and ^{14}C-ß-PEA, as determined in saturation experiments, were calculated by non-linear regression analysis, using the GraphPad Prism statistics software package (23). For the calculation of the IC_{50}'s for lazabemide and Ro 41-1049, the parameters of the equation for one site inhibition were fitted to the experimental data (23). K_i's were calculated as defined by Cheng and Prusoff (24) for competitive inhibition. Arithmetic means are given with S.E.M. and geometric means with 95% confidence values. Statistical analysis of the saturation curves was done with a one-way analysis of variance (ANOVA) followed by Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference.

Drugs

Drugs used were: ^{14}C-ß-phenylethylamine hydrochloride (50 Ci/mmol) (NEN Chemical), ^{3}H-5-hydroxytryptamine creatinine sulphate (23.6 Ci/mmol) (NEN Chemicals), Ro 41-1049 [N-(2-aminoethyl)-5-(m-fluorophenyl)-4-thiazole carboxamide hydrochloride] (RBI, Natick, USA), Ro 19-6327 (generic name lazabemide) [N-(2-aminoethyl)-5-chloro-2-pyridine carboxamide hydrochloride] (F. Hoffmann-La Roche Ltd).

RESULTS

Deamination of ß-PEA, considered a specific MAO-B substrate, in the rat heart was found to present an unusual pattern, as can be observed in Fig. 1.

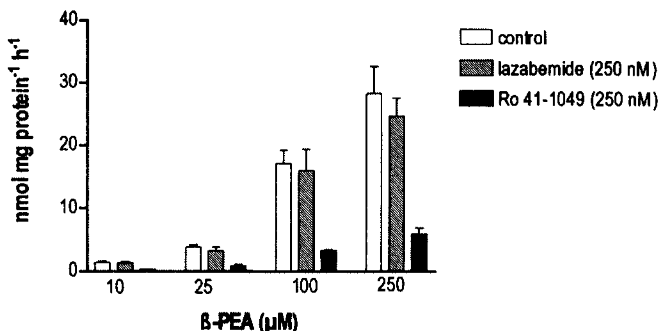

Fig. 1. Deamination of ß-PEA in rat heart homogenates in the absence (□) and the presence of lazabemide (250 nM; ▨) or Ro 41-1049 (250 nM; ■). Symbols represent means of four experiments per group and vertical lines show SEM

The saturation curve for deamination of ß-PEA in the rat heart was not affected (V_{max} = 53±10 vs 42±6 nmol mg protein^{-1} h^{-1}) by the selective MAO-B inhibitor lazabemide (250 nM), but was significantly reduced (V_{max} = 10±1 nmol mg protein^{-1} h^{-1}) by the selective MAO-A inhibitor Ro 41-1049 (250 nM). As shown in Table 1, deamination of ß-PEA in the rat heart is a low affinity process (when compared with that in the kidney) with high K_m values (244±98 vs 19±6 µM). On the other hand, deamination of 5-HT in the rat heart and renal cortex revealed high K_m values, which were similar to those for ß-PEA in the heart (Table 1).

Inhibition studies (see Fig. 2) show that deamination of ß-PEA (1000 µM) in the rat heart was inhibited in a concentration-dependent manner by Ro 41-1049 (the selective MAO-A inhibitor) with a K_i value of 32 nM (22, 48; 95% confidence limits), but not by the selective MAO-B inhibitor lazabemide (up to 500 nM). Inhibition of 5-HT (1000 µM) deamination in rat heart homogenates by Ro 41-1049 was also concentration-dependent with a K_i value of 21 (16, 26) nM, while there was no effect for lazabemide.

As shown in Fig. 3, deamination of 5-HT (1000 μM) in the rat renal cortex was inhibited in a concentration-dependent manner by Ro 41-1049 with a K_i value of 12 (8, 17) nM. Deamination of ß-PEA in the renal cortex, in the presence of 250 nM Ro 41-1049, was inhibited by lazabemide with a K_i of 5 (3, 7) nM.

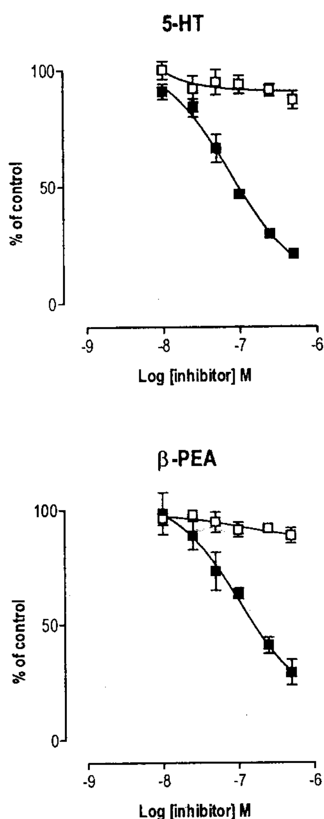

Fig. 2. Effect of increasing concentrations of Ro 41-1049 (■) and lazabemide (?) on rat heart deamination of 5-HT and ß-PEA. Symbols represent means of four experiments per group and vertical lines show SEM

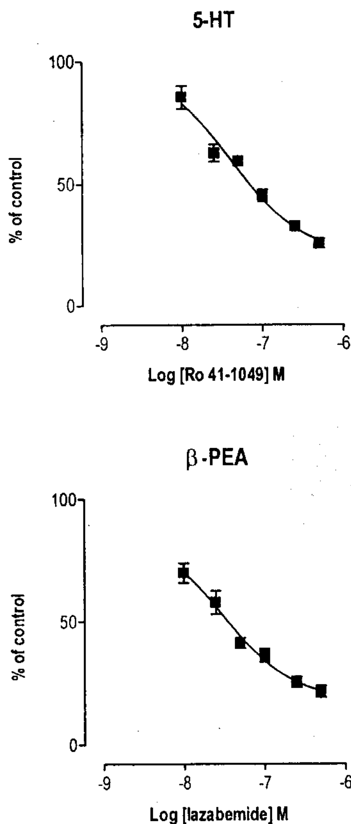

Fig. 3. Effect of increasing concentrations of Ro 41-1049 and lazabemide on the deamination of 5-HT and ß-PEA in rat renal cortex homogenates. Symbols represent means of four experiments per group and vertical lines show SEM

Table 1. V_{max} (in nmol mg protein^{-1} h^{-1}) and K_m (in μM) values for deamination of ß-PEA and 5-HT in homogenates of rat heart and renal cortex.

ß-PEA	V_{max}	K_m
	Heart	
Control	53±10	244±98
Lazabemide (250 nM)	42±6	191±59
Ro 41-1049 (250 nM)	10±1 *	229±68
	Renal cortex	
Control	9±1	19±6
5-HT		
	Heart	
Control	179±15	274±75
	Renal cortex	
Control	18±1	220±21

Values are means ± S.E.M. of four experiments per group.* significantly different from corresponding control values ($P<0.05$)

DISCUSSION

Classical classification of MAO forms has been made on the basis of substrate specificity and inhibitor sensitivity (6, 7). The suggestion that the two MAOs differ in their amino acid sequences came from peptide mapping analysis (25) and from immunoaffinity studies (26). The view that these two forms could arise from differences in post-translational modifications of a common enzyme precursor coded by a single structural gene locus (25), or even older assumptions that they resulted from a single enzyme existing in different membrane environments (27), were only abandoned after the isolation of MAO A and B cDNAs (3, 4). Although these isozymes have some different compositions they catalyze the same reaction, only preferring distinct amine substrates (19). However, it is also well known that substrate specificities vary with the species considered and with the tissue studied (2, 8). It has been previously shown (13) that the pattern of ß-PEA deamination in the rat heart is somewhat different from the usual one in that it is inhibited by the "A" inhibitor clorgyline. If tissue specificities simply reflect the ratio of MAO-A/MAO-B content, in the rat heart the specific binding of Ro 41-1049 and lazabemide in radioautography studies show A:B ratios ranging from 23 to 458 (9), or if they are the consequence of differences in the MAOs is not clear. In this study we tried to clarify some of these issues by using different substrates and inhibitors.

MAOs are flavoenzymes in that they require a flavin coenzyme to be active (1). For each MAO form there is a region containing a Cys residue (Cys406 and Cys397 in MAO-A and MAO-B, respectively) which covalently anchors flavin-adenine diphosphate (FAD) (28). In the rat heart, the investigation and characterisation of the active sites of both MAO forms using inhibitors has greatly relied on the use of the acetylenic inhibitors, clorgyline for MAO-A and selegiline for MAO-B (29), which form a 1:1 adduct by a covalent (irreversible) attachment to that same coenzyme (30). The use of another type of inhibitors, the N-(2-aminoethyl)arylcarboxamide derivatives lazabemide and Ro 41-1049, as probes for the biochemical characterisation of rat heart MAOs was, as far as we know, not done before. These compounds being converted into a product that is generally reactive and able to inactivate the enzyme, typically by covalent bond formation (19), turn to be very useful tools to study MAOs mechanisms of action.

As shown here, deamination of ß-PEA, classically considered a specific MAO-B substrate, in the rat heart was found to have an unusual pattern. This unusual pattern is mainly evidenced by high K_m values, approximately 10-fold that described for the same substrate in other tissues (2), and its insensitivity to lazabemide. Comparatively in the rat kidney and intestine, using this same substrate, we have recently shown that in both isolated renal epithelial cells and renal tubules and jejunal mucosa, deamination of ß-PEA proceeds through a high affinity mechanism (low K_m) and highly sensitive to lazabemide (20, 21). Another type of evidence suggesting that heart MAO deaminates ß-PEA by a process different from that observed in other tissues endowed with MAO-B activity is that K_i values for Ro 41-1049 using ß-PEA as a substrate were similar to those when 5-HT was used as substrate. This also indicates that Ro 41-1049 can no longer be regarded as a selective inhibitor of deamination of MAO-A substrates in the rat heart. However, if the A form in the rat heart has some affinity for the substrate ß-PEA, then we should have a similar sensitivity for the inhibitor lazabemide. Lazabemide has a very high degree of similarity with ß-PEA and shares the normal catalytic pathway, hence its presence should have some effect on ß-PEA deamination in this tissue. Because lazabemide lacked inhibitory effect on the deamination of ß-PEA, the real nature of the MAO form engaged in ß-PEA deamination in the rat heart remains questionable. However, K_m values for deamination of 5-HT, a specific MAO-A substrate, and its sensitivity to Ro 41-1094 in the heart were found not to differ from those in the kidney. This fits well the suggestion that MAO-A is the predominant MAO form in the rat heart (14, 29).

In conclusion, although these results suggest that in the rat heart the specific MAO-B substrate, ß-PEA, is deaminated by a form of MAO which most probably correspond to MAO-A, it may be worthwhile to further characterise the MAO isozymes in this tissue using a molecular approach.

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PUBLICAÇÃO 6

"Differential substrate specificity of monoamine oxidase in the rat heart and renal cortex"

(Enviado para publicação)

DIFFERENTIAL SUBSTRATE SPECIFICITY OF MONOAMINE OXIDASE IN THE RAT HEART AND RENAL CORTEX

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Abstract

Although it is known that substrate specificities differ with species and within each species with the tissues, in the rat heart no natural substrate was found for MAO-B. β-phenylethylamine (β-PEA) has always been considered the "endogenous" substrate of MAO B. We thought worthwide to evaluate the effect of Ro 41-1049 and lazabemide, both members of a class of highly selective, mechanism-based and reversible inhibitors for MAO-A and MAO B, respectively on the metabolization of β-PEA by the rat heart. Also the lack of molecular data on rat heart MAOs, prompted us to better characterize rat heart MAOs, both kinetically and using molecular biology techniques. K_m values for deamination of ß-PEA in the rat heart were 13-fold those in the kidney, by contrast, K_m values for deamination of 5-HT were quite similar in both tissues. Unexpectedly, the selective MAO-A inhibitor Ro 41-1049 was by far the most potent inhibitor of ß-PEA (20 μM) deamination in the rat heart, while clorgyline, another MAO A inhibitor, and lazabemide, a MAO B inhibitor, had intermediate efficacy; selegiline was found unable to inhibit deamination of ß-PEA. In the rat renal cortex lazabemide and selegiline both inhibited ß-PEA deamination. The reduction of ß-PEA concentration to just 200 nM, the use of heart membranes instead of tissue homogenates or the use of heart membranes pre-treated with 1% digitonine failed to change this pattern of inhibition. Semicarbazide was found not to alter deamination of ß-PEA. Western blot showed the presence of both isoforms (55 kd and 61 kd) in the renal cortex. In the heart there was a predominance of the A form, the B form being undetected. The RT-PCR products for both MAO-A and MAO-B, were found to have the expected sizes. In conclusion, we found mRNA for MAO-B but were unable to detect the protein itself or its activity when using ß-PEA as the substrate.

Key Words: rat, heart, renal cortex, β-PEA, MAO-A, MAO-B,

Introduction

The heart is a target organ for the actions of catecholamines and hydrogen peroxide (H_2O_2). Monoamine oxidase (MAO, amine-oxygen oxidoreductase, EC 1.4.3.4), an

enzyme located in the outer mitochondrial membrane, is known to be involved in these situations. On the one hand, it catalyses the oxidative deamination of catecholamines to their corresponding aldehydes; on the other hand, it is a source for H_2O_2 generation and hydroxyl radical formation [33,41]. MAO exists in two forms, A and B, classically differentiated on the basis of substrate specificity [24], inhibitor sensitivity [26], and tissue and cell distribution [32,37]. Each type of the enzyme contains two subunits with similar sizes [30], which differ in that only one of them contains covalently bound flavin [25].

Although it is known that substrate specificities differ with species and within each species with the tissues, in the rat heart no natural substrate was found for MAO-B. Concurrently the distribution and proportions of both types of MAO varies widely between species and with the tissue in question [14]. Still the substrate and inhibitor specificities should not change from tissue to tissue, or from species to species, provided there are no modifications in the molecular structure of the enzymes.

In spite of this, β-phenylethylamine (β-PEA) has always been considered the "endogenous" substrate of MAO B [39]. In the rat heart the metabolization of this substrate has been the reason for some uncertainty [15,34], and the amount of MAO B in this organ is said to be very small [32]. This unusual metabolization of β-PEA could be just related to different ratios of MAO forms or substrate concentrations [11,29,35]. However, all studies on the inhibitory kinetic in the rat heart were done in the seventies and early eighties using clorgyline and selegiline, two acetylenic inhibitors which bind to the FAD binding-site [40]. We thought worthwide to evaluate the effect of Ro 41-1049 and lazabemide, both members of a class of highly selective, mechanism-based and reversible inhibitors for MAO A and MAO B, respectively [9] on the metabolization of β-PEA by the rat heart. Indeed, lazabemide bears a remarkable structural resemblance to β-PEA, forming adducts with the active site [5]. These two molecules share the aromatic ring and the amine, and the conversation of β-PEA proceeds by the normal catalytic mechanism. Using this same pathway it turns to be a very useful tool to study MAO B mechanisms of actions.

Also, at the time of these previous studies in the rat heart, there was still the general idea that these MAO forms could arise from differences in posttranslational modifications of a common enzyme precursor coded by a single structural gene locus [4], or even older assumptions that they resulted from a single enzyme existing in different membrane environments [22]. These views were only abandoned after the isolation of MAO cDNA clones [1].

These genes are closely linked and located in the X chromosome and this knowledge enabled a much more molecular approach on MAOs, allowing the typification and characterisation of its coding regions. As an example of this, it is nowadays well known that exon 12, which is the most conserved exon between human MAOs, codes for the covalent flavin adenine dinucleotide (FAD)- binding site [16] or that the C-terminal and middle portions of MAO B are essential for the maintenance of its activity [6,18]. Also, recently, Ito et al found that a single amino acid, Phe-208 in MAO A and Ile-199 at the corresponding position in MAO B, has a vital role in determining the substrate selectivity of MAOs [36].

Whether these substrate preferences in the rat heart just reflect differences in the MAOs tissue distributions or may also reflect structural differences in the isozymes themselves is

not clear. This and the lack of molecular data on rat heart MAOs, prompts us to better characterize rat heart MAOs, both kinetically and using molecular biology techniques.

Materials and methods

Male Wistar rats, 45 to 60 days old and weighing 200 to 280 g were used in the experiments. Animals were kept two per cage under controlled environmental conditions (12 hours light/dark cycle and room temperature 24°C). Food and tap water were allowed *ad libitum*. The experiments were all carried out during daylight hours.

Preparation of homogenates

In brief, rats were killed by decapitation under ether anaesthesia, the kidneys and heart removed through a midline thoraco-abdominal incision and immediately washed in ice-cold saline. The kidneys were decapsulated, cut in half and placed in ice-cold saline. The outer cortex was cut out with fine scissors and homogenised in 67 mM phosphate buffer, pH 7.2, at 4°C with a Thomas Teflon homogeneiser kept continuously on ice. As for the heart, after being very well rinsed with ice-cold saline, a portion of ventricle was used for the homogenisation in the same conditions described for the kidney.

Membrane preparation

Tissues were homogenised in phosphate buffer pH 7.4, 50 mM, with protease inhibitors (bacitracine 1 mg/mL, soybean 1 mg/mL and phenyl methyl sulfonyl fluorure [PMSF] 100 mM). After centrifugation (18,000 r.p.m., 20 min, and 4° C), the pellet was resuspended in phosphate buffer plus protease inhibitors solution, and the process repeated once more. The final pellet was stored in the phosphate/inhibitors solution at – 80°C, until used.

Determination of MAO-A and B activities

MAO-A and MAO-B activities were determined using, respectively, [^{3}H]-5-hydroxytrytpamine (^{3}H -5-HT) (50 to 2000 μM) and [^{14}C]-ß-phenylethylamine (^{14}C-ß-PEA) (5 to 500 μM) as preferential substrates. The reaction mixture contained 50 μL of homogenate and 50 μL of 67 mM phosphate buffer and increasing concentrations of each substrate (^{3}H -5-HT for MAO A and ^{14}C-ß-PEA for MAO B). After 5 or 20 minutes of incubation, respectively for ß-PEA and 5-HT, at 37°C with continuous oxygenation and shaking, the tubes were transferred to an ice-water bath and the reaction stopped by the addition of 10 μL of 3 M HCl. The deaminated products were then extracted with a mixture of ethyl acetate and toluene 1:1 (2 mL) and measured by liquid scintillation

counting. The MAO activity is expressed in nanomoles of substrate metabolised per mg of protein per hour of incubation (nmol/mg protein/h).

The sensitivity of [^{14}C]-ß-PEA (0.2, and 20 µM) deamination, to selective MAO A and MAO B inhibitors, respectively, Ro 41-1049 and clorgyline for MAO A, and lazabemide and selegiline for MAO B was also studied. Homogenates were pre-incubated for 60 min at 37°C in the presence of increasing concentrations of each inhibitor, ranging from 0.5 to 2000 nM.

Semicarbazide-sensitive amine oxidase studies

Homogenates were pre-incubated for 60 min at 37°C in the presence of 1 mM of semicarbazide. Afterwards, homogenates were incubated for 5 minutes with ß-PEA, 20 and 200 µM, and the deamination products extracted and measured as described above.

Solubitization studies

Homogenates were prepared in the presence of a solubilization solution containing 1 % digitonine, 3-[N-morpholine] propane sulfonic acid (MOPS) 25 mM, ethylene glycol bis (β-aminoethyl ether)- N, N, N', N'- tetracetate (EGTA) 2 mM and PMSF 0.1 mM. The ratio detergent/protein was 3:1. After homogenisation the samples were centrifuged at 13.500 r.p.m. for 20 minutes, after which the pellet was resuspended in the same solubilization solution and shaked for 30 minutes at 4°C. A second centrifugation followed, 30 minutes at 18.000 r.p.m. and 4°C, the supernatant of which was stored at –80°C until used.

Western blot analysis

Membrane or homogenate proteins, with normalised protein contents (20 µg), were solubilized in loading buffer (40 mM Tris-HCl, pH 8.0, containing 8 % sodium dodecyl sulfate (SDS), 40 % glycerol, ethylene diamino tetracetate (EDTA) 10 mM and 0.05 % bromophenol blue) at 95 °C for 5 min and subjected to 10 % SDS polyacrilamide gel electrophoresis (20 mA and 200 V). Next, proteins were transfered to a nitrocellulose membrane (polyvinylidene difluoride transfer membrane- PolyScreen® NEN Research Products, MA, USA) with a semidry electroblotter (Trans-blot, Biorad) for 1h at 450 mA and 20 V. The blots were blocked with 5 % non fat dried milk in washing buffer (phosphate buffer saline, pH 7.5 and 0.1 % Tween 20) overnight at 4 °C. Afterwards, the blots were washed twice for 15 min and incubated for 1 h at room temperature with a rabbit polyclonal antiserum obtained from rabbits immunized with the peptide TNGGQERKFVGGSGQ corresponding to amino acids 211-225 in MAO A and 202-216 in MAO B. Blots were then incubated for 40 minutes with a horse secondary anti-rabbit antibody marked with horseradish peroxidase. After several washings the blots were reaveled (ECL kit, Pharmacia) and exposed to a photografic film. In some experiments, and in order to be quantitative, after incubation with the rabbit antiserum the blots were

incubated with a second antiserum against the ATPase β-subunit. Finally, the blots were incubated with [^{125}I]-protein A (0.14 μCi/ml) and the radioactivity visualised using a 445 SI phosphoimager.

Preparation of RNA

Heart and kidney total RNA was extracted according to the protocol of Chomczynski and Sacchi [8], using sterile conditions and always in ice. In brief, after the sacrifice of the animal and the removal of the organs, the tissues were rapidly and abundantly rinsed and cleaned with ice-cold saline and immediately frozen in liquid nitrogen. For RNA extraction, 300 μL of 2 M sodium acetate pH 4, 3 mL of phenol and 600 μL of a 24:1 mixture of chloroform/isopropylic acid were added in succession. After resting for 20 minutes in acid the tubes were centrifuged for 30 minutes at 9500 r.p.m., and the supernatant removed. This was followed by the precipitation and washing of the RNA pellet. The final pellet was stored at -20°C until used.

Reverse Transcription -Polymerase Chain Reaction (RT-PCR)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was carried out in the RNA samples previously prepared. Pre-treatment with DNase was followed by the addition of EDTA, then by 10 minutes in a dry-bath at 65°C, the addition of 1 μL of oligo(dT)$_{12\text{-}18}$ (0.1 mg/mL), and finally the mixture was heated to 65°C for 7 minutes and quickly chilled on ice. The Reverse Transcription (RT) mixture, containing DTT 0.1 M, dNTP 10 mM, RNAsine (10 a 30 u/l), Superscript™ II RNase H$^-$ Reverse Transcriptase (200 u/l)(Life Technologies) and buffer, was added to each vial for a final volume of 20 μL. They were then incubated, first for 1 hour at 37 °C and then for 5 minutes at 95 °C. The Polymerase Chain Reaction (PCR) mixture contained Tp PCR, MgCl2, TaqPolymerase, dNTP and water for a final volume of 50 μL. The primers couples, from Life Technologies, were for MAO A Sense 15/Antisense 578 (586 bp), Sense 509/Antisense 1318 (829 bp) and Sense 1537/Antisense 2037 (500 bp). For MAO B the couples were Sense 72/Antisense 1147 (1075 bp), Sense 894/Antisense 2315 (1421 bp) and Sense 1478/Antisense 2315 (837 bp). The PCR was run in a Perkin Elmer DNA Thermal Cycle 480 for 2 minutes at 94 °C, followed by 30-35 cycles of 1 minute at 94 °C, 1 minute at 58 °C and 1.5 minutes at 72 °C.

Protein assay

The protein content in homogenates and in membranes was determined according to the method of Bradford [2] with human albumin as a standard.

Data analysis

The V_{max} and K_m values for the deamination of [^{3}H]-5-HT and [^{14}C]-β-PEA, as determined in saturation experiments, were calculated by non-linear regression analysis, using the GraphPad Prism statistics software package [31]. For the calculation of IC_{50} 's for lazabemide, selegiline, Ro 41-1049, clorgyline and semicarbazide the parameters of the equation for one site inhibition were fitted to the experimental data [31]. K_i 's were calculated as defined by Cheng and Prusoff [7] for competitive inhibition. Arithmetic means are given with S.E.M. or geometric means with 95% confidence values.

Drugs and chemicals

Drugs used were:, ß-phenylethylamine hydrochloride (Sigma), 5-hydroxytryptamine hydrochloride (Sigma), [^{14}C]-ß-phenylethylamine hydrochloride (50 Ci/mmol) (NEN Chemical), [^{3}H]-5-hydroxytryptamine creatinine sulphate (23.6 Ci/mmol) (NEN Chemicals), Ro 41-1049 [*N*-(2-aminoethyl)-5-(*m*-fluorophenyl)-4-thiazole carboxamide hydrochloride] (RBI), Ro 19-6327 (generic name lazabemide) [N-(2-aminoethyl)-5-chloro-2-pyridine carboxamide hydrochloride] (F.Hoffmann-La Roche Ltd), L-ß-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma), selegiline (Research Biochemicals Int. Natick, MA) and clorgyline and semicarbazide. All other chemicals were with the highest purity available.

Results

Deamination of β-PEA and 5-HT in rat heart and renal cortex homogenates

In contrast to that occurring in the renal cortex where deamination of ß-PEA was a high affinity process, K_m values for deamination of ß-PEA in the rat heart were 13-fold those in the kidney. By contrast, K_m values for deamination of 5-HT were quite similar in both tissues (Table 1). It is also interesting to note that in the rat heart K_m values for deamination of ß-PEA were of the same magnitude to those of 5-HT.

Table 1. V_{max} and K_m values for deamination of ß-PEA and 5-HT in homogenates of rat heart and renal cortex. Values are means ± S.E.M. of four experiments per group.

Heart	V_{max} (nmol mg protein^{-1} h^{-1})	K_m (uM)
ß-PEA	51±9	228±86
5-HT	173±16	265±81
Renal cortex		
ß-PEA	9±1	18.2±6.2
5-HT	19±2	237±23

Effects of inhibitors on the deamination of β-PEA in rat heart and renal cortex homogenates

Quite unexpectedly, the selective MAO A inhibitor Ro 41-1049 was by far the most potent inhibitor of ß-PEA (20 μM) deamination in the rat heart. Clorgyline, another MAO A inhibitor, and lazabemide, a MAO B inhibitor, had intermediate efficacy; selegiline was found unable to inhibit deamination of ß-PEA (figure 1a). By contrast, in the rat renal cortex lazabemide and selegiline, two selective inhibitors of MAO B, were found to effectively inhibit ß-PEA deamination, whereas inhibitors of MAO A, Ro 41-1049 and clorgyline, had no effect on the deamination of the amine (figure 1b).

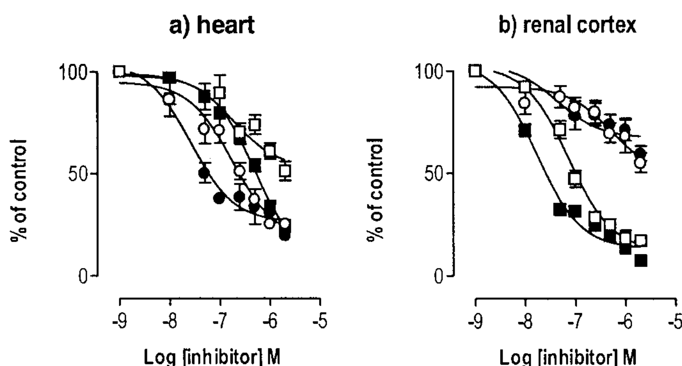

Fig. 1a) e b)

Deamination of 20 μM of ß-PEA by the rat heart a) and rat renal cortex b) in the presence of increasing concentrations of the MAO A inhibitors Ro 41-1049 (filled circles), clorgyline (open circles) and of the MAO B inhibitors lazabemide (filled squares) and selegiline (open squares). Symbols represent the means of four experiments per group and vertical lines show SEM.

In the heart the reduction of ß-PEA concentration to just 200 nM, taking in consideration that perhaps for this tissue the concentration we were using was still very high, produced no effective change. The relative potency of these inhibitors remained unchanged (Table 2), Ro 41-1049 being the most potent inhibitor and selegiline without approaching the 50% inhibition level. Again, clorgyline and lazabemide showed a similar pattern of inhibition, which did not differ from that obtained with 20 μM ß-PEA.

Table 2. IC_{50}'s for MAO of inhibitors (Ro 41-1049; clorgiline; lazabemide; selegiline) on the deamination of 20 µM ß-PEA (condition I) and 200 nM ß-PEA (condition II) by rat heart homogenates. Values of IC_{50}'s, in nM, represent the mean of 4 experiments per group with 95% confidence intervals.

	Ro 41-1049	Clorgiline	Lazabemide	Selegiline
I	26 (15;45)	175 (95;319)	485 (319;736)	nd
II	36 (20;63)	183 (119;281)	271 (179;412)	nd

nd: less then 50 % inhibition

Effects of inhibitors on the deamination of ß-PEA in heart mebranes

In order to rule out the possibility that mitochondrial membrane composition played any role in MAO substrate specificity, deamination of ß-PEA (20 µM) was performed in a new series of experiments using heart membranes instead of tissue homogenates. The results obtained (figure 2) show no significant alteration of inhibitory profile previously observed. Again, Ro 41-1049 was the most potent inhibitor, while clorgyline and lazabemide behaved quite similarly. In another set of experiments, different dilutions of the homogenates were used, in order to obtain different protein content, but without modification of the inhibitory potency (data not shown). When heart membranes were pre-treated with 1% digitonine, in order to alter the membrane phospholipid environment, the results obtained were similar to those observed with non-solubilized heart membranes (table 3).

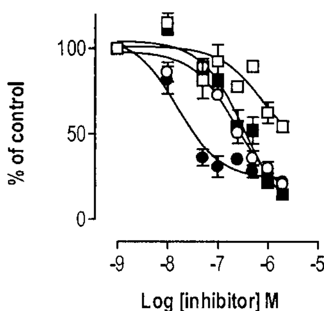

Fig. 2

Deamination of 20 µM of ß-PEA by the rat heart membranes in the presence of increasing concentrations of the MAO A inhibitors Ro 41-1049 (filled circles), clorgyline (open circles) and of the MAO B inhibitors lazabemide (filled squares) and selegiline (open squares). Symbols represent the means of four experiments per group and vertical lines show SEM.

Table 3. IC_{50}'s of inhibitors (Ro 41-1049; clorgiline; lazabemide; selegiline) on the deamination of 20 µM ß-PEA by homogenates of non-solubilized (condition I) and solubilized (1% digitonine; condition II) rat heart membranes. Values of IC_{50}'s, in nM, represent the mean of 4 experiments per group with 95% confidence intervals.

	Ro 41-1049	Clorgiline	Lazabemide	Selegiline
I	16 (9;29)	227 (146;351)	345 (190;628)	nd
II	22 (15;32)	256 (163;403)	593 (358;983)	nd

nd: less then 50 % inhibition

Semicarbazide-sensitive amine oxidase studies

Because semicarbazide-sensitive amine oxidase (SSAO) activity has been described in the rat heart (Lyles, 1996), it was decided to evaluate the possibility of its involvement on the metabolism of ß-PEA. Semicarbazide (1 mM) was used in the presence of two concentrations of ß-PEA (20 µM and 200 µM). As shown in figure 3, semicarbazide was found not to alter deamination of ß-PEA, excluding the possibility that deamination of ß-PEA in the rat heart was performed through SSAO rather than MAO.

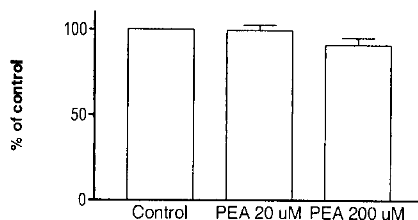

Fig. 3

Deamination of ß-PEA (20 µM and 200 µM) by the rat heart membranes in the presence of 1 mM of semicarbazide. Bars represent means of four experiments per group and vertical lines show SEM.

Western blot and RT-PCR results

Using a polyclonal antibody directed against a sequence that is similar in both MAO A and MAO B forms we performed SDS Western Blot that showed the presence in the renal cortex of both isoforms (55 kd and 61 kd), while in the heart it only showed the A form (figure 4). In RT-PCR studies, a series of primers chosen to cover the full length cDNA

encoding for each MAO form were used, in order to evaluate the presence of multiple products of the MAO gene that might suggest structural and functional heterogeneity of MAO protein. As is shown in figure 5a and 5b, respectively for MAO A and for MAO B, the RT-PCR products had the expected sizes.

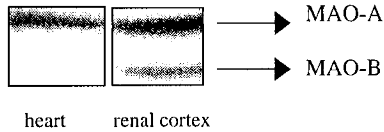

Fig. 4
Western blot of heart membranes. The arrows indicate proteins with molecular masses of approximately 61 and 55 kDa, corresponding to MAO-A and MAO-B respectively. Data are representative of 6 experiments.

Fig. 5a

RT-PCR products for MAO-A had the expected sizes: 586 bp, 829 bp and 500 bp.

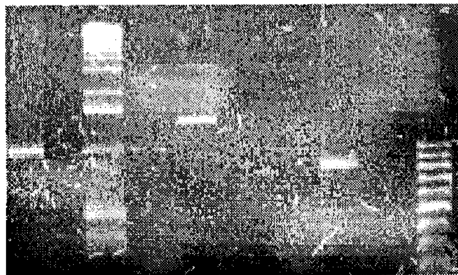

Fig. 5b

RT-PCR products for MAO-B had the expected sizes: 1075 bp, 1421 bp and 837 bp.

Discussion

Heart substrates and inhibitors

Although it is known that substrate specificity differ between species and within each species between different tissues, in the rat heart no natural substrate was found for MAO B. In the MAO B knock out mice urinary β-PEA excretion showed an 8-fold increase, when compared to corresponding controls and in contrast to the unchanged levels of other monoamines [17]. This strongly favours the view that β-PEA is the natural substrate of MAO B. Previous work from our laboratory has shown in kidney and intestine from different rat strains, and in different cell lines (OK cells and CACO-2 cells) that β-PEA is a substrate for MAO-B, this being sensitive to selective inhibitors for the enzyme [13,20,21]. In a preliminary set of experiments in the rat heart, the inhibitory effect of Ro 41-1049 on the deamination of β-PEA and 5-HT was found to be quite similar [19]. In these experiments, however, the concentrations of both substrates used (1000 μM) were 5-fold the corresponding K_m values. These concentrations are quite high and may lack substrate specificity, especially for β-PEA, which may explain why selective MAO A inhibition with Ro 41-1049 was endowed with similar IC_{50} values against 5-HT and β-PEA. To overcome this problem, the concentration of β-PEA used in the present series of experiments was reduced down to 20 μM. The results obtained were rather mixed in their nature, but favouring a MAO A type of degradation (Ro 41-1049 as the most potent inhibitor, whereas lazabemide and clorgiline were found to exert similar inhibitory profiles). By contrast, the inhibitory profile of MAO A and B inhibitors upon β-PEA in the renal cortex was typical of the B type, being lazabemide more potent than selegiline, whereas Ro 41-1049 and clorgyline were devoid of inhibitory effects. Because some authors suggested that in order to specifically determine MAO B activity the concentration of β-PEA should be around 10 μM or even lower, to exclude deamination by MAO A [11], we have repeated these experiments in the presence of 0.2 μM β-PEA. The results obtained clearly showed that the pattern of inhibition was not different from that obtained with a high concentration of β-PEA. Therefore, it is suggested that in the rat heart β-PEA, independently of the concentrations used, is deaminated by a form of MAO which is both sensitive to mechanism-based MAO A and MAO B inhibitors. However, the sensitivity of the form of MAO to the selective MAO A inhibitor Ro 41-1049 is considerably greater than that for lazabemide, the selective MAO B inhibitor. IC_{50} values for Ro 41-1049 against deamination of β-PEA in the rat heart are in the low nM range, which is in agreement with the figure obtained against 5-HT in both the heart, kidney and intestine [19,20]. On the other hand, IC_{50} values for lazabemide against deamination of β-PEA in the rat heart are ten-fold those obtained in tissues endowed with the classic type MAO B [19,20].

The reason for this apparent discrepancy, β-PEA on the one hand is the substrate but, on the other hand, lazabemide and selegiline are not the inhibitors, remained unsolved. If for selegiline the mechanism of action may account for its inhibitory incapacity, for

lazabemide that is much more difficult. In fact, these two molecules, β-PEA and lazabemide, share the aromatic ring and the amine, and the conversation of lazabemide proceeds by the normal catalytic mechanism. If, instead of heart homogenated we prepared heart membranes, once more we found no difference in the inhibitory profile. The tentative linking of MAO's activities to the composition of the mitochondrial membrane, specially the suggestion by White that the enzymatic activity of MAO A is critically dependent on associated phospholipids [38], was tested by changing the experimental conditions used. This time we used a 1% digitonine solubilization solution in order to to try to change the membrane phospholipid environment, and the results obtained were again similar. The possibility of having any kind of "inhibitory factor" in the membrane environment was also discarded by the inexistance of any changes when using diluted samples.

The presence of a SSAO activity in the rat heart, is well documented [28]. Since the seventies that there are reports of a "clorgyline-resistant" monoamine metabolizing activity in the rat heart [29]. In order to exclude the possibility of having SSAO metabolizing, at least partially, β-PEA we did some experiments using 1 mM of semicarbazide, a dose that completely inhibits SSAO activity [28]. For both concentrations of β-PEA used, 20 and 200 μM, there was no effect of semicarbazide, which excludes a role for SSAO.

Molecular aspects

Where these only differences in the distribution of the enzymes, or are there any molecular differences in rat heart enzymes that may justify them? In fact the western blot results, using a polyclonal antibody directed against a sequence that is similar in both forms, only showed the presence of MAO-A. The search for multiple products of MAO genes, which would suggest structural and functional heterogeneity of rat MAOs, was done with RT-PCR. The products obtained had the expected sizes, excluding, once more this possibility. This results show that the gene is being transcripted, as the presence of mRNA confirms, but the protein is not synthetised in a detectable quantity. Still, changes at the molecular level as the ones described by Ito, a single amino acid change, Phe-208 and Ile-199, in MAO A and B respectively, can modify the substrate selectivity of MAOs [36], can not be excluded by the studies we did.

Although both forms share certain similarities, peptide mapping analysis [3,4] and immunoaffinity studies [10] already suggested that the two MAOs differ in their amino acid sequences, which was confirmed, later on, with the isolation of MAO cDNA clones [1]. In human liver the isoenzymes resulting from the MAO A and MAO B cDNAs differ in their size, the MAO A being a protein with 527 amino acid residues (*M* 59700 Da) and the MAO B a 520 amino acid residues protein (*M* 58800 Da) [1]. For rat liver MAO B cDNA there was a 88 % amino acid sequence identity with human liver, the regions of amino acid sequence identity being randomly distributed over the entire protein [23]. As for liver MAO A cDNA, the deduced amino acid sequence had 88 % and 86 % identity to the sequences of human liver and bovine adrenal medulla, respectively [27]. The analysis of the cDNA and the genomic sequences demonstrate that the coding regions of both the MAO A and B genes are comprised of 15 exons and 14 introns and possess identical

exon-intron organization [16]. With all this relative homology on the one side, and heterogeneity on the the other, we may never be able to exclude the possibility of an alternative splicing taking place, and generating heart-specific forms of MAO, as for other enzymes [12].

In conclusion, using low ß-PEA concentrations that would favour specific deamination of the substrate by MAO-B, we found a degradation profile that is compatible with a MAO-A activity. This appears to be specific of the heart and is neither related to the membrane environment, nor related to major biochemical differences with classical MAO. This may indicate that MAO may be much more heterogeneous than we think today and that this kind of ß-PEA degradation may also be useful to explain certain findings in other tissues.

Acknowledgements

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Capítulo V

Avaliação da metabolização da Noradrenalina em tecidos de rato. Estudo do efeito da maturação e envelhecimento.

PUBLICAÇÃO 7

"Influence of maturation and ageing on the biotransformation of noradrenaline in the rat"

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Influence of maturation and ageing on the biotransformation of noradrenaline in the rat

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Summary. The present investigation was undertaken to study the influence of maturation and ageing on the disposition of noradrenaline by the aorta, heart (ventricle), liver and kidney of the rat. Slices of these tissues taken from rats aged less than 18h, 2.5–3 months or 18–24 months were incubated with 0.1 μmol.l^{-1} ^{3}H-amine during 30min. At the end of this period, the accumulation of the intact amine in the tissue, as well as the ^{3}H-metabolites formed (3,4-dihydroxyphenylethylglycol, 3,4-dihydroxymandelic acid, normetanephrine and O-methylated deaminated metabolites) were determined by scintillation counting. The results obtained show that in the rat: 1) at any age, noradrenaline is preferentially deaminated; 2) while the capacity of the sympathetic nerve terminals in accumulating noradrenaline is rather well developed at birth, the metabolic system for its degradation is still immature; 3) aldehyde dehydrogenase activity or that of its co-factor (or both) of the heart is apparently missing at birth; 4) removal of noradrenaline by the liver and the kidney did not change with ageing, while that by the aorta decreased and that by the heart increased.

Introduction

The mechanisms for adrenergic function do not develop at the same time (Blatchford et al., 1976; Su et al., 1977). For example, the pathways for adrenergic transmitter inactivation, transmitter action on smooth muscle cells and neuronal transmitter release develop in that sequence in the fetal lamb (Su et al., 1977). On the other hand, ageing is associated with a variety of changes in cardiovascular function, as well as with an increased incidence of cardiovascular disorders (Docherty, 1990). The present investigation was undertaken to look for the influence of maturation and ageing on the role played by monoamine oxidase (MAO) and catechol O-methyl transferase (COMT) activities in the inactivation of noradrenaline in the rat.

Material and methods

Three groups of Wistar rats were used: one of animals less than 18h old (body weight = 6.0 ± 0.3g; n = 5), another of animals 2.5–3 months old (body weight = 351 ± 8g; n = 4) and a third group of animals 18–24 months old (body weight = 841 ± 24g; n = 6). The animals of either sex were anaesthetized with pentobarbital sodium (30mg.kg^{-1}, i.p.) and the aorta, the heart, the liver and the kidney were dissected free and placed in aerated (95% O_2; 5% CO_2), modified Krebs-Henseleit solution (Guimarães et al., 1978) of the following composition (mmol.l^{-1}): NaCl, 118.6; KCl, 4.70; $CaCl_2$, 2.52; KH_2PO_4, 1.18; $MgSO_4$, 1.23; $NaHCO_3$, 25.0; glucose, 10.0. Then, slices of these organs were cut and placed for 30min in small beakers containing 2.5ml of modified Krebs-Henseleit aerated solution at 37°C. After this period of stabilization 0.1μmol.l^{-1} ^{3}H-noradrenaline was added to the incubation medium for a further period of 30min. To avoid autoxidation of the ^{3}H-amine, EDTA 0.027 and ascorbic acid 0.57mmol.l^{-1} were added to the medium which was maintained at 37°C. At the end of this incubation, the tissues were immersed in 3ml of 0.2mol.l^{-1} perchloric acid and kept overnight for extraction of radioactivity.

Aliquots of the eluates and the tissue extracts were passed through alumina and Dowex 50WX4 columns, as described by Graefe et al. (1973). A second Dowex column was used to remove traces of ^{3}H-amine from the DOMA-containing alumina eluate (Trendelenburg et al., 1983). Five fractions were isolated: noradrenaline, 3,4-dihydroxyphenylglycol (DOPEG), 3,4-dihydroxymandelic acid (DOMA), normetanephrine (NMN) and O-methylated-deaminated metabolites (OMDA) (which represents 3-methoxy-4-hydroxyphenylglycol plus 3-methoxy-4-hydroxymandelic acid). The recovery of radioactivity in the chromatographic procedure (sum of radioactivity found in the five fractions/total radioactivity in the sample) was 0.92 ± 0.02 (n = 22). Results were not corrected for recovery.

Radioactivity was measured by liquid scintillation counting (liquid scintillation counter 1209 Rackbeta, LKB Wallac, Turku, Finland) in 2ml aliquots of eluate (or 0.5ml of tissue extract +1.5ml of Krebs solution) after addition of 8ml of scintillation mixture (OptiPhase "HiSafe" 3, LKB, Loughborough, Leics, England).

Endogenous noradrenaline content of the tissue

The endogenous noradrenaline content of the tissues was measured by HPLC with electrochemical detection (Bioanalytical Systems, West Lafayette, Ind., USA). Immediately after removal of the tissues, a sample of each one was collected in perchloric acid (0.2mol.l^{-1}) and kept overnight. Aliquots of the extract were directly injected in a HPLC system with a 5μm ODS reverse-phase column. The mobile phase — which was a degassed solution of 50mmol.l^{-1} KH_2PO_4, 1.7mmol.l^{-1} sodium heptane sulphonate, 0.09mmol.l^{-1} EDTA, 10% methanol v/v, pH 3.5 adjusted with perchloric acid — was pumped at a flow-rate of 1ml.min^{-1}. Quantification was carried out with a carbon paste electrode at 0.75V vs a Ag/AgCl reference electrode.

Determination of the extracellular space

Slices of heart (ventricle), liver and kidney or segments of aorta were incubated during 5min in 3ml of modified Krebs-Henseleit solution containing 0.2μmol.l^{-1} of ^{3}H-sorbitol and radioactivity was measured by liquid scintillation counting as described above.

Statistics

The results are presented as arithmetic means with standard errors. One-way analysis of variance was used to test differences between unpaired results. A probability level of 0.05 or less was considered statistically significant.

Drugs used

^{3}H-7-(−)-noradrenaline (10.4 Ci.mmol^{-1}, NEN Dupont, Dreieich, FRG); ^{3}H-sorbitol (12.9 Ci.mmol^{-1} NEN Dupont).

Results

1. Noradrenaline content of the different organs

The noradrenaline contents of the different organs at different ages are shown in Table 1. The liver, at any age, had the lowest noradrenaline content of all the organs studied. The noradrenaline contents of the aorta and liver in neonates were not different from those in adults. In contrast, there was a marked increase of noradrenaline contents in the heart and kidney during maturation. There was a marked reduction of the noradrenaline content of the aorta with ageing.

2. Metabolism of noradrenaline in the different organs

Aorta

As shown in Table 2, in the aorta of newborn rats the amount of noradrenaline accumulated was not significantly different from that which was metabolically degradated (182 vs. 234 pmol.g^{-1}; $P > 0.05$). Deamination was the mechanism largely predominant. There was practically no formation of O-methylated-deaminated metabolites. In adult rats, the total amount of metabolites formed from noradrenaline was more than twice that formed in the

Table 1. Noradrenaline content of some organs of the rat at different ages

	Aorta	Heart	Kidney	Liver
Newborns	1.16 ± 0.12	0.59 ± 0.06*	0.35 ± 0.08*	0.18 ± 0.05
Adults	1.67 ± 0.34	3.48 ± 0.06	1.88 ± 0.11	0.24 ± 0.05
Old rats	0.90 ± 0.07*	3.74 ± 0.18	2.15 ± 0.22	0.32 ± 0.01

Values are expressed as nmol.g^{-1} of tissue and represent mean ± s.e.m of 4–6 experiments. * Significantly different from the value obtained in adults in the same organ

Table 2. Metabolism of noradrenaline in the rat

	OMDA	NMN	DOPEG	DOMA	Noradrenaline	Total metabolites	Removal
Aorta							
Newborns	4 ± 2*	25 ± 9*	141 ± 35	64 ± 39	182 ± 21	234 ± 31*	456 ± 43*
Adults	256 ± 51	267 ± 93	124 ± 29	25 ± 20	198 ± 49	672 ± 169	870 ± 177
Old rats	138 ± 24	79 ± 3*	52 ± 16*	11 ± 8	83 ± 15*	280 ± 29*	363 ± 11*
Heart							
Newborns	3 ± 0*	15 ± 4*	41 ± 1*	6 ± 4*	87 ± 13*	65 ± 3*	152 ± 7*
Adults	62 ± 3	3 ± 1	361 ± 93	267 ± 115	260 ± 43	693 ± 52	953 ± 17
Old rats	105 ± 25	2 ± 0	375 ± 171	480 ± 120	203 ± 23	962 ± 143	1165 ± 142
Kidney							
Newborns	26 ± 2*	69 ± 4*	19 ± 5*	4 ± 4	54 ± 14	118 ± 7*	172 ± 18
Adults	88 ± 13	234 ± 26	38 ± 3	21 ± 9	99 ± 41	381 ± 46	480 ± 57
Old rats	102 ± 18	255 ± 23	39 ± 11	20 ± 9	148 ± 30	416 ± 45	564 ± 79
Liver							
Newborns	114 ± 7	37 ± 5*	35 ± 2	32 ± 13	1 ± 1	218 ± 14*	219 ± 12*
Adults	148 ± 32	4 ± 1	108 ± 42	62 ± 33	2 ± 1	322 ± 40	324 ± 43
Old rats	152 ± 23	5 ± 1	78 ± 35	128 ± 25	13 ± 3*	363 ± 33	376 ± 32

Shown are the metabolites formed during 30 min incubation with 0.1 $\mu mol.l^{-1}$ ^{3}H-noradrenaline in the four tissues indicated in the table. Values are expressed as $pmol.g^{-1}$ and represent mean ± s.e.m of 4–6 experiments. Removal represents accumulation plus total metabolites. * Significantly different from the value obtained in adults in the same organ

aorta of newborn animals, while the amine accumulated in newborn and adult rats was not different (182 vs. 198 $pmol.g^{-1}$; $P > 0.05$). Noradrenaline was both deaminated and O-methylated, the O-methylated metabolites predominating. While in newborns the ratio total metabolites/amine accumulated was 1.3, in adults it was 3.4.

In old rats, the amounts of the accumulated amine and of the total metabolites were only about 40% of those observed in adults. The amounts of O-methylated and of deaminated metabolites were not different. As in adults, O-methylation predominated over deamination (Table 2).

Heart

As shown in Table 2, the heart of newborn rats showed the lowest capacity to degradate noradrenaline among the organs studied. Noradrenaline was predominantly deaminated and the accumulation of intact amine was less than 50% that observed in aorta. In adults, deaminated metabolites (DOPEG + DOMA) represented more than 90% of the total metabolites formed. The total amount of metabolites formed was 11 times higher in adults than in newborns. In old rats, the cardiac capacity to degradate noradrenaline reached its maximum. The amount of metabolites formed was 15 times higher

in old rats than in newborns. In old rats, the heart was the tissue possessing the highest capacity to degradate noradrenaline. Also in the heart of old rats, there was practically no O-methylation of noradrenaline. DOPEG + DOMA represented more than 90% of total metabolites formed and DOMA was the predominant metabolite, representing 50% of the total amount of metabolites formed.

Kidney

The kidney of newborn rats had a relatively low capacity to accumulate noradrenaline. The accumulation represented 31% of the removal. In contrast to what was observed in other tissues, the deamination of noradrenaline in the kidney was very low at any age, O-methylation predominating largely. These peculiar characteristics of the renal tissue were also observed in adult and old rats: a relative low accumulation of the intact amine and a large predominance of O-methylation over deamination. Both in adult and old rats, the total amount of metabolites formed was much higher than in newborns: 3.2 and 3.5 times higher in adult and old rats, respectively.

Liver

In newborn, adult and old rats the liver was the tissue with the lowest capacity to accumulate the intact amine (Table 2). As for the heart NMN practically did not appear in adult and old rats. On the contrary, similar amounts of NMN, DOPEG and DOMA were formed by the liver of newborns. As far as the metabolic degradation of noradrenaline is concerned, the liver was apparently the most mature tissue at birth. The ratio total metabolites formed in adults /total metabolites formed in newborns was: heart > kidney > aorta > liver (Table 2).

Discussion

The present results confirm that noradrenaline is preferentially inactivated by uptake into the sympathetic nerve terminals followed by accumulation or deamination. This has been already shown for adult animals in the canine saphenous vein (Guimarães, 1975; Guimarães and Paiva, 1977; Paiva and Guimarães, 1978) in the canine mesenteric artery (Garrett and Branco, 1977; Guimarães and Paiva, 1977) and in the rat heart (Fiebig and Trendelenburg, 1978). In vivo, it was also shown in adult rats, that noradrenaline is preferentially inactivated by neuronal pathways of metabolism (Eisenhofer, 1994; Eisenhofer and Finberg, 1994). The present results very clearly show that in the aorta (in neonates), in the heart (at any age), and in the liver (at any age), noradrenaline is preferentially deaminated at the concentration of $0.1\,\mu mol.l^{-1}$.

The amount of noradrenaline accumulated in the aorta, kidney and liver of newborns was not significantly different from that accumulated in the same organ of adult rats. This shows that at birth the sympathetic nerve endings are already developed and endowed with an active neuronal uptake. The presence of an active neuronal uptake at birth was previously reported for the carotid artery of the lamb (Su et al., 1977), for the rabbit aorta (Guimarães et al., 1991) and for the canine saphenous vein (Moura et al., 1993). However, the presence of neuronal uptake at birth does not necessarily means maturity of the sympathetic nerves since the development of the neuronal uptake precedes effective adrenergic transmission (Su et al., 1977). The transmembrane transport system is associated with the neuronal membrane of the new neurons, while the synthesis and storage capacities are linked to enzymes and vesicles which depend on the cell body (Dahlström and Häggendal, 1967; Laduron and Belpaire, 1968). According to the low noradrenaline content of the heart of newborn rats which was about 14% that of the heart of adult animals, the accumulation of noradrenaline was also lower in newborns than in adults, indicating that at birth the sympathetic nerves of the heart are either not yet fully present or not yet fully mature. Furthermore, in the heart, the enzymatic degradating capacity is even more underdeveloped at birth than the accumulating capacity, since the accumulation in the newborn represents about 35% that of the adult, while the total metabolites formation in the newborn reaches only about 10% of the value in the adult.

In sharp contrast to the capacity of the sympathetic nerve terminals in accumulating noradrenaline, the metabolic system for its degradation is still immature at birth. In all tissues, the amount of metabolites formed from both MAO and COMT activities is much smaller in newborns than in adults. The ratio metabolites formed/amine accumulated was about 2 times higher in adults than in newborns in the aorta, kidney and liver and about 3.5 times higher in adults than in newborns in the heart.

In adult and old rats, the predominant metabolites formed from noradrenaline in the heart were DOPEG and DOMA, while in newborns DOMA was practically not formed. The lack of DOMA formation at a moment at which DOPEG was the metabolite largely predominant favours the view that, at birth, there is a failure of the dehydrogenating process which can be due to either a failure of the aldehyde dehydrogenase or to a failure of its co-factor or to a failure of both the enzyme and the co-factor.

At any age, the accumulation of noradrenaline in the liver was very poor, as expected on the basis of the low noradrenaline content of this tissue. The low noradrenaline content and the very low amine accumulation in the liver indicate that the sympathetic innervation is very scarce and that deamination takes place predominantly at extraneuronal sites. In the isolated perfused rat liver, the extraneuronal uptake inhibitor corticosterone reduced the ^{3}H-metabolites without changing the accumulation of ^{3}H-noradrenaline (Steinberg et al., 1988) confirming that the metabolic degradation occurs mainly extraneuronally. While isolated hepatocytes possess a very high capacity to remove and metabolize both adrenaline and noradrenaline

(Martel et al., 1993), liver slices showed the lowest capacity to remove and degradate those amines among the four organs included in the present study.

At any age, the kidney showed an apparent lack of MAO activity, since noradrenaline was more O-methylated than deaminated and since more NMN than OMDA was formed. The ratio NMN/OMDA was about 3.

In adult and old rats, there was practically no NMN formation in the heart and in the liver. This unexpected finding might be ascribed to a high deaminating activity of the heart and the liver which so quickly transformed NMN into MOPEG or VMA that no NMN was accumulated. Alternatively one may admit that noradrenaline has a low affinity for COMT in these tissues.

With ageing (adult vs. old animals) there was a marked decline (by about 60%) in the capacity of the aorta to metabolize noradrenaline, while in the heart the capacity to degradate the same amine was markedly enhanced (a 2.5-fold increase). Most probably, the reduction in the metabolic capacity of the aorta is linked to degenerative changes occurred in the vessel wall dependent on age. Regarding the enhancement of this capacity in the heart it is tempting to speculate that this may be related to the high noradrenaline content of the hearts of old rats candidates to or already suffering from heart failure.

In summary, we conclude that in the rat: 1) at any age, noradrenaline is preferentially deaminated; 2) at birth, the capacity to metabolize noradrenaline is still underdeveloped, while that to accumulate it is already mature; 3) aldehyde dehydrogenase activity or that of its co-factor (or both) of the heart is apparently missing at birth; 4) removal of noradrenaline by the liver and the kidney did not change with ageing, while that by the aorta decreased and that by the heart increased.

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Capítulo VI

Discussão e Conclusões

Papel das MAOs no rim

Na primeira parte deste trabalho, a que corresponde o Capítulo II, avaliou-se o papel das MAOs no metabolismo de várias aminas em tecidos renais. Isolaram-se e estudaram-se túbulos contornados próximais e células do epitélio tubular renal de Rato, bem como células provenientes de uma linha celular epitelial derivada do rim de Opossum (células OK). O rim é considerado o mais importante sistema monoaminérgico periférico, sobretudo tendo em conta o papel que a DA desempenha na regulação da excreção do sódio. A grande abundância em descarboxílase dos ácidos aminados aromáticos (AAAD), justifica a grande capacidade do rim para sintetizar DA a partir da descarboxilação da di-hidroxifenilalanina (L-DOPA) circulante ou filtrada, conforme demonstram vários trabalhos feitos, quer no Homem, quer no Rato (Baines et al., 1980; Hayashi et al., 1990; Wolfovitz et al., 1993). Essa DA de origem renal exerce as suas funções, quer nas células onde é sintetizada (função autócrina), quer nas células que lhes são adjacentes (função parácrina). A modulação da actividade da DA é condicionada, por um lado pela disponibilidade do precursor imediato – L-DOPA – e, por outro lado, pelo modo como ela é metabolizada ou inactivada. Embora o transporte da L-DOPA para dentro da célula possa ser um factor condicionante, a modulação da actividade da DA faz-se, essencialmente, pela sua metabolização ou inactivação. Como anteriormente referido, a DA pode ter 5 destinos metabólicos: 1) desaminação por acção das MAOs; 2) metilação por acção da COMT; 3) sulfoconjugação por acção da PST; 4) hidroxilação pela hidroxílase da DA originando NA; 5) glicurono-conjugação pela glicuronídase. A importância relativa de cada um destes destinos varia de tecido para tecido, conforme as frequências relativas de cada um dos gru-

pos de enzimas. O rim é um dos tecidos que apresenta maior actividade das MAOs, pelo que não é de estranhar ser a desaminação a principal via de metabolização da DA (Kopin, 1985; Fernandes et al., 1993). A MAO-A e a MAO-B estão presentes no cortex e na medula renal e a sua localização é essencialmente extra-neuronial (Caramona et al., 1990; Soares-da-Silva et al., 1992b).

No estudo realizado (**publicação 1**) procurou-se isolar, o mais possível, as unidades funcionais do sistema monoaminérgico renal, de modo a que sofressem a menor influência possível das estruturas envolventes. Para isso foram isoladas células e túbulos contornados proximais, que se revelaram bons sistemas para estudar a actividade de formação de DA e respectiva desaminação pelas MAOs, originando o ácido di-hidroxifenilacético (DOPAC). Nestas células tubulares renais isoladas verificou-se haver produção de DA e DOPAC dependente da duração da incubação, da concentração do substrato (L-DOPA) e também da composição do meio. Para os mesmos tempos de incubação e concentração de substrato os estudos feitos em meio de Hanks demonstraram uma maior capacidade de sintetizar DA e de a desaminar em DOPAC do que os estudos em meio DMEM (Dulbeco's Modified Eagle Medium). A esta diferença não será provavelmente indiferente a maior riqueza em aminoácidos aromáticos do segundo destes meios, o que poderá implicar uma competição pelo transporte para dentro da célula entre esses a.a. e a L-DOPA. Em meio de Hanks, a presença do Ro 41-1049 (250 nM) (um inibidor da MAO-A) reduziu a formação de DOPAC, sem afectar a formação de DA. Na presença da lazabemida (250 nM), inibidor da MAO-B, não houve diferenças, quer na formação de DA, quer na formação de DOPAC, em relação à situação de controlo. Quando na presença simultânea dos dois inibidores, Ro 41-1049 e lazabemida (ambos na concentração de 250 nM), os valores encontrados foram sobreponíveis aos encontrados nas experiências em que se utilizou, apenas, Ro 41-1049. É de salientar que a formação de DOPAC é um processo dependente do tempo, uma vez que a relação DOPAC/DA aumentou com a duração da incubação. O Ro 41-1049

inibiu este aumento. É de referir ainda que a utilização da lazabemida não modificou essa relação nem a sua expressão temporal e que, mesmo o Ro 41-1049, não aboliu totalmente a desaminação da DA. Todas estas experiências foram realizadas na presença de tolcapone, um inibidor da COMT. Estes resultados articulam-se com os obtidos em estudos anteriores que indicavam que a DA de origem renal era transformada em DOPAC por ambas as formas de MAO e sugeriam a existência de uma desaminação preferencial pela MAO-A. Estes mesmos estudos, feitos em fatias de rim de Rato, demonstraram, igualmente, que a inibição da MAO-A pelo Ro 41-1049 causava uma redução da formação de DOPAC e uma acumulação de DA, enquanto a inibição da MAO-B pela lazabemida não causava qualquer efeito (Fernandes et al., 1990; Fernandes et al., 1991; Pestana et al., 1994).

Estudos feitos em ratos confirmaram estes mesmos resultados ao mostrarem que só havia redução na formação de DOPAC quando se usava o Ro 41-1049 (Vieira-Coelho et al., 1994). Por outro lado, nesse mesmo tecido, verificou-se a existência, quer de actividade desaminativa da MAO-A, quer de actividade desaminativa da MAO-B (Fernandes et al., 1992). Por tudo isto, estes autores sugeriram como explicação para este fenómeno a teoria "multi-compartimental", i.e., a MAO-A estaria localizada no compartimento onde decorre a síntese da DA, enquanto a MAO-B estaria localizada fora desse compartimento. Assim, no seu conjunto, estes resultados obtidos nas células tubulares isoladas reforçam, também, a teoria "multi-compartimental", pondo mesmo em questão a presença de MAO-B neste tipo de células, dada a total ineficácia do inibidor respectivo. Daí a relevância dos estudos efectuados nos homogeneizados de túbulos contornados proximais onde se avaliou a cinética da actividade das MAOs perante os substratos 5-HT e PEA, marcados radioactivamente. A análise das curvas de saturação respectivas revelou um K_m de 351±71 μM para a 5-HT com uma V_{max} de 25±2 nmol mg proteína^{-1}h^{-1}, e para a PEA um K_m de 58±12 μM com uma V_{max} de 24±2 nmol mg proteína^{-1}h^{-1}. O Ro 41-1049 provocou uma inibição da desaminação da 5-HT com um K_i de 24 nM, enquanto que o K_i da lazabemida

para a desaminação da PEA foi de 17 nM. Estes resultados obtidos em homogeneizados de túbulos não deixam qualquer dúvida quanto à presença de ambas as formas de MAO nos túbulos contornados proximais do rim de Rato. Temos, deste modo, demonstrada a presença de MAO-B nos túbulos renais, embora esta forma de MAO pareça não ter importância na metabolização da DA pelas células tubulares isoladas. Uma hipótese interpretativa para esta diferença de resultados quando o estudo é feito em homogeneizados ou quando se usam células isoladas ou fatias de tecido é a de que a MAO-A e a MAO-B estejam em compartimentos diferentes que condicionam diferentes acessibilidades ou da amina ou do substrato às duas formas da enzima. Quando se usam homogeneizados de tecidos, todas as barreiras – membranas celulares ou outras – são desfeitas e, por isso, o acesso às MAOs é directo. Quando se usam células isoladas ou fatias de tecido, pode haver barreiras que criem compartimentos físicos ou funcionais que perturbem esse acesso. Esses diferentes compartimentos tanto podem dizer respeito a diferentes células, como a diferentes estruturas dentro de uma mesma célula. No primeiro caso, não é difícil admitir que, num tecido como o córtex renal, com tanta variedade de células haja um acesso diferente da DA ou do substrato utilizado ao citoplasma. Alternativamente não é inconcebível admitir a existência de barreiras intracelulares funcionais ou físicas que condicionem o acesso da DA ou de outro substrato às MAOs.

Desde há vários anos que se sabe que as MAOs são enzimas intracelulares que se encontram localizadas na membrana externa da mitocôndria (Greenawalt et al., 1970). Todas as proteínas com esta localização são codificadas por genes situados no núcleo e sintetizadas a partir dos respectivos mRNA em ribossomas citoplasmáticos (Zubay, 1993a; Stryer, 1994c). Sabe-se que as MAOs são sintetizadas por esse mesmo processo (Sagara et al., 1982) e que as cadeias nascentes vão sendo flaviniladas à medida que se formam (Zhou et al., 1995). Esta junção da molécula de FAD é um processo autocatalítico (Weyler et al., 1990; Hirashiki et al., 1995). O modo como as MAOs, recém-formadas, se dirigem para o seu destino final dentro da célula e a

relevância que as proteínas chaperão (como as proteínas de choque térmico – "heat shock proteins") têm na preservação da sua estrutura não são ainda conhecidos. Sabe-se, contudo, que as MAOs, ao contrário de outras proteínas com localização semelhante, não contêm uma sequência hidrofóbica na sua extremidade amina (Bach et al., 1988), a qual é normalmente a responsável pela condução das proteínas para o seu destino final na membrana externa da mitocôndria (Schatz, 1987). Mais ainda, no caso da MAO-B sabe-se que a perda dos 55 a.a. da extremidade amina não tem qualquer consequência sobre o seu precurso até à membrana (Mitoma et al., 1992). Outra particularidade importante neste processo reside no facto de a inserção das MAOs na membrana externa, embora estando dependente do ATP e da ubiquitina, não necessitar nem da existência de potenciais de membrana, nem da presença da molécula de FAD (Schatz, 1987; Zhuang et al., 1988; Zhaung et al., 1989; Zhuang et al., 1992; Zhou et al., 1995). Os gráficos de hidropatia da MAO-A e da MAO-B são quase iguais, surgindo em cada um sete regiões hidrofóbicas, das quais apenas duas (resíduos 7-30 e 491-extremidade carboxílica) poderão corresponder a domínios transmembranares e ser responsáveis pela fixação na membrana (Bach et al., 1988; Hsu et al., 1988).Estes dois domínios são codificados pelos exões 1 e 15, respectivamente, e partilham uma identidade de 62.5% da sequência peptíca entre as duas formas de MAO (Grimsby et al., 1991). Embora a inserção na membrana esteja dependente da extremidade carboxílica, mais uma vez haverá diferenças entre as MAOs, pois a perda dos 24 a.a. da extremidade carboxílica da MAO-A humana não altera a localização da mesma (Weyler, 1994), enquanto que para a MAO-B a perda dos 28 a.a. terminais resulta numa proteína inactiva (Mitoma et al., 1992). Também a MAO da truta cuja extremidade carboxílica é mais curta em 29 a.a. mantem a sua localização e actividade (Chen et al., 1994). Será então o modo como as MAOs se fixam na membrana uma das justificações para as diferenças encontradas entre ambas? De facto, se as sequências que permitem a fixação das MAOs à membrana são distintas, então também o modo como essas enzimas se comportam pode-

rá ser diferente, residindo aqui uma possível explicação para a teoria "multi-compartimental".

Trabalhos anteriores apontavam já para essas diferentes localizações dos dois tipos de MAO na membrana externa. Usando diferentes anti-soros Russell e col. (Russell et al., 1979a) encontraram a actividade de oxidação da PEA na superficie citosólica da membrana externa, a actividade de oxidação da 5-HT na superficie interna da membrana externa e a actividade de oxidação da tiramina em ambas os lados da membrana externa da mitocôndria. Sendo assim, o acesso da DA a cada uma das formas de MAO estaria condicionado pela posição relativa de cada qual em relação à superfície da membrana mitocôndrica externa. Contudo, este trabalho levantou dúvidas dada a discutível especificidade dos anticorpos usados (Weyler et al., 1990). Estudos de outros autores têm vindo a sugerir a existência, de facto, de diferenças no modo como estas duas proteínas se encontram fixadas à membrana externa. Sendo a MAO-B mais resistente à acção de proteases (Zhuang et al., 1988), ao tratamento com neuramínidase (Houslay et al., 1980) e ainda à acção da fosfolipase A2 (White et al., 1984), estaria numa posição "mais protegida" na membrana mitocôndrica do que a MAO-A. O estabelecimento definitivo da localização relativa das MAOs está dependente da interferência que os próprios métodos usados na sua avaliação têm (Kandaswami et al., 1977; Achee et al., 1981; Bancells et al., 1987).

Uma segunda possibilidade a considerar, ainda no nível intra-celular, é a da existência de diferentes tipos de mitocôndrias, eventualmente designáveis como de tipo "A" e de tipo "B", e que teriam diferentes densidades ou localizações dentro da célula. De facto há alguns resultados obtidos em estudos feitos em cérebro de Rato em que foi possível separar três tipos distintos de mitocôndrias, com afinidades para os substratos 5-HT e PEA, e com sensibilidades aos inibidores clorgilina e selegilina, claramente distintas (Bourne et al., 1975; Owen et al., 1977). Porém, no que se refere às MAOs, nunca foi demonstrada a existência de populações de mitocôndrias com características próprias nem essa parece ser uma possibilidade plausível,

muito embora se saiba que coexistem mitocôndrias com DNA mitocôndrico (mtDNA) normal e mutante numa mesma célula, fenómeno designado de heteroplasmia e que permite a persitência de mutações do mtDNA que de outro modo seriam letais (Johns, 1995;1996).

Finalmente, e considerando ainda o nível intracelular, é de referir uma outra possibilidade, bastante mais especulativa, mas nem por isso menos interessante. Sendo as células epiteliais células com grande polaridade elas são muito dependentes, para o seu funcionamento, de uma estrutura do citoesqueleto bastante bem definida. Isto é comprovado pela grande sensibilidade a agressões que, alterando os microfilamentos de actina, causam uma perda da relação tão característica entre estrutura e função nestes epitélios (Soares-da-Silva, 1992a; Fish et al., 1994). Simultâneamente, o funcionamento metabólico das células tem sido interpretado de acordo com duas hipóteses: a tradicional, que representa a célula como um "saco de água e enzimas", onde as interacções enzima-substrato são dominadas por processos de difusão, e um segundo modelo que partindo da estrutura tridimensional intracelular concebe a existência de sistemas de circulação intracelular (Hochachka, 1999). Este segundo modelo apoia-se no citoesqueleto das células, nomeadamente nos seus filamentos de actina, para justificar um sistema onde a ordem e a estrutura prevalecem. As enzimas estariam, mesmo no citoplasma, estruturalmente localizadas e não seriam livres para difundir e os substratos teriam, também, zonas preferenciais por onde se distribuir (Hochachka, 1999). Além disso é importante referir que as próprias mitocôndrias se movimentam dentro das células, utilizando os referidos filamentos de actina como suporte para essas deslocações (Boldogh et al., 1998). Dada a importância do citoesqueleto e do sistema de circulação intracelular, não será difícil imaginar que nos epitélios com grande polaridade, como é o tubular renal, a localização da AAAD relativamente às mitocôndrias (que poderão não ser todas iguais) bem como o precurso que a DA recém-sintetizada tem de fazer, poderão ser determinantes na forma de MAO que vai actuar. Importa referir que os dados obtidos no estudo da

captação de L-DOPA por células Caco-2 sugerem que esta não tem uma distribuição homogénea na célula (Vieira-Coelho et al., 1998).

O outro nível de explicação possível diz respeito à variedade de células existentes no cortex renal e mesmo na totalidade do rim (e que designámos de nível intercelular). Os estudos realizados no cérebro, de diferentes espécies, demonstram que a co-localização numa mesma célula de ambas as formas de MAO é extremamente rara (Westlund et al., 1985; Thorpe et al., 1987; Westlund et al., 1988; Arai et al., 1997). Nos tecidos periféricos há, contudo, dados demonstrando a existência de co-localização no pâncreas exócrino e no cortex da supra-renal (Rodriguez et al., 2000) ou ainda nos adipócitos (Pizzinat et al., 1999c). Os estudos de Saura, utilizando lazabemida marcada radioactivamente e técnicas de auto-radiografia, demonstraram que no rim de Rato a MAO-A se distribui homogeneamente, enquanto que a MAO-B se distribui de modo heterogéneo (Saura et al., 1992). Detalhando essas distribuições, verifica-se que a MAO-B apenas está presente nos túbulos corticais renais e que a MAO-A é 1.2 vezes superior à MAO-B. Esta relação está de acordo com a encontrada em estudos cinéticos feitos em fatias de rim (Fernandes et al., 1992) e é, também, concordante com os valores encontrados em estudos de "western blot" (Pizzinat et al., 1999b). Nas outras zonas do rim individualizadas nesse estudo – restante cortex, medular interna e externa – a relação MAO-A/MAO-B é próxima de 20. Esta localização da MAO-B é confirmada pelos presentes resultados em que a PEA foi usada como substrato e não explica as diferenças encontradas para a lazabemida em relação ao Ro 41-1049. Atendendo a que nas presentes condições experimentais as células tubulares isoladas e os túbulos contornados proximais eram, microscopicamente, as estruturas predominantes, a única explicação plausível será a da presença de células mesangiais contaminantes, as quais contém apenas MAO-A (Pizzinat et al., 1999b). A possibilidade de se estar a estudar uma mistura de célular tubulares e mesangiais, permitiria compreender o menor efeito da lazabemida sobre a metabolização da DA recém-sintetizada. Por outro lado, a existência destas

células também é compatível com o efeito inibitório que, quer a lazabemida, quer o Ro 41-1049, tiveram sobre a metabolização de PEA e 5-HT, respectivamente, o que está de acordo com a presença de ambas as formas de MAO nos túbulos renais. Como as preferências pelos substratos variam com os tecidos e também com as espécies, outra explicação para as diferenças encontradas poderá ser o tipo de substrato utilizado nos estudos feitos com células tubulares isoladas. Embora a DA possa ser metabolizada por ambos os tipos de MAO no cérebro humano (O'Carroll et al., 1983), ela é considerada essencialmente um substrato da MAO-A no cérebro de Rato (Waldmeier et al., 1976; Garrett et al., 1990). Neste sentido apontam também os resultados obtidos, quer em ratinhos KO para o gene da MAO-A (KO MAO-A), os quais têm níveis cerebrais de DA aumentados e menores concentrações de DOPAC (Cases et al., 1995), quer em ratinhos KO para o gene da MAO-B (KO MAO-B), os quais não acumulam DA (Grimsby et al., 1997). Contudo, nestes últimos ratinhos KO MAO-B a administração de doses elevadas de L-DOPA causa um aumento dos níveis de DA (Fornai et al., 1999). Atendendo à grande semelhança de sequências existente entre as MAOs A e B do Homem e as correspondentes do Rato, não é muito provável que os locais activos das MAOs cerebrais humana e de Rato sejam significativamente diferentes. Uma explicação possível será a existência de diferenças nas estruturas secundárias e terciárias das enzimas que condicionem estas preferências. Nesse mesmo sentido apontam os resultados obtidos num estudo em que se verificaram grandes diferenças na sensibilidade aos mesmos inibidores entre a MAO-B de Rato e de Boi (Krueger et al., 1995). Finalmente, será sempre de admitir que esta variabilidade na metabolização da DA seja apenas condicionada pela abundância relativa de MAO-A e de MAO-B encontrada nos tecidos.

A avaliação comparativa da capacidade das células tubulares renais do Rato e das células OK de sintetizar e degradar DA resultou do interesse deste modelo para o estudo das acções renais das monoaminas. De entre as caracterísiticas das células OK que as tornam um bom modelo de estudo de

sistemas monoaminérgicos destacam-se as seguintes: a capacidade para descarboxilar a L-DOPA transformando-a em DA, a não acumulação da DA endógena, a presença de receptores D_1, a capacidade para transportar os precursores da DA (L-DOPA) e da 5-HT (5-HTP) e ainda a resposta quer à DA quer à 5-HT (Murphy et al., 1989; Cheng et al., 1990; Perrichot et al., 1995; Vieira-Coelho et al., 1997; Vieira-Coelho et al., 2001). Também pelo facto de estarmos a considerar uma linha celular, muitas das dúvidas colocadas pelas suspensões de túbulos, quanto ao seu grau de "pureza", não se colocam quando se usam estes modelos celulares. Os estudos cinéticos em que foram utilizadas a 5-HT e a PEA como substratos, cujos resultados são apresentados no capítulo II (**publicação 2**), demonstraram que as células OK não dispõem de MAO-B, pelo menos funcionalmente significativa, dado que é praticamente nula a desaminação da PEA. Por outro lado os valores de K_m (408 μM) e de V_{max} (38.7 nmol mg protein^{-1}h^{-1}) determinados para a MAO-A não são significativamente distintos dos encontrados para as células tubulares renais. Quando comparadas com as células tubulares, as células OK demonstraram, ainda, menor capacidade para descarboxilar a L-DOPA (AAAD), embora o seu K_m seja semelhante ao dos túbulos. Além disso, mostraram uma actividade de metilação (COMT) considerável, traduzida numa V_{max} superior e num K_m quase cinco vezes inferior. Foi ainda possível verificar uma quase ausência de fosfatase alcalina e de gama-glutamil-transférase das células OK. Estes resultados demonstram que esta linha celular dispõe de todo o sistema enzímico necessário para sintetizar e desaminar a DA. Contudo, a utilização deste modelo celular para estudar a actividade dos sistemas monoaminérgicos renais deverá ter em conta a falta de MAO-B.

Papel das MAOs no intestino

O jejuno que é um tecido com características de certo modo semelhantes ao rim, no que diz respeito aos aspectos monoaminérgicos, foi um

dos objectos da presente dissertação e constitui o assunto do Capítulo III. É grande a evidência acumulada ao longo dos últimos anos demonstrando a importância que a DA e a 5-HT têm na modulação de múltiplas actividades intestinais, com particular ênfase no caso do metabolismo hidro-electrolítico (Lefebvre, 1988). É de considerar que, no intestino, estas duas aminas têm um papel semelhante ao que têm no rim, onde através da activação de receptores específicos causam efeitos opostos sobre o transporte de electrólitos pelo epitélio, mediados pela ATPase Na^{+}/K^{+} (Soares-da-Silva et al., 1996a; Soares-da-Silva et al., 1996b). A mucosa jejunal possui uma grande actividade da AAAD (Vieira-Coelho et al., 1993), pelo que é de prever que seja capaz de sintetizar DA e 5-HT a partir da descarboxilação, respectivamente, da L-DOPA e da 5-HT circulantes ou luminais. Esta capacidade das células da mucosa jejunal é evidenciada pelos estudos que demonstram que nem a DA intestinal tem origem em estruturas neuroniais, nem a sua transformção em NA tem significado (Esplugues et al., 1985; Bertorello et al., 1993). Também aqui a disponibilidade relativa destas aminas e, consequentemente, a capacidade de exercerem as suas acções depende, por um lado da síntese a partir do precursor respectivo e, por outro lado, do grau de metabolização que sofrem. Sendo a mucosa intestinal rica em MAO e em COMT, são estas as principais responsáveis pela terminação da acção das monoaminas (Kopin, 1985). Assim, se reunem no intestino todas as condições para que se possa definir um sistema monoaminérgico intestinal não- neuronial, o qual poderá ser no seu funcionamento e regulação semelhante ao renal.

Na **publicação 3** procurou-se caracterizar as MAOs presentes em homogeneizados de células de jejuno, quer pela determinação das suas actividades, quer pelo estudo das suas sensibilidades aos inibidores selectivos Ro 41-1049 e lazabemida. Os resultados obtidos que mostram a presença de ambas as formas de MAO, mostram também para cada uma delas, valores de V_{max} e K_m muito próximos dos descritos, no capítulo II, para as mesmas formas nos túbulos renais. Poderá considerar-se apenas que, a presença de MAO-B é ligeiramente mais significativa no jejuno do que no rim.

As actividades da MAO-A (substrato 5-HT) e da MAO-B (substrato PEA), não foram modificadas pela presença de 250 nM de lazabemida ou de 250 nM de Ro 41-1049, respectivamente. Ambos estes compostos, Ro 41-1049 e lazabemida, revelaram ser potentes inibidores da respectiva forma de MAO. Verificou-se, ainda, que a mucosa jejunal possui COMT, a qual é fortemente inibida pelo tolcapone. Estes resultados sublinham a importância dos sistemas de metabolização de monoaminas, particularmente dos de desaminação, na mucosa jejunal. A natureza do intestino como grande estrutura de inter-face com o meio externo torna, ainda, mais relevantes estes resultados uma vez que à existência das MAOs corresponde, seguramente, um papel metabólico de destaque.

Os estudos por "northern blot" revelaram que, para a MAO-A e com excepção do intestino delgado e da placenta, que exprimem um mRNA adicional com 2 kb, a maioria dos tecidos exprimem um mRNA com 4.4-5.1 kb (Bach et al., 1988; Hsu et al., 1988; Grimsby et al., 1990; Shih et al., 1990). Para a MAO-B o mRNA tem 2.8-3 kb e é o mesmo em todos os tecidos estudados (Bach et al., 1988; Grimsby et al., 1990). Qual será o significado da presença de um segundo transcripto mais pequeno para a MAO-A intestinal e placentária? Procurando estabelecer um paralelismo entre estes tecidos, a maior semelhança reside no facto de ambos serem tecidos de interface – no intestino com o meio exterior e na placenta entre a mãe e o feto – e como tal estarem envolvidos em funções de metabolização de aminas xenobióticas e/ou em excesso vindas de outro meio. A vantagem para a MAO-A estaria na possibilidade de dispôr de um modo de transcrição mais "versátil" permitindo-lhe ser rapidamente sintetizada para responder às necessidades (Shih et al., 1990). As MAOs desempenham funções na primeira linha da metabolização das aminas da dieta sobretudo da tiramina, da PEA e da L-DOPA. São desde há vários anos conhecidos os problemas criados pela administração de aminas e a.a. constituintes do queijo (tiramina, triptamina, PEA, L-DOPA) em doentes medicados com inibidores clássicos das MAOs ("cheese effect"– que se manifesta por crises hipertensivas) (Youdim et al.,

1988). Recentemente tem sido apontados os alimentos ricos em PEA (como chocolate, queijo ou vinho) como factores desencadeantes de enxaqueca em individuos susceptíveis dado o papel que a PEA terá na modulação da circulação cortical cerebral (Scremin et al., 1999). Para além destas funções de interface o jejuno poderá estar envolvido em mecanismos de regulação e contra-regulação envolvendo a L-DOPA e a DA. A L-DOPA da dieta é metabolizada no tubo digestivo e a L-DOPA plasmática é captada pelos músculos em resposta à subida dos níveis de insulina após uma refeição (Eldrup et al., 1998). A L-DOPA plasmática vai também aumentar a actividade das MAOs pancreáticas ao provocar a acumulação de DA neste tecido. Esta maior actividade de desaminação da DA resulta numa maior produção de H_2O_2 e estes dois fenómenos tomarão parte na diminuição da libertação de insulina induzida pela glicose (Lundquist et al., 1991; Pizzinat et al., 1999a). Desta forma as MAOs intestinais e pancreáticas poderão, ao modular a desaminação da DA formada a partir da L-DOPA da dieta e da plasmática, tomar parte na regulação da glicemia. As MAOs intestinais poderão ainda, estar envolvidas na diminuição da absorção jejunal de sódio mediada pela DA como resposta à uninefrectomia (Vieira-Coelho et al., 2000).

A necessidade de caracterizar melhor o sistema monoaminérgico intestinal motivou a procura de outros modelos experimentais. Nesse sentido, um primeiro passo foi dado quando, do mesmo modo que se utilizaram as células OK para estudar o rim, se passou a utilizar uma linha celular, as células Caco-2, para estudar o intestino. Estas representam uma linha de células epiteliais, com origem num adenocarcinoma de cólon humano e que, em cultura, se diferenciam em enterócitos. Quando se utiliza esta linha celular está-se a recorrer a um modelo totalmente homogéneo com todas as vantagens que daí resultam. Um outro passo, foi o desenvolvimento de uma técnica de isolamento de células jejunais e não de mucosa, as quais foram caracterizadas morfologicamente (por microscopia óptica e electrónica), tendo-se verificado uma pureza em enterócitos de 99.9%. As restantes 0.01% das células apresentaram características de células enterocro-

mafins, as quais pela sua riqueza em 5-HT, poderão dificultar a avaliação qualitativa dos resultados.

Na presente **publicação 4** o objectivo foi a caracterização da actividade desaminativa nestes dois modelos celulares descritos – células Caco-2 e células jejunais isoladas. Pelo seu carácter original deu-se maior relevo, ao primeiro destes modelos e usou-se o segundo como termo de comparação. Os resultados obtidos nas células Caco-2 demonstram a existência de actividade de ambas as formas de MAO nessa linha celular. Muito embora os valores de V_{max} encontrados para a 5-HT e para a PEA sejam menores do que os obtidos nas células epiteliais jejunais, os valores de K_m para ambos os tipos de MAO são semelhantes aos encontrados nos homogeneizados de mucosa jejunal (**publicação 3**) ou mesmo nos estudos realizados nos túbulos renais e células OK (nestas apenas para a MAO-A). Nas células jejunais isoladas a V_{max} da MAO-B foi menor do que a obtida nos homogeneizados de mucosa de jejuno. Uma vez que as condições experimentais utilizadas foram as mesmas, nomeadamente quanto ao tempo de incubação e ao substrato, a única explicação plausível é a de que no processo de obtenção dos enterócitos se tenham retirado elementos celulares mais ricos em MAO-B (por exemplo células enterocromafins). Os estudos de metabolização da DA demonstraram que as células jejunais isoladas têm consideravelmente maior actividade (11 vezes) do que as células Caco-2. Contudo e porque não se fizeram estudos com inibidores selectivos das MAOs, não foi possível determinar qual das duas formas prevalece na desaminação da DA por estas linhas celulares. As células Caco-2 revelaram possuir razoável actividade da AAAD, o que não é de estranhar dada a capacidade já antes demonstrada por estas células para captarem L-DOPA e 5-HTP (Vieira-Coelho et al., 1998). Nesta capacidade demonstrada pelas células Caco-2 para descarboxilar, quer L-DOPA originando DA, quer 5-HTP originando 5-HT, a afinidade da AAAD para os substratos é semelhante à verificada nas células do jejuno. Finalmente, refere-se o facto de as células Caco-2 não apresentarem actividade da COMT. Fica assim demonstrado o grande interesse das

células Caco-2 para estudos monoaminérgicos intestinais, na medida em que essas células dispõem das enzimas necessárias para sintetizar e metabolizar a DA e a 5-HT. É, ainda, importante lembrar que nestes estudos em que se recorre a linhas celulares não se deve esquecer a origem das mesmas e os seus estadios de diferenciação celular (definidos pelo tempo e passagem da cultura celular). Isto porque, por exemplo, a actividade da MAO-A em fibroblastos humanos é tanto maior, quanto maior a proporção de células senescentes existentes na cultura e estas células são em tanto maior número quanto maior for a idade do dador (Denney et al., 1999).

Metabolização da PEA e características próprias do coração

A informação existente na literatura aponta para um predomínio da MAO-A no coração de Rato (Kuwahara et al., 1990; Saura et al., 1992). Simultaneamente, conhece-se que a PEA é considerada um substrato preferencial da MAO-B (Yang et al., 1973), embora essa preferência possa variar com o tecido e com a espécie animal (Suzuki et al., 1981b; Fowler et al., 1984). Na **publicação 5** caracterizou-se o comportamento cinético das MAOs no coração, utilizando como substratos a 5-HT e a PEA e, como inibidores selectivos o Ro 41-1049 e a lazabemida, respectivamente. A curva de saturação para a PEA mostrou uma V_{max} de 53±10 nmol mg proteina^{-1}h^{-1}, valor que não era afectado pela presença do inibidor selectivo da MAO-B, a lazabemida (V_{max}=42±6 nmol mg proteina^{-1}h^{-1}), mas era significativamente reduzido pelo inibidor selectivo da MAO-A, o Ro 41-1049 (V_{max} de 10±1 nmol mg proteina^{-1}h^{-1}). Os valores de K_m encontrados em qualquer uma das três situações são muito próximos, e claramente diferentes dos encontrados com o mesmo substrato, PEA, no cortex renal. A desaminação da 5-HT pela MAO-A, quer no cortex renal quer no coração, revelou valores de K_m eleva-

dos e da mesma ordem de grandeza dos encontrados para a PEA. Esta diferença manifesta-se pelos elevados valores de K_m encontrados para a PEA no coração, que são cerca de 10 vezes superiores aos dos túbulos renais e do jejuno, bem como aos referidos na literatura (Youdim et al., 1988). Estes valores de K_m são da mesma ordem de grandeza dos determinados para a 5-HT em qualquer dos tecidos referidos. Quando se olha para o efeito exercido pelos inibidores selectivos, verifica-se que, o Ro 41-1049 (inibidor selectivo da MAO-A) inibiu de igual modo a desaminação da PEA e da 5-HT e, por outro lado, a lazabemida (inibidor selectivo da MAO-B) não causou qualquer inibição, não afectando nem a desaminação da PEA, nem a da 5-HT. Estes resultados, demonstram um padrão de metabolização da PEA que foge completamente ao padrão clássico que a descreve como substrato da MAO-B. Perante estes resultados colocaram-se várias questões.

A PEA tem sido considerada como substrato específico da MAO-B (Yang et al., 1973) e nesse sentido apontam alguns estudos realizados. É sabido que a inibição da MAO-B cerebral (no Rato e no Ratinho) resulta num aumento das concentrações de PEA e consequente potenciação das respostas neuroniais dopaminérgicas (Juorio et al., 1988; Berry et al., 1994). De igual modo, a inibição da MAO-B no cérebro de Rato resulta na inibição da fixação de PEA exógena (Li et al., 1992). Mas talvez o mais relevante de todos seja o conhecimento de que em ratinhos KO MAO-B os níveis cerebrais de PEA estão aumentados cerca de 8 vezes, sem haver qualquer alteração dos níveis de DA, NA ou 5-HT (Grimsby et al., 1997). É assim claro que a PEA pode ser considerada um substrato natural da MAO-B. Contudo, a concentração de amina utilizada na **publicação 5** (1000 μM) poderá ser excessiva e, assim, perder a especificidade de tipo, tornando-se também substrato da MAO-A (Fowler et al., 1984). Isso explicaria, também, o comportamento idêntico do Ro 41-1049 perante a PEA e a 5-HT. Para ultrapassar esta perda de especificidade analizou-se o efeito de vários tipos de inibidores sobre a metabolização de 20 μM de PEA por homogeneizados de coração e de cortex renal (**publicação 6**). O inibidor mais potente da desaminação da PEA foi o Ro 41-

1049, seguindo-se, com potências intermédias próximas, a clorgilina e a lazabemida, sendo a selegilina o menos potente, ao não chegar a inibir 50% da actividade. Contrariamente, no cortex renal o padrão das potências inibitórias encontrado foi o esperado para o substrato em causa. Enquanto que a lazabemida e a selegilina tiveram efeito inibitório marcado, o Ro 41--1049 e a clorgilina não manifestaram qualquer efeito. Perante estes resultados levantou-se, de novo, a questão da relevância da concentração de PEA. Conhece-se que no cérebro humano a PEA em concentrações de 20 μM tem um comportamento próprio de substrato da MAO-B (O'Carroll et al., 1983). Há contudo vários autores que consideram essa concentração excessiva referindo como mais adequados valores de 10 μM a 12.5 μM para o cérebro de Rato (Suzuki et al., 1979; Suzuki et al., 1981b) e de 1 a 10 μM para o ventrículo da mesma espécie animal (Dial et al., 1979). Poderá mesmo acontecer que, para concentrações de PEA tão elevadas como as utilizadas inicialmente (1000 μM), possa ocorrer inibição da MAO-B pelo próprio substrato (Suzuki et al., 1981b). Contudo, nas nossas condições experimentais, a redução da concentração de PEA para 0.2 μM não alterou o padrão de inibição inicialmente descrito para a concentração 20 μM. Seria contudo de esperar que a lazabemida, pelo facto de ser estruturalmente muito semelhante à PEA, tivesse um maior efeito inibidor. De facto, a geração da forma activa deste inibidor apenas se dá após a ocupação do centro activo da enzima, pelo que seria de esperar que esta partilha parcial da via catalítica aumentasse muito a especificidade desta forma de inibição (Cesura et al., 1989; Da Prada et al., 1990). Também a utilização de membranas em lugar de homogeneizados não alterou esse padrão inibitório. Em face deste dado, considerou-se, novamente, o problema da localização das MAOs na membrana externa da mitocôndria. Agora não se punha em causa a hipótese de haver uma diferente localização das formas de MAO, mas sim saber de que modo a composição (sobretudo lipídica) da membrana poderia contribuir para a especificidade dos substratos (Houslay et al., 1973; Russell et al., 1979b; White et al., 1982). A repetição dos mesmos estudos em membranas pré-tratadas

com digitonina, de forma a alterar o ambiente lipídico das membranas, não modificou os resultados obtidos. De igual modo, a diluição das amostras não resultou em qualquer alteração da ordem relativa, acima descrita, para a potência dos inibidores.

Haverá qualquer outra actividade enzímica que possa justificar este comportamento relativamente à PEA? Nos anos setenta foi descrita, no coração de Rato, uma enzima com capacidade para metabolizar monoaminas, a qual teria como característica a "resistência" à inibição pela clorgilina (Lyles et al., 1975). Essa actividade corresponde à oxídase de aminas sensível à semicarbazida ("semicarbazide-sensitive amine oxidase"- SSAO) que existe, entre outros tecidos, no coração de Rato (Lyles, 1996). A SSAO está principalmente localizada nos vasos sanguíneos e corresponde à proteína de adesão vascular ("vascular adhesion protein-1"- VAP-1) recentemente clonada no Homem e no Rato (Bono et al., 1998; Smith et al., 1998). A VAP-1 é uma glicoproteína endotelial que toma parte na adesão dos linfócitos ao endotélio vascular e no recrutamento de granulócitos, para além de evidenciar actividade metabolizadora de monoaminas (Salmi et al., 2000; Tohka et al., 2001). A SSAO poderá ainda ter interferência no transporte de glicose nos adipócitos (Enrique-Tarancon et al., 1998). Além disso, há tecidos onde está descrita a possibilidade de a SSAO utilizar como substrato a PEA (Lyles, 1996). Tendo presentes estes dois factos – a existência de SSAO no coração de Rato e a possibilidade de a PEA ser substrato desta enzima – estudamos a metabolização de PEA na presença de 1 mM de semicarbazida, uma concentração que está descrita como inibindo completamente a actividade da SSAO (Lyles, 1996; Pino et al., 1997). Contudo, para as duas concentrações de PEA usadas, 20 μM e 200 μM, não se verificou qualquer variação em relação à situação de controlo o que excluí a participação, mesmo que parcial, da SSAO na metabolização da PEA nestas condições experimentais.

Esta forma atípica de metabolização da PEA verificada no coração parece, contudo, não ser exclusiva deste tecido. Outros autores descreveram no fígado de Rato a existência de uma pequena actividade da MAO-A sobre

a PEA, com um K_m cerca de 10 vezes superior e uma V_{max} cerca de 8 vezes inferior à da MAO-B (Suzuki et al., 1981a) e, no cérebro humano e no de Rato, os mesmos autores demonstraram haver uma pequena parte da metabolização da PEA sensível à inibição pela clorgilina (Suzuki et al., 1981b). No fígado humano, Denney e col. verificaram que, após passagem da mistura proteica pela coluna de imuno-afinidade, com retenção da MAO-B pelos anticorpos monoclonais para ela específicos, era ainda detectável uma ligeira oxidação da PEA parte da qual, por ser sensível à clorgilina, seria catalizada pela MAO-A (Denney et al., 1982). Também nas células mesangiais do rim de Rato, a MAO-A metaboliza a PEA com constantes de afinidade aparentemente idênticas às encontradas para a MAO-B (Pizzinat et al., 1999b). No mesmo sentido, apontam os resultados obtidos, quer com células de rim de Macaco transfectadas para a MAO-A, as quais apresentam actividade de desaminação da PEA (10 μM) (Lan et al., 1989a), quer com a MAO-A placentária humana, a qual oxida PEA e é mais sensível à clorgilina do que à selegilina (Hsu et al., 1988).

Ainda no Capítulo IV, **publicação 6**, caracterizaram-se molecularmente, as formas de MAO presentes no coração do Rato, tendo sido colocada a possibilidade de existirem modificações nos mRNAs ou na próprias proteínas. Os estudos realizados por RT-PCR ("Reverse transcriptase - polymerase chain reaction") revelaram a presença de fragmentos para a MAO-A e para a MAO-B com as dimensões esperadas e correspondentes ao descrito na literatura. Porém, a separação das proteínas por "western blot", utilizando um anticorpo policlonal dirigido para uma sequência comum a ambas as formas, demonstrou claramente a presença de MAO-A, mas não revelou a presença de MAO-B. Estes resultados levantam alguns problemas e sugerem algumas explicações. Como explicar que existindo o mRNA para a MAO-B, por um lado não seja detectada a proteína, mas que, por outro lado, a marcação para a MAO-B utilizando técnicas histológicas seja positiva? Uma primeira hipótese é a da possibilidade de o anticorpo policlonal não identificar o epitopo correspondente na MAO-B cardíaca. De facto, se a MAO-B

cardíaca apresentar alterações na sequência dos seus a.a. na região de ligação do anticorpo policlonal, este não a reconhecerá, não se ligando à proteína. Esta situação foi descrita em estudos de mutagénese directa, em que a troca de um único a.a. (aspartato por glutamato na posição 227) foi suficiente para impedir o seu reconhecimento por um anticorpo monoclonal (Zhou et al., 1998). Uma outra situação desta natureza está descrita para algumas quimeras que embora detectadas a nível do mRNA não o eram a nível proteico (Gottowik et al., 1995). Nessa circunstância a explicação dada pelos autores foi a da incapacidade dos anticorpos utilizados para reconhecerem esses mutantes. Esta possiblidade não pode ser excluída no nosso caso, embora ela não seja provável dado que não estão descritos problemas deste tipo para este anticorpo quando utilizado noutros tecidos. Nesse estudo em que se utilizaram quimeras ficou demonstrado, para a MAO-B humana, que os a.a. da sequência 146-220 são bastante mais relevantes na definição da potência da lazabemida do que na da afinidade para a PEA (Gottowik et al., 1995). Estes dados permitem-nos considerar a possibilidade de haver alterações da estrutura da enzima que condicionem modificações na interacção com o substrato sem modificarem a interacção com o inibidor e vice-versa. Uma segunda explicação tem como base a existência de modificações pós-translacionais. O papel das modificações pós-translacionais nestas enzimas não tem sido muito estudado, embora a evidência acumulada não favoreça muito esta hipótese. Ambas as formas demonstram a existência de locais potenciais de N-glicosilação. Se na MAO A [asparagina(Asn)-valina-treonina(Thr), posições 181-183] não parece muito relevante por ficar numa região hidrofóbica, já na MAO B (Asn-Met-Thr, 145-147) não sabemos o papel que poderão ter (Bach et al., 1988). Contudo é de referir que, quer num caso, quer no outro, a glicosilação não deverá ter papel de relevo atendendo ao facto de as proteínas sintetizadas em ribossomas citoplasmáticos não passarem pelo aparelho de Golgi no seu precurso intra-celular (Stryer, 1994c). Uma outra hipótese diz respeito à intensidade com que se dá a translação da proteína MAO-B. Esta translação poderá estar sujeita a facto-

res de regulação que façam com que varie com as circunstâncias e tenha pouca expressão na situação basal. Deste modo se explicaria a não detecção da proteína no "western blot". Esta terceira alternativa está mais de acordo com os resultados obtidos por vários autores (Saura et al., 1992). Assim, o gene é transcrito, como a existência do mRNA atesta, mas a sua expressão proteíca não é a normalmente encontrada.

O facto de se ter encontrado para a MAO-A, quer os fragmentos de mRNA quer a proteína propriamente dita no "western blot", não invalida a hipótese de esta enzima ser, no coração de Rato, distinta da encontrada em outros locais. O papel de alguns a.a. específicos poderá ser determinante na especificidade dos substratos e na sensibilidade a certos inibidores. Na MAO-B, a troca da isoleucina (Ile) na posição 199 por fenilalanina (Phe) resulta num mutante - B(Ile199Phe) – com elevada afinidade para a 5-HT, ficando com um K_m semelhante ao da MAO-A, e menor afinidade para a PEA (Tsugeno et al., 1997). A troca do a.a. correspondente na MAO-A, Phe na posição 208 por Ile - A(Phe208Ile) – resulta numa perda de afinidade para a 5-HT, que desce para níveis semelhantes aos da MAO-B, sem causar modificações na afinidade para a PEA (Tsugeno et al., 1997). Estes dados vieram demonstrar o papel determinante que a posição de um só a.a. pode desempenhar no grau de selectividade dos substratos para as formas A ou B. Nesse mesmo estudo demonstrou-se que a substituição da Phe (por outro a.a. aromático) ou da Ile (por outros a.a. alifáticos com cadeias laterais) tinha as mesmas consequências sobre as afinidades relativas para os substratos que as dos mutantes descritos (Tsugeno et al., 1997). Embora apresentando uma preferência pelos mesmos substratos que a MAO-A, o mutante B (Ile199Phe) foi inibido pela selegilina, que é um inibidor da MAO-B. Esta discrepância, que é ter afinidade para os substratos da MAO-A e ser sensível aos inibidores da MAO-B, apenas em consequência da troca de um a.a. poderá servir de explicação para a metabolização da PEA por nós descrita para o coração de Rato. A MAO presente neste tecido apresentou, de facto, uma afinidade para os substratos da MAO-B e uma sensibilidade aos

inibidores da MAO-A, o que poderá ser explicado por uma simples troca de um a.a., embora de sentido contrário à descrita por Tsugeno e col.. Contudo, a variabilidade a este nível é considerável pois estudos feitos no Homem, com a mutação recíproca desses mesmos a.a., não demonstraram que isso possa determinar qualquer tipo de comportamento perante substratos e inibidores (Geha et al., 2000). Estes mesmos autores atribuiram as diferenças entre a MAO-A e a MAO-B humanas à existência de Ile na posição 335 no caso da primeira e Tyr na posição 326 no caso da segunda (Geha et al., 2001). Estes dados são interessantes na medida em que são opostos aos descritos por Tsugeno e col., uma vez que no Homem, o a.a. alifático está na MAO-A e o a.a. aromático está na MAO-B. Verificou-se, ainda, que na MAO-B, a modificação Thr por alanina (Ala), na posição 158 (Thr158Ala), resulta na perda total de actividade da enzima (Cesura et al., 1996). Do mesmo modo a simples mutação dos a.a. responsáveis pelas ligações covalente e não-covalente à molécula de FAD tornam estas enzimas inactivas (Gottowik et al., 1993; Kwan et al., 1995; Zhou et al., 1995). Também a importância dos a.a. não é sempre a mesma pois alguns, como por exemplo a prolina, que facilita a existência de "dobras" na estrutura secundária, ou a Cys, que torna possível a existência de pontes dissulfureto, são bastante mais determinantes da estrutura secundária e terciária do que outros a.a. (Zubay, 1993b). Todos estes dados sugerem que, muito embora a estrutura tri-dimensional das MAOs seja provavelmente semelhante nas diferentes espécies, bastam pequenas modificações dos a.a. nas regiões envolvidas na fixação dos substratos para determinarem comportamentos distintos.

A possibilidade de existirem outras formas de MAO para além das A e B tem sido sugerida por alguns dados experimentais. A sequênciação do cDNA da MAO da Truta (MAO-T) mostrou a existência de uma molécula com 499 a.a., e uma identidade com a MAO-A e a MAO-B humanas de 70% e 71%, respectivamente (Chen et al., 1994). Nesse mesmo estudo verificou-se que esta MAO tinha um comportamento peculiar, na medida em que apresentava um K_m para a 5-HT semelhante ao da MAO-A humana e, simulta-

neamente, um K_m para a PEA semelhante ao da MAO-B humana. Além disso, apresentava uma sensibilidade maior à clorgilina do que à selegilina, independentemente do substrato ser a 5-HT ou a PEA. Finalmente, esta nova MAO apresentava menor sensibilidade à clorgilina (usando a 5-HT como substrato) e à selegilina (usando a PEA como substrato) do que as formas humanas A e B, respectivamente. Embora esta MAO-T apresente uma estrutura da sua extremidade carboxílica diferente, ela mantém outras características que estão presentes em todas as formas, como por exemplo, a sequência pentapeptídica responsável pela ligação covalente do cofactor FAD. É então de esperar que pelo conhecimento dos a.a. conservados entre a MAO-T e a MAO-A e entre a MAO-T e a MAO-B, seja possível caracterizar melhor aqueles que são determinantes na definição das características da MAO-A e da MAO-B, respectivamente. Outra hipótese é a de existirem diferentes subtipos de cada uma das formas da MAO. Estudos efectuados com diversos compostos imidazolínicos sugerem a existência de uma população de MAO-B mista, na qual se distinguem isoformas, umas com e outras sem um lugar de ligação imidazolínico de alta afinidade (Raddatz et al., 2000). Já anteriormente se tinham verificado diferenças de acesso ao local de ligação imidazolínico da MAO-B em diferentes tecidos, bem como discrepâncias estequiométricas entre a densidade de locais de ligação imidazolínicos de tipo I_2 e de MAO-B, que sugeriam a existência dessas sub-populações enzímicas (Sastre et al., 1993; Raddatz et al., 1995). A existência, de mais de uma forma de MAO-B obrigaria a rever alguns conceitos, nomeadamente, quanto à possibilidade de existirem sub-populações como resultado de modificações, quer na transcrição inicial ("editing of the primary transcript"), quer pós-translacionais, muito embora não haja qualquer evidência em apoio desta hipótese.

A MAO E A METABOLIZAÇÃO DA NA EM DIFERENTES IDADES

O conhecimento de que as características das MAOs se modificam com a idade levou-nos a estudar as consequências dessa variação. Para tal, recorreu-se ao estudo da actividade das MAOs em alguns tecidos de Rato de diferentes idades – rim, coração, aorta e fígado – utilizando a NA como substrato. Os resultados dessa avaliação são apresentados no Capítulo V desta dissertação (**publicação 7**).

No grupo de ratos recém-nascidos (menos de 18 horas após o nascimento), a capacidade desaminativa pelas MAOs está pouco desenvolvida, embora a capacidade para acumular a amina esteja já perfeitamente desenvolvida. O total de metabolitos formado em consequência da actividade das MAOs e da COMT é sempre menor nos recém-nascidos, para qualquer dos tecidos estudados. Em qualquer dos 3 grupos etários considerados a NA é preferencialmente desaminada, com excepção do rim onde é preferencialmente metilada. A actividade das MAOs, expressa pela formação de di-hidroxifeniletilglicol (DOPEG) e de ácido di-hidroximandélico (DOMA), aumenta do nascimento até à idade adulta (2-3 meses de vida), quer no rim, quer no coração. Enquanto que no rim a actividade das MAOs permanece constante desde os 2-3 meses (ratos adultos) até aos 18-24 meses (ratos idosos), no coração essa actividade aumenta à medida que se avança na idade. Este aumento tanto poderá ser devido ao maior teor em NA do coração dos ratos idosos, como a uma alteração da actividade das MAOs com a idade. Ainda no coração é de referir que nos recém-nascidos parece haver uma deficiente actividade da desidrogenase aldeídica, que tanto poderá dever-se a uma menor quantidade de enzima, a uma deficiência dos seus co-factores (nomeadamente o FAD), como a uma variação das proporções de cada uma das formas de MAO. Esta última hipótese obrigar-nos-ia a admitir que a afinidade das diferentes MAOs para a NA não é a mesma. Se assim for e se se considerar que, ao nascer, há mais MAO-B com menor afinidade para a NA e que, com o desenvolvimento, a relação MAO-B/MAO-A se alte-

ra, favorecendo a MAO-A que tem maior afinidade para a NA, então o aumento de actividade ficaria justificado apenas pela variação das proporções entre as formas de MAO sem qualquer dependência de variações da actividade total. No Rato, sabe-se que com a idade há um aumento da actividade das MAOs cardíacas (Meco et al., 1987). Para além deste aumento, está demonstrado que a proporção de cada uma das MAOs no coração também varia com a idade: às 3 semanas, predomina a MAO-B, às 8 semanas, há equilíbrio entre ambas as formas e às 15 semanas, apenas se encontra MAO-A (Edwards et al., 1979). No rim, não há qualquer variação da actividade total das MAOs entre os 3 meses e os 24 meses de idade (Strolin-Benedetti et al., 1992). Todas estas modificações dependentes da idade ocorrem também no cérebro, onde ao nascer a quantidade de MAO-B é muito reduzida, predominando a MAO-A. Com a idade verifica-se um aumento da MAO-B mantendo-se a MAO-A sem grande variação. Contudo, este aumento de actividade não se verifica para todos os substratos (Strolin-Benedetti et al., 1980). A existência de variações na quantidade relativa da actividade das MAOs com a idade poderá justificar que alguns efeitos que não são significativos numa determinada faixa etária o sejam noutras. Um outro factor a ter em conta nestes estudos da actividade enzímica dependente da idade é a espécie animal em que o estudo é feito (Lewinsohn et al., 1980; Strolin-Benedetti et al., 1994). Também é de considerar a variação do estado hormonal, desde a vida embrionária e fetal, passando pela adolescência, até à idade adulta, sendo várias as influências sofridas pelas MAOs a este nível (Youdim et al., 1989; Ma et al., 1995; Carlo et al., 1996). Finalmente o modo como os co-factores modulam a actividade das enzimas nos recém-nascidos tem de ser considerado, nomeadamente em relação à flavinização da MAO (Zhou et al., 1995).

Distribuição tecidular e relação com funções e substratos – perspectiva teleológica

A procura de uma relação entre a localização das formas de MAO nos diferentes tecidos e as funções fisiológicas exercidas por essas mesmas enzimas tem sido uma preocupação constante. No sistema nervoso central (SNC) a MAO-A está localizada nos neurónios adrenérgicos e noradrenérgicos e não nos neurónios serotoninérgicos, como seria de esperar pelo conhecimento de que a 5-HT é o seu substrato natural. Contudo, numerosos autores tem interpretado este facto atribuindo à MAO-A, nestas condições, funções de protecção dos neurónios catecolaminérgicos ao inactivar a 5-HT que neles se acumularia como falso neuro transmissor (Levitt et al., 1982; Westlund et al., 1985; Westlund et al., 1993). De igual modo, na opinião dos mesmos autores, o papel da MAO-B nos neurónios serotoninérgicos seria equiparável, prevenindo a acumulação de substratos naturais como a DA que pudessem interferir com o armazenamento, libertação e captação da 5-HT. Além destas funções, a MAO-B das estruturas circum-ventriculares teria ainda funções de protecção do cérebro em relação a aminas residuais, de que são exemplo alguns dos seus substratos naturais preferenciais como a PEA e triptamina que poderiam actuar como falsos neurotransmissores, ao ser captadas pelos neurónios (Saura et al., 1992). A presença de MAO nas células epêndimais e no endotélio vascular protegeria ainda o cérebro de monoaminas provenientes do liquído cefalo-raquídeo ou da circulação sanguínea, respectivamente (Kalaria et al., 1987; Saura et al., 1992; Westlund et al., 1993). Esta mesma relação entre a topografia e função poderia também explicar algumas situações patológicas, como a da toxicidade do MPTP. Esta neurotoxina uma vez oxidada pela MAO-B (presente nas células da glia e neurónios serotoninérgicos) daria origem a MPP+, que seria selectivamente concentrada nos neurónios dopaminérgicos da substância nigra, onde exerceria os seus efeitos tóxicos (Langston et al., 1983; Chiba et al., 1984; Langston et al., 1984).

Analogamente, se poderia admitir a possibilidade de existir uma relação entre a topografia e a função das MAOs nos sistemas monoaminérgicos periféricos. Se se considerar que para o organismo é primordial a natriurese em relação à anti-natriurese, é natural a existência de um predomínio da MAO-A no rim, dado que a 5-HT é um substrato preferencial desta forma de MAO. Estaria assim assegurada a existência de um "tonus" natriurético basal no epitélio tubular renal, da responsabilidade da DA, ficando a acção anti-natriurética da 5-HT bastante mais limitada no seu âmbito. Concordante com esta hipótese estará o facto de a metabolização da L-DOPA pela AAAD ter preferência em relação à descarboxilação da 5-HTP (Soares-da-Silva et al., 1996a). Também no jejuno, a existência de um maior equilíbrio entre a MAO-A e a MAO-B poderá estar relacionada com as funções que estas aqui desempenham. A presença simultânea na dieta de aminas consideradas "fisiológicas" para o organismo, que serão preferencialmente metabolizadas pela MAO-A, e de aminas xenobióticas, que serão preferencialmente metabolizadas pela MAO-B, justificará esse equilíbrio. Finalmente e no que diz respeito ao coração, este tipo de raciocíneo é mais difícil de estabelecer, porque o predomínio de MAO-A no Rato não tem correspondência noutras espécies, especialmente na espécie humana. No Homem a forma de MAO predominante no coração é a MAO-B, pelo que a relação entre topografia e função observada no Rato, não pode ser transferida para a espécie humana. Pode sempre considerar-se que para a placenta (apenas com MAO-A) e para as plaquetas (apenas com MAO-B) esta relação entre topografia e função tem significado fisiológico. Na placenta só há MAO-A pois o importante é defender o feto de excessos de aminas circulantes, nomeadamente NA, 5-HT e DA. Nada interessará que haja MAO-B pois a placenta apenas é atingida pelas monoaminas que circulam no plasma. No plasma, predomina a forma B que está muito mais ligada à depuração de xenobióticos, pouco interferindo na metabolização das aminas endógenas.

Não deve ser esquecido o papel de outras enzimas que, como a COMT, a PST e a glicuronídase, são capazes de degradar os mesmos substratos. A

existência de vias alternativas para esta metabolização de aminas permitirá a compensação requerida quando necessário. Finalmente o facto de as diferentes formas de MAO ocorrerem nos mesmos tecidos e serem capazes de degradar os mesmos substratos pode ser uma vantagem para o organismo, porque assim a MAO-A e a MAO-B poderão suprir, em caso de necessidade, a falta uma da outra.

Conclusões

O tipo de modelo experimental (células intactas, células em cultura, homogeneizados) e a diferente natureza dos vários tecidos são fundamentais para a análise e interpretação da capacidade de desaminar monoaminas.

O tipo de MAO que intervém na metabolização dos diferentes substratos depende mais da proporção relativa de MAO-A e de MAO-B existente nos tecidos, do que da natureza do próprio substrato.

No coração de Rato:

- a metabolização da PEA é diferente daquilo que era de esperar de um substrato da MAO-B;
- a metabolização da PEA e a sua sensibilidade aos inibidores das MAOs é independente da concentração do substrato e das condições experimentais;
- verifica-se que ocorre a transcrição, quer da MAO-A, quer da MAO-B, embora não seja detectável a expressão proteíca da MAO-B;
- a PEA é metabolizada pela MAO-A;

No rim de Rato:

- existem os dois tipos de MAO que exprimem actividade desaminativa qualquer que seja o modelo experimental usado;
- o papel da MAO-B na metabolização da DA é menor do que o da MAO-A, o que sugere uma localização diferente dos dois subtipos de MAO;
- as células OK não dispõem de MAO-B, embora possuam uma elevada capacidade de metabolizar monoaminas;

No jejuno de Rato:

- existe uma grande capacidade para metabolizar monoaminas, especialmente no que diz respeito à capacidade desaminativa;
- as células Caco-2 dispõe de um sistema para metabolizar monoaminas o que as torna um bom modelo experimental para os estudos desta natureza;

A capacidade desaminativa do Rato recém-nascido é muito reduzida, mas aumenta rapidamente nas primeiras semanas de vida.

A evolução dessa actividade em função da idade não é paralela nos diferentes orgãos.

Capítulo VII

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Capítulo VIII

Resumo / Summary

CONTRIBUIÇÃO PARA O ESTUDO DAS MONOAMINOXÍDASES A E B

No presente estudo apresentam-se os resultados obtidos na caracterização das monoaminoxídases (MAOs) A e B em vários tecidos periféricos. A relevância desta actividade de desaminação de monoaminas advém do papel que os sistemas monoaminérgicos periféricos, sobretudo renal e intestinal, desempenham. Em todos os tecidos estudados - rim, jejuno e coração - se verificou a existência de MAOs, embora em proporções variáveis e, consequentemente, com importância fisiológica distinta. No rim, que é considerado o principal sistema monoaminérgico periférico, existem ambas as formas de MAO. Contudo, a forma como cada uma das MAOs se evidencia vai depender da preparação experimental utilizada. A actividade da MAO-B não é evidenciável nos túbulos contornados próximais isolados, apenas o sendo em homogeneizados dos mesmos. Isto demonstra a localização em compartimentos distintos de cada uma das formas de MAO. No intestino, mais especificamente no jejuno, as MAOs, além de um papel regulador da quantidade de monoaminas que é absorvida, possuem, também, como elemento de uma rede inter-ligada, a capacidade de actuar em função do "interesse" geral do organismo. Neste tecido encontram-se ambas as formas de MAO em quantidades que são mais próximas entre si do que em qualquer um dos outros tecidos estudados. No coração de Rato a existência de MAO-B é posta em causa pelo modo como é metabolizado o seu substrato a β-feniletilamina (PEA). Esta metabolização não é sensível à inibição pelo inibidor da MAO-B a lazabemida, mas é inibida pelo inibidor da MAO-A, o Ro 41-1049. A modificação das condições experimentais por meio do recurso à utilização de membranas em lugar de homogeneizados, solubilizando

essas mesmas membranas ou diminuindo a concentração do substrato para aumentar a especificidade para a MAO-B, não modificou os resultados obtidos. Embora tenha sido detectado o mRNA da MAO-B é muito pouco provável a existência desse subtipo de MAO activo no coração de Rato, uma vez que a presença dessa enzima não é detectada no "western blot", nem funcionalmente verificável através da metabolização dos seus substratos preferidos. Por outro lado, a metabolização da PEA pela MAO-A não pode ser considerada normal. Verificou-se, também, que as linhas celulares estudadas, células OK e Caco-2, são bons modelos para o estudo das actividades das MAOs, embora nas primeiras não seja detectada actividade da MAO-B. Verificou-se, também, que a actividade desaminativa varia com a idade, aumentando desde o nascimento até aos 24 meses de vida e que nem a evolução desse aumento nem a sua expressão máxima é igual nos diferentes tecidos. A utilização de diferentes substratos permitiu verificar que a actividade desaminativa difere de tecido para tecido, assim como depende das condições experimentais utilizadas. Os resultados obtidos demonstram que a abundância relativa de cada uma das formas nos diferentes tecidos é o principal factor na determinação do tipo de MAO envolvida na metabolização dos substratos. Poder-se-á considerar a existência de uma relação entre o tipo de distribuição das MAOs pelos diferentes tecidos e as funções que aí desempenham.

Contribution to the study of Monoamine oxidase A and B.

The results of the study of monoamine oxidases in different peripherial tissues are presented in this thesis. The importance of these enzymes comes from the role renal and intestinal monoamines play. All of the tissues studied – kidney, intestine and heart – showed the existance of MAOs, although in different proportions and with different physiologic relevance. In the kidney, which is considered the most important peripherial monoaminergic system, there are both forms of MAO. However, the way each MAO type is evident depends on the experimental conditions used, with MAO-B not shown in isolated proximal renal tubules but present in renal tubules homogenates. This shows that in this tissue MAOs are present in different cellular compartments. In the intestine, more precisely in the jejune, MAOs, being responsible for monoamines levels, also take part in multiple activities of regulation and counter-regulation with other tissues. In this tissue both forms exist and the differences in its proportions are smaller then in the other tissues. In the rat heart, the way β-phenylethylamine is metabolized does not look compatible with the presence of MAO-B. This activity is not inhibited by MAO-B inhibitor, lazabemide, but it is inhibited by MAO-A inhibitor Ro 41-1049. There were no changes in the results obtained when we used membranes, solubilized membranes or reduced concentrations of the substrate. We consider that the presence of MAO-B in the rat heart is challenged by these results, because although its mRNA is present, the enzyme itself neither is detected in western blot studies, nor metabolizes one of its prefered substrates. On the other hand the way it is

metabolized is not typical of MAO-A. The cell cultures used, OK cells and Caco-2 cells, were shown to be good models for the study of MAOs, although in OK cells MAO-B activity was not present. Ageing studies also demonstrated that deaminating activity increases from birth onwards and that this changes are not the same in all tissues studied. The use of different substrates alowed us to put in evidence the differences in metabolic activities among tissues. This activity is also very dependent on the experimental conditions. Results show that the relative abundance of each type of MAO in the different tissues is the prime determinant in the form of MAO that will be engaged in the metabolization of substrates. We shall also considere the existance of a relation between the distribution of MAOs through the different tissues and the functions they play in these same tissues.