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Permutador Na+/H+ em Tecido Renal: Estudo Molecular e Funcional

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A meus alunos,

Ao Cassola,

A meu filho, Diogo.

Indice:

Introdução

O permutador Na^+/H^+ é um dos mecanismos de transporte iônico acoplado mais bem caracterizados até o momento. Historicamente, estudos sobre este mecanismo de transporte remontam à hipótese quimiosmótica de Mitchell. Em 1961, Mitchell propos a necessidade da existência da troca de cátions por H^+ e experimentalmente demonstrou sua presença em mitocôndrias [80]. Em 1976, a existência do permutador na membrana citoplasmática de células de mamíferos foi descrita pela primeira por Murer e col., em experimentos realizados com vesículas de membrana luminal de túbulos proximais renais [86]. Desde então, a existência deste transportador tem sido descrita em virtualmente todos os tipos celulares incluindo procariotas e eucariotas. O pH intracelular (pHi) é mantido próximo a 7,2 – muito próximo da neutralidade - em condições fisiológicas, sendo estes valores de pH muito mais altos que os calculados para uma distribuição de equilíbrio eletroquímico dos ions H (~ 6,2). Este pHi elevado é mantido por vários sitemas de extrusão de protons presentes na membrana citoplasmática incluindo o permutador Na^+/H^+.

Esse sistema de transporte iônico é eletroneutro, com uma rígida estequiometria de 1:1 para a troca de Na^+ por H^+. Essa reação de troca é reversível e movida pelos gradientes químicos transmembrana para Na^+ e H^+, sem envolvimento direto de energia metabólica tal como hidrólise de ATP. O gradiente de Na^+ dirigido para o interior da célula, que é gerado pela Na^+/K^+-ATPase, fornece uma força movente constante para a extrusão de H^+. Além de constituirem um sistema muito eficiente de proteção contra a acificação intracelular, os permutadores Na^+/H^+ são importantes na regulação do volume celular e são prontamente ativados em resposta a uma variedade de sinais mitogênicos e

não mitogênicos, tais como fatores de crescimento, transformação oncogênica, neurotransmissores, hormônios, estresse mecânico etc, evidenciando a participação desse mecanismo de transporte no adequado funcionamento celular nas mais diferentes condições [123].

Em células epiteliais, os permutadores Na^+/H^+ estão também envolvidos no transporte transepitelial de $NaHCO_3$ e NaCl. Os túbulos proximais de rins de mamíferos reabsorvem aproximadamente 80% do bicarbonato e cerca de 60-65% do NaCl filtrado [5]. Todo o transporte transcelular de NaCl e cerca de dois terços da absorção transcelular de $NaHCO_3$ são mediados pelo permutador Na^+/H^+ presente em membrana apical das células dos túbulos proximais [97]. Além disso, a absorção transcelular de $NaHCO_3$ fornece a força movente para a reabsorção paracelular de NaCl [100]. Quantitativamente, isso significa que a massa total de NaCl e $NaHCO_3$ contida no meio extracelular passa mais de dez vezes através das moléculas dessa única proteína de transporte ao longo de 24 horas (aprox. 24.500 mEq de Na^+/dia). Obviamente, uma regulação sofisticada, fina e versátil do permutador Na^+/H^+ presente em membrana apical de túbulos proximais é de fundamental importância para a homeostase ácido-base e e para a regulação do volume do meio extracelular.

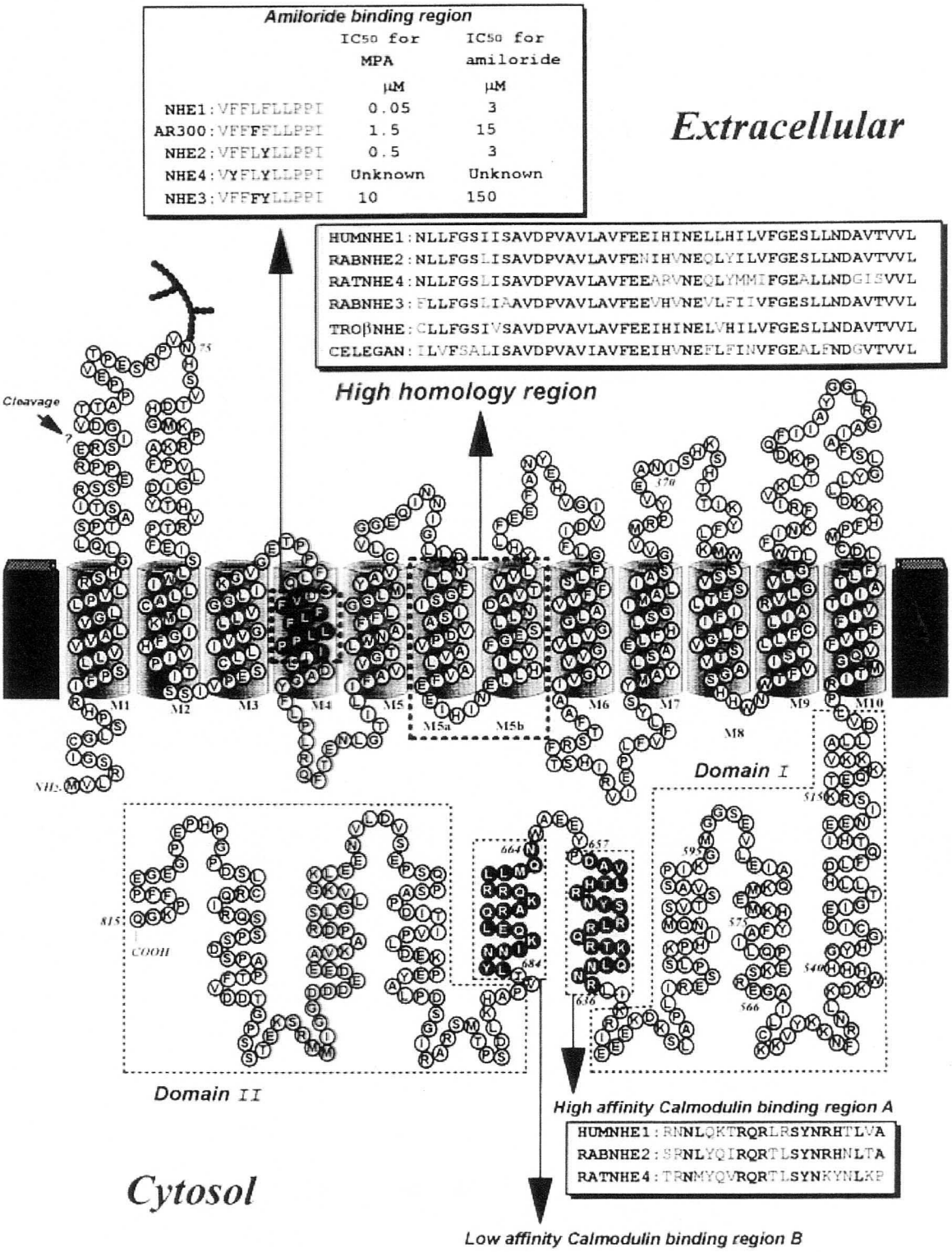
Embora as propriedades básicas do permutador Na^+/H^+ já fossem bem conhecidas já no início da década de 1980, suas características moleculares só começaram a ser desvendadas a partir da clonagem de seu DNA, em 1989, pelo grupo de pesquisadores franceses liderado por Pouysségur [103]. O isolamento dessa proteína vinha sendo arduamente perseguido em alguns laboratórios já há algum tempo, mas a ausência de ligantes específicos e a baixa expressão dessa proteína tornavam a possibilidade de sucesso bastante pequena. Assim, a clonagem realizada por Sardet e col. constituiu

realmente um "breakthrough" na história da investigação nessa área. A elegante estratégia utilizada para a clonagem consistiu em três etapas: 1) seleção de uma linhagem de fibroblastos de camundongo deficientes em permutador Na^+/H^+, através de uma técnica denominada "próton suicida", desenvolvida por esse grupo de pesquisadores, que consistia em induzir uma acificação prolongada e letal em células que expressavam o permutador Na^+/H^+ na membrana citoplasmática [96] – esta estratégia permitia, portanto, o isolamento de colônias de células que *não espressavam* o permutador; 2) transfecção dessas células mutantes com DNA genômico humano e seleção das células com hiperexpressão funcional do permutador, explorando a capacidade destas células de se recuperarem facilmente de uma sobrecarga ácida induzida pela exposição a NH_4Cl [103], e 3) clonagem molecular do gene humano transfectado que codificava o permutador Na^+/H^+. O clone obtido consistia de uma região 5' não codificante com 407 pares de bases (bp), uma região codificante com 2.445, e uma região 3' não codificante de 1.125 pb. Esse cDNA, ligado a um vetor de expressão, ao ser transfectado em fibroblastos desprovidos de trocador Na^+/H^+, restaurava a capacidade destas células de se recuperarem de uma sobrecarga ácida [103]. A sequência de nucleotídeos previa uma proteína com 815 amino ácidos, com peso molecular calculado de 90.704. Este primeiro permutador Na^+/H^+ clonado mostrou ser o tipo de distribuição ubíqua, responsável pela regulação do pHi nos diversos tipos de células, denominado "housekeeping type" ou isoforma 1 (**Na/H E**xhanger 1 – NHE-1). O plot de hidropatia da sequência de amino ácidos através do método de Engelman e col. [42] prevê uma proteína com 10 segmentos transmembrana, e através do método de Kyte e Doolittle [67], 12 segmentos transmembrana, seguidos de um longo segmento citoplasmático carboxi-terminal.

Utilizando a estratégia de clonagem por homologia, com base neste primeiro clone de cDNA, um total de seis membros dessa família de genes foram identificados em mamíferos até o momento (NHE-1 a 6) [28;61;103;114-116]. Quatro dessas isofomas (NHE1-4) estão presentes na membrana plasmática de células tubulares renais [123]. NHE1, como já comentado anteriormente, está presente em praticamente todas as células de mamíferos investigadas e em células epiteliais renais está presente em membrana basolateral [22;87]. As outras formas dessa proteína têm uma distribuição tecidual mais restrita. Destas, NHE3 é a mais abundante em tecido renal, está confinada à membrana apical das células de alguns segmentos tubulares, especialmente túbulos proximais e segmento espesso ascendente da alça de Henle [21;23;87], exercendo um papel crucial na absorção transepitelial de Na^+ e HCO_3^-. NHE-5 não está presente em epitélios. Ao contrário das demais isoforma do permutador Na^+/H^+, NHE-6 não se expressa na membrana citoplasmática, mas em mitocôndrias [89]. As várias isoformas se distinguem funcionalmente por sua sensibilidade a amiloride e seus análogos, pela diferente afinidade por Na^+ e sensibilidade a pHi, pela modulação por proteínas quinases, pela distribuição tecidual e pelo padrão de localização em estruturas subcelulares.

Na página seguinte está anexado o esquema proposto para ilustar a disposição da proteína NHE-1 na membrana (Esquema apresentado na Revisão feita por Wakabayashi e col. [123]). Uma série de outras informações sobre as quais discorreremos mais detalhadamente em seguida, estão resumidas nesta figura.

Human NHE1


Amiloride binding region
IC50 for MPA µM
IC50 for amiloride µM
NHE1: VFFLFLLPPI 0.05 3
AR300: VFFFFLLPPI 1.5 15
NHE2: VFFLYLLPPI 0.5 3
NHE4: VYFLYLLPPI Unknown Unknown
NHE3: VFFFYLLPPI 10 150
Extracellular
HUMNHE1: NLLFGSIISAVDPVAVLAVFEEIHINELLHILVFGESLLNDAVTVVL
RABNHE2: NLLFGSLISAVDPVAVLAVFENIHVNEQLYILVFGESLLNDAVTVVL
RATNHE4: NLLFGSLISAVDPVAVLAVFEEARVNEQLYMMIFGEALLNDGISVVL
RABNHE3: FLLFGSLIAAVDPVAVLAVFEEVHVNEVLFIIVFGESLLNDAVTVVL
TROβNHE: CLLFGSIVSAVDPVAVLAVFEEIHINELVHILVFGESLLNDAVTVVL
CELEGAN: ILVFSALISAVDPVAVIAVFEEIHVNEFLFINVFGEALFNDGVTVVL
High homology region
Cleavage
M1
M2
M3
M4
M5
M5a
M5b
M6
M7
M8
M9
M10
Domain I
Domain II
COOH
High affinity Calmodulin binding region A
HUMNHE1: RNNLQKTRQRLRSYNRHTLVA
RABNHE2: SRNLYQIRQRTLSYNRHNLTA
RATNHE4: TRNMYQVRQRTLSYNKYNLKP
Cytosol
Low affinity Calmodulin binding region B

Análise da estrutura molecular dos permutadores Na/H

Quando realizamos a comparação de sequências entre as diferentes isoformas de NHEs, observamos que o módulo transmembrana exibe identidade de amino ácidos entre 45 e 60%, mas este índice cai para 25-30% quando comparamos o módulo citoplasmático. Wabakabayashi e col. [122], em trabalho de 1992, em que realizou a expressão de proteínas quiméricas parcial ou completamente amputadas do módulo citoplasmático, mostraram que o módulo transmembrana completo, compreendendo os primeiros 500 amino ácidos, é necessário e suficiente para a realização da troca de Na^+ por H^+. Cabendo, portanto, ao módulo citoplasmático uma função regulatória da atividade de transporte. Uma análise mais detalhada dos "clusters" de sequências homólogas revela a presença de duas subfamílias de isoformas que provavelmente divergiram mais tarde no processo evolutivo: NHE-2 / NHE-4 e NHE-3 / NHE-5 [37]. A parte central do módulo transmembrana (os segmentos transmembrana 5a e 5b do modelo proposto) é praticamente idêntico em todas as isoformas de NHE. Essa parte do polipeptídeo possui resíduos com carga negativa imersos em um trecho de sequência altamente hidrofóbica (aspartatos 226, 238 e 267, e glutamatos 247, 248, 253 e 262 em NHE-1 humano). A substituição do Glu-262 em NHE-1 resulta em inativação do transportador [43]. Embora não se possa excluir um efeito indireto desta mutação, este resultado associado ao alto índice de conservação desta região entre os membros da família de permutadores sugere que esses dois segmentos transmembrana (5a e 5b) constituem o núcleo catalítico dos permutadores Na^+/H^+.

O primeiro segmento transmembrana e a primeira alça extracelular são bem pouco conservadas. Diferem inclusive entre isoformas idênticas de diferentes espécies

[34]. Uma análise mais detalhada do primeiro trecho de resíduos hidrofóbicos revela que o primeiro segmento transmembrana tem características de peptídeo-sinal, incluindo a extremidade amino carregada positivamente e um trecho hidrofóbico relativamente curto [118].

A alça compreendida entre os segmentos transmembrana 1 e 2 são glicosiladas nas isoformas NHE-1 e NHE-2 [38].

Características cinéticas e farmacológicas

A análise das características bioquímicas dos permutadores Na^+/H^+ mostra que para a isoformas 1, 2 e 3 de NHE a ligação do Na^+ extracelular ocorre em um sítio que não pode ser distinguido do sítio de transporte; aparentemente, o Na^+ se liga ao sítio e é translocado. A dependência da atividade de troca de Na^+ por H^+ em relação à concentração de Na^+ extracelular segue a cinética hiperbólica simples de Michaelis-Menten [7;70;92]. Para a maioria da isoformas analisadas, a ligação do Na^+ ao sítio externo ocorre com um *Km* medido que é pelo menos de 3 vezes menor que a concentração extracelular de Na^+ . Em isformas de ratos, foram oservados os seguintes K_m para o Na^+: NHE-1 = 10 mM; NHE-2 = 50 mM, NHE-3 = 4,7 mM . Assim, os permutadores Na^+/H^+ fisiologicamente estão próximos da saturação, no que se refere ao Na^+ externo. Pequenas variações na concentração extracelular de Na^+ praticamente não têm efeito sobre a atividade dos permutadores. Comportamento curioso exibe a isoforma 4 em relação ao Na^+ externo: é ativada por Na^+ externo com uma cinética cooperativa [25]. H^+, Li^+ e K^+ (este último apenas para NHE-1) também se ligam ao sítio externo,

inibindo competitivamente o transporte de Na^+. A ordem de afinidade aparente por cátions externos para as isoformas NHE-1 , -2 e -3 é $H^+ >> Li^+ > Na^+ >> K^+$ [135].

A dependência da atividade de troca de Na^+ por H^+ em relação à concentração de H^+ intracelular não segue uma cinética hiperbólica simples, mas é mais abrupta, com um coeficiente de Hill maior que 2. Tal dependência do pHi sugere a existência de mais de um sítio de ligação para H^+. Aronson e col. [8], com base na análise do transporte de Na^+ em vesículas de bordo em escova de túbulos proximais, propuseram a exixtência de um sítio alostérico para ligação de H^+ na face citosólica da proteína. Coeficiente de Hill ~ 2 para H^+ interno foi observado para todas as isoformas analisadas (NHE 1-3) [123]. Embora a hipótese da existência de um sensor de prótons tenha fornecido um paradigma valioso no estudo da regulação da atividade do permutador Na^+/H^+, outras hipóteses podem ser aventadas para essa ativação cooperativa. Os permutadores estão na membrana em forma de dímeros e vários modelos envolvendo a ligação coordenada de íons H^+ a cada monômero têm sido construídos para explicar esse comportamento cooperativo [43]. Embora pareça claro que os permutadores existam em formas de homodímeros, não existe evidência conclusiva de que a unidade funcional dessa proteína na membrana citoplasmática seja dimérica.

A pH_i em valores fisiológicos, NHE-1 e NHE-2 estão virtualmente inativos, mas são prontamente ativados quando o pH_i cai abaixo da neutralidade [92]. NHE-3 mostra uma maior afinidade por prótons intracelulares, estando ativo em pH_i fisiológico. Células transfectadas com NHE-3 apresentam pH_i de repouso mais elevado [119].

Uma característica relevante dos permutadores Na^+/H^+ é a sensibilidade a amiloride e seus derivados. A forma mais sensível é NHE-1, embora NHE-2 e NHE-3 também mostrem sensibilidade. NHE-3 é a forma menos sensível. Basicamente, todos os

derivados alkyl 5 N-substituídos do amiloride, tais como 5-N-dimethyl amiloride (DMA) or 5-N-ethyl isopropyl amiloride (EIPA) possuem alta especificidade por NHE-1, inibindo esta isoforma com k_i 10 – 100 vezes mais alto que o próprio amiloride ($IC_{50\text{-}amiloride}$ = 3 μM; $IC_{50\text{-}DMA}$ = 0,05 μM), embora interajam bem mais fracamente com as demais isoformas. Uma nova classe de inibidores competitivos derivados de phenacyl guanidinium (HOE694, o cariporide, e HOE642) inibem NHE-1 de forma ~ 4.000 mais eficiente que NHE-3 [35]. Análise de proteínas mutantes têm mostrado que os segmentos transmembrana 4 e 9 são os sítios mais prováveis de interação com esses inibidores [36;90]. Mais recentemente, foi desenvolvido um outro derivado guanídinico, 3-[2- (3-guanidino-2-methyl-3-oxo-propenyl) –5 –methyl- phenyl]-N-isopro pylidene-2-methyl-acrylamide dihydro-chloride, ou S3226, que é o inibidor mais específico para NHE-3 até agora desenvolvido. Apresenta IC_{50} de 0.02 μM para NHE-3, e de 3.6 e 80 μM para NHE-1 e NHE-2 respectivamente [106]

Distribuição tecidual e subcelular dos permutadores Na^+/H^+

NHE-1 se expressa em praticamente todos os tipos celulares, em todas as espécies. O quantidade de RNA mensageiro de NHE-1, no entanto, não é uniforme entre os diferentes tecidos [55]. Esta é a forma da proteína considerada "housekeeping", com papel crucial na regulação do pH_i e do volume celular. Em células epiteliais, NHE-1 se localiza em membrana basolateral [22]. Apenas em placenta NHE-1 foi observado em membrana apical [62].

NHE-2, em coelhos, se expressa predominantemente em rins, intestino e em glândula adrenal, mostrando um baixo nível de expressão em músculo esquelético e em traquéia [113]. No trato gastrointestinal, o RNA mensageiro de NHE-2 é relativamente abundante (jejuno > ileum > duodeno > colon descendente). Em ratos, a mensagem de NHE-2 se expressa predominantemente em intestino delgado, colon e estômago, mas em quantidades bem menores em músculo esquelético, rins, cérebro, testículos, útero, coração e pulmões [124]. Em humanos, a mensagem de NHE-2 é especialmente abundante em músculo esquelético, colon e rins, com níveis mais baixos em testículos, próstata, ovário e intestino delgado [75]. Embora NHE-2 tenha sido observado consistentemente em intestino, sua presença nos rins é controversa [26].

NHE-3, em coelhos, em ratos e em humanos, se expressa de forma abundante rins e intestino, com nível de expressão um pouco menor em estômago [28;91;111]. O RNA mensageiro de NHE-3 é também encontrado em outros tecidos, seguindo mais ou menos a seguinte distribuição: rins >> intestino delgado >> testículo > ovário > colon = próstata > timo > leucócitos periféricos = cérebro > baço > placenta [91].

Estudos com imunohistoquímica mostram que em rins NHE-3 se localiza na membrana apical de células tubulares, especialmente em túbulos proximais e em segmento espesso ascendente da alça de Henle [21]. Além de ser observado na membrana plasmática, NHE-3 é também visualizado em endossomas subapicais [20].

O RNA mensageiro de NHE-4 é observado em estômago, intestino, rins e em hipocampo. Nos rins, NHE-4 é mais abundante em ducto coletor medular interno e se distribui heterogeneamente em membrana basolateral das células de túbulos corticais [27].

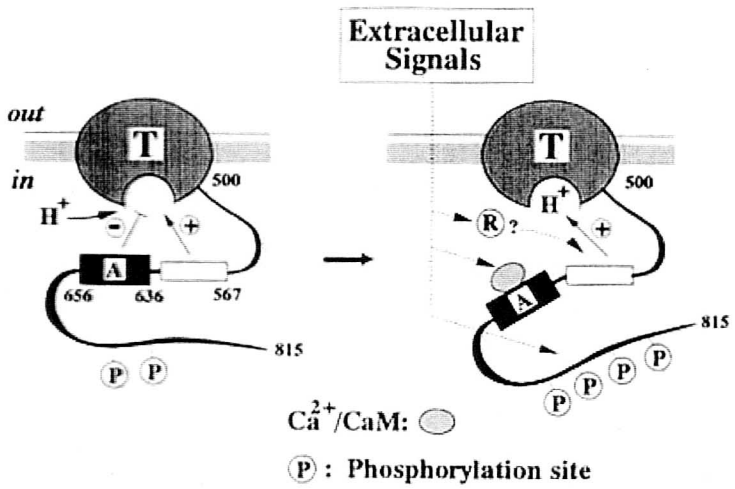
O RNA mensageiro de NHE-5 tem sido detectado predominatemente em cérebro, mas também em testículos, baço e músculos esqueléticos [61]. Não é detectado em epitélios. NHE-6, que se expressa em mitocôndria, tem distribuição ubíqua.

Modulação funcional da atividade dos permutadores Na^+/H^+

As diferentes isorformas de NHE mostram também mecanismos específicos de modulação, que estão relacionados especialmente à porção citoplasmática N-terminal desses transportadores, ou módulo regulatório. O modelo que tem sido proposto para NHE-1 é que a cauda citoplasmática coopera com o sensor de pH_i, modificando o valor de limiar de pH_i capaz de ativar o permutador Na^+/H^+. Assim, a cauda ciplasmática seria um integrador de sinais capaz de transmitir informações (hormonais e outras) ao sensor de pH_i na membrana. A exemplo da região promotora que regula a taxa de transcrição dos genes, a porção citoplasmática dessa família de proteínas aparenta ter reunido, ao longo do processo evolutivo, módulos regulatórios ("regulatory boxes") que interpretam sinais extra e intracelulares específicos. Embora a sequência da cauda ciplasmática seja pouco conservada entre os diversos membros da família de trocadores Na^+/H^+, métodos de alinhamento que se baseiam na presença de estruturas secundárias hidrofóbicas mostram que a porção citosólica dos diferentes membros da família exibe similaridades estrurais [47]. Experimentalmente, foi observado através de medidas de dicroismo circular que a porção citosólica de NHE-1 possui um elevado grau de organização estrutural [47].

NHE-1 é ativado por uma grande variedade de estímulos extracelulares, incluindo hormônios, integrinas, e virtualmente todos os fatores de crescimento. O sensor de pH de NHE-1 se mostrou regulado pela interação com uma região compreendida entre os aminoácidos 567-635, que aparentemente é essencial para a manutenção da sensibilidade ao pH_i, e por uma região autoinibitória, compreendida entre os amino ácidos 636-656, que liga calmodulina (CaM) com alta afinidade ($kd \sim 20$ nM) [15]. O modelo proposto é que esse domínio da proteína manteria baixa, tonicamente, a sensibilidade de NHE-1 ao pH_i. A ligação de Ca-CaM promoveria a liberação da inibição, aumentando a atividade de NHE-1. Isso explicaria a rápida ativação de NHE-1 induzida por Ca^{2+}, observada primeiramente por Villereal e col. [117]. Mutação nos quatro amino ácidos positivos presentes nesta região torna NHE-1 constitutivamente ativo, por aumentar sua sensibilidade ao pH_i [120]. Muitos dos amino ácidos dessa região que liga Ca^{2+}-CaM estão conservados em NHE-2 e NHE-4, mas não em NHE-3. Foi observado que as isoformas 2 e 4 também se ligam a CaM, de modo dependente de Ca^{2+}.

A figura abaixo resume o modelo proposto para a modulação do sensor de pH_i de NHE-1 pela cauda citoplasmática da proteína. (Esquema de Wakabayashi e col. [123]) . As explicações sobre as anotações da figura estão no texto.



A ativação mais consistente e sustentada de NHE-1 pode também se dever a fosforilação de resíduos da própria proteína ou de outras proteínas que com ela venham a interagir. Ambos os mecanismos foram documentados. NHE-1 é uma fosfoproteína e o nível de fosforilação está aumentado em células estimuladas com agentes mitogênicos [102]. O mapeamento dos fosfopeptídeos evidenciou que os sítios de fosforilação estão localizados na região citoplasmática C-terminal [121].

Existem numerosas evidências de que NHE-1 seja ativado por proteína kinase C (PKC) [123], embora não se tenha demonstrado inequivocamente que esta ativação dependa diretamente de fosforilação de NHE-1. NHE-1 não é modulado por proteína kinase A (PKA). Ficou recentemente demonstrado que a serina 703 é fosforilada *in vivo* pela p42/p44 MAPK (Mitogen Activated Protein Kinase), sendo este um resíduo importante na resposta de ativação por soro [110]. Mutação na serina 703, no entanto, não abole totalmente a ativação por soro (ativação residual de 50%) [16]. Esta observação levou ao modelo de ativação por interação com outras proteínas que

poderiam ser fosforiladas quando da estimulação celular com soro [1]. Foram identificadas algumas proteínas candidatas a essa interação: p24 NHE-1 [48], CHP (Calcineurin B Homologous Protein) [71] e outras.

NHE-1 é ativado por estímulos mecânicos, tais como choque osmótico, espalhamento celular, adesão celular etc. Grinstein e col. mostraram que a ativação por estímulos mecânicos é independente de fosforilação de NHE-1 [49]. Tendo esses mesmos autores observado que NHE-1 está associado com actina do citoesqueleto em placas de adesão focais [50], eles sugeriram que a ativação mecânica poderia ser mediada pelo contato direto com proteínas do citoesqueleto. É interessante observar que a ativação ótima de NHE-1 depende de níveis elevados de ATP no meio intracelular, embora o nível de ATP não interfira com o grau de fosforilação da proteína [40]. Além disso a depleção de ATP leva a uma distribuição mais homogênea de NHE-1 na membrana citoplasmática. NHE-1, portanto, poderia estar interagindo, de forma dependente de ATP, com proteínas acopladoras que mediariam a interação de NHE-1 com elementos do citoesqueleto.

O mecanismo de regulação de NHE-3 é diferente do de NHE-1, uma vez que alterações na atividade de NHE-3 se manifestam por modificações do V_{max}, ao invés de modificação na afinidade aparente pelo H^+ intracelular (K_{H^+}), observada em NHE-1. Embora o paradigma atual dos domínios estruturais e funcionais aceito para os permutadores Na^+/H^+ (domínio transmembrana e domínio citoplasmático) aparentemente seja correto para a maioria das isoformas, a realidade pode ser mais complexa. Utilizando anticorpos específicos contra trechos de amino ácidos da extremidade carboxi, Biemesderfer e col. observaram que, embora a maior parte da metade C-

terminal da proteína seja citosólica, a extremidade final pode, na verdade, estar no extracelular [18].

Estudos com imunohistoquímica, nos quais foram utilizados vários anticorpos muito bem caracterizados, mostram que, no rim, NHE-3 está presente em membrana apical das células de túbulos proximais e de segmento espesso ascendente [21]. Embora haja controvérsia a respeito de ser esta a única isoforma de permutador Na^+/H^+ presente em membrana apical de túbulos proximais, esta é, seguramente, a isoforma predominante neste local.

NHE-3 é regulado por uma série de agonistas cujos receptores específicos ativam proteínas quinases. A porção carboxi-terminal de NHE-3, por sua vez, possui vários sítios que são alvos possíveis de fosforilação; em células intactas, NHE-3 é uma fosfoproteína e além disso, existem fortes evidências experimentais de que este permutador seja fosforilado, e em geral inibido, tanto por PKC como por PKA [82]. Kurashima e col. mostraram que as serinas 605 e 634 são fosforiladas por PKA [65]. Zhao e col. observaram fosforilação por PKA das serinas 552 e 605, mas não da serina 634 [138]. É possível que haja redundância nesse sistema, sendo fosforilados resíduos diferentes em diferentes circunstâncias. NHE-3 é também modulado por PKC e por quinase dependente Ca^{2+}-CaM. No entanto, os sítios de fosforilação por essas quinases in vivo não estão claros [129]. Além da fosforilação, existem evidências experimentais de que outros mecanismos acessórios estejam operando na regulação de atividade de NHE-3 quando da ativação dessas proteínas quinases, pois nem sempre a modificação de atividade da proteína está associada a fosforilação da mesma [129;134].

Ainda não se sabe como a fosforilação de NHE-3 e eventuais mecanismos acessórios a serem ainda elucidados levam a alterações na atividade do permutador. O

tráfico da proteína entre vesículas endossomais e a membrana citoplasmática certamente é um mecanismo importante no controle de sua atividade. Através da utilização de técnica de imunofluorescência e microscopia confocal, é possível detectar acúmulo de NHE-3 em endossomas. NHE-3 parecer ser funcionalmente ativo em endossomas que estão reciclando, e a população de NHE-3 em membrana citoplasmática depende da dinâmica de endocitose e reciclagem, que é regulada pela via de sinalização da fosfatidilinositol-3 quinase [64] [63]. Mais recentemente, Kurashima e col. observaram que a atividade ótima de NHE-3 requer que a via de sinalização envolvendo a GTPase solúvel RhoA e a quinase I a ela associada (RhoA/ROK) esteja funcional, e atuando, pelo menos parcialmente, na fosforilação da cadeia leve da miosina, e consequentemente, na organização do citoesqueleto de actina [109]. Biemesderfer e col., por sua vez, observaram a associação de NHE-3 com megalina [19], uma proteína que aparentemente tem uma função genérica na capacidade endocítica em túbulos proximais.

Em diversas situações associadas a aumento (depleção de K^+ e acidose) ou redução (exposição a paratormônio - PTH) da expressão de NHE-3 em membrana apical, não se observou alteração da quantidade total de NHE-3 em córtex renal, sugerindo que esteja ocorrendo apenas a redistribuição de NHE-3 entre vesículas endossomais e membrana plasmática [6;44;93;133]. Akhter e col. demonstraram que os 142 amino ácidos C-terminais de NHE-3 englobam determinantes endocíticos, pois uma vez removidos da proteína observa-se um aumento significativo de sua inserção constitutiva na membrana, com o aumento do V_{max} do transportador [3].

A reciclagem de NHE-3, no entanto, não é o único meio de modular a atividade desse transportador. A própria fosforilação por PKA inibe a atividade da proteína quando NHE-3 é isolado de vesículas de membrana apical e reconstituído em lipossoma,

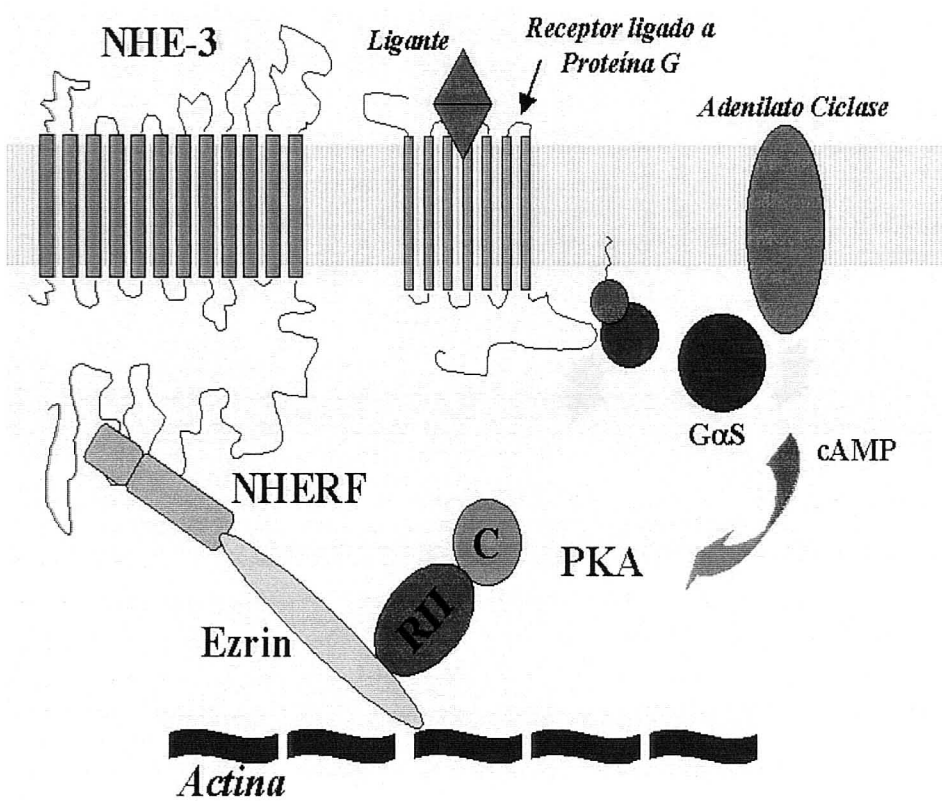
situação em que a possibilidade de reciclagem está totalmente abolida [125]. Isso mostra que a atividade intrínseca do permutador pode ser modificada. Miller e Pollock também observaram que a inibição aguda de NHE-3 por PTH envolve redução em V_{max} e modificação na afinidade por H^+ (K_H), sem alteração em K_{Na} [77]. Uma questão não esclarecida é se a própria fosforilação altera a cinética do transportador ou se proteínas acessórias estariam envolvidas no processo de inibição.

Weinman e Shenolikar foram os primeiros a propor e a isolar um fator regulador de NHE-3 [127;128]. A adição deste fator era necessária para restaurar a inibição de NHE-3 por PKA após reconstituição da proteína em lipossomas. A proteína clonada recebeu o nome de NHERF (Na/H exchanger regulator factor) [128]. Posteriormente, Yu e col., ao buscarem a identificação de proteínas que interagem com a porção citoplasmática de NHE-3 através da utilização de "yeast two-hybrid system", clonaram E3KARP (exchanger-3 kinase A regulatory protein), que é homóloga a NHERF (52% de identidade) [137]. Em células desprovidas de NHERF ou E3KARP, NHE-3 não é responsivo a PKA. No entanto, a sensibilidade a PKA é observada se estas células são transfectadas com uma destas proteínas regulatórias [137].

NHERF e E3KARP são membros de uma família de proteínas que possuem dois domínios PDZ em série (domínio PDZ é uma sequência conservada de amino ácidos que medeia um tipo de interação proteína-proteína [53]. A proteína humana homóloga de NHERF (EBP50) se liga a ezrin, uma proteína do citoesqueleto presente em microvilus [101]. Ezrin, por sua vez, pertence à família de proteínas ERM (ezrin-radixin-moesin) que exercem um papel regulatório e estrutural na formação de microvilus, devido a sua capacidade de ligar o citoesqueleto a proteínas da membrana plasmática [4]. Yun e col. mostraram que os 26 amino ácidos C-terminais de E3KARP são fundamentais para sua

interação com ezrin. A interação de E3KARP com NHE-3 se faz através do segundo domínio PDZ presente nos 200 amino ácidos C-terminais de E3KERP e os amino ácidos 585-660 de NHE-3 [136]. Tendo em vista a capacidade de ezrin de se ligar à subunidade regulatória da PKA do tipo II [41], Lamprecht e Yun propuseram um modelo no qual o complexo NHERF/ezrin funcionaria como uma AKAP (A kinase anchoring protein) para NHE-3 [68]. Atualmente já estão identificadas cerca de 40 proteínas que funcionam como AKAP, ou seja, proteínas que possuem módulos estruturais que trazem quinases e fosfatases específicas para próximo de seus substratos, de modo a permitir reações catalíticas espacialmente restritas ao alvo específico [69].

Modelo proposto por Lamprecht e Yun para a modulação de NHE-3 por PKA:



NHERF existe como uma fosfoproteína na célula. Existem sítios potenciais de fosforilação por PKA próximos a sua extremidade carboxílica, no entanto, a importância da fosforilação de NHERF para a regulação de NHE-3 ainda não foi elucidada. Uma hipótese de trabalho é que NHERF estaria no citosol na forma oligomérica e que a fosforilação seria necessária para a impedir a oligomerização, tornando NHERF disponível para interação com NHE-3 [79].

Embora NHERF seja mais abundante em rins e intestinos, seu RNA mensageiro é também observado em fígado, coração, músculo esquelético, e outros tecidos. E3KARP, a proteína homóloga a NHERF para a qual tem sido proposto o nome de NHERF-2, é mais abundate em coração. NHERF tem sido observado em associação com outras proteínas de transporte iônico, tais como co-transportadores Na-HCO_3 ou Na-PO_4, canais para K^+ e CFTR. Assim, embora essa proteína tenha sido primeiramente identificada em associação com NHE-3, seu papel na fisiologia celular certamente é muito mais amplo que a simples modulação de NHE-3 [2].

O estudo da interação de NHERF com outras proteínas tem contribuido para a compreensão de um paradoxo que incomoda os fisiologistas renais há longo tempo: a atividade de NHE-3 é aumentada com a estimulação de receptores β-adrenérgicos, enquanto a estimulação dos demais receptores ligados a produção de AMPc inibe esse transportador. Hall e col. [51] demonstrou que receptores β2-adrenérgicos quando estimulados por seu ligante, interagem com NHERF através de seus três amino ácidos C-terminais que se ligam a um dos domínio PDZ de NHERF. A ativação β2-adrenérgica, portanto, sequestra NHERF, liberando NHE-3 da inibição por ele favorecida.

Modulação da expressão gênica dos permutadores Na^+/H^+

As regiões promotoras dos genes de NHE-1, NHE-2 e NHE-3 já foram sequenciadas em algumas espécies. Na região promotora de NHE-1, clonada e sequenciada em humanos e em coelhos [24;78], se observa as seguintes sequências consensuais para ligação de fatores de transcrição: uma "TATA" box, a 25 nucleotídeos à montante do ponto de início de transcrição (TATAAGT), onde se liga o fator de transcrição TFIID, importante na orientação do local para a ligação da RNA polimerase II); três GC boxes (CCCGGG), que são sítios de ligação para o fator de transcrição Sp-1; duas CAT boxes (CAATT); três sítios AP-1 (TGA(G/C)T(C/A)A), que são sítios de ligação para dímero *fos-jun* em resposta a ativação de proteínas quinases C; cinco hemi-sítios para ligação de receptores para glicocorticóides (TGTTCT, AGTTCT, TGTCCT); cinco CACCC boxes, e um elemento responsivo a AMPc (GTGACGT(A/C)(A/G)). Muitos desses sítios são comuns a seres humanos e coelhos, estando ausentes nestes últimos dois dos sítios AP-1 e os dois sítios Sp-1. O significado funcional de cada uma dessas sequências consenso que, teoricamente, podem interagir com fatores de transcrição não foi ainda bem explorado. Algumas situações induzidas experimentalmente resultam em alterações na quantidade de mRNA específico de NHE-1 em alguns tecidos, sugerindo modulação transcricional da expressão do gene. Rao e col [99] e Berk e col [14] mostraram que exposição a fatores que induzem proliferação celular (estímulo hiperplásico), tais como soro, "platelet derived growth factor" (PDGF) e "fibroblast growth factor" (FGF), aumentam os níveis estacionários de mRNA de NHE-1 e a atividade desse permutador, em células musculares lisas vasculares, enquanto que a exposição a angiotensina II (estímulo hipertrófico) não tem qualquer efeito sobre a

expressão do permutador. A ativação de proteína quinase C (PKC) parece ser importante para a ação dos agentes hiperplásicos sobre a expressão do permutador [81], provavelmente através da fosforilação do fator de transcrição AP-1, cuja sequência consenso foi identificada na região promotora do gene *NHE-1* [24]. Foi observado também que células musculares lisas de vasos, quando cultivadas em meio com elevados níveis de glicose apresentam aumento dos níveis de mRNA de NHE-1, de forma dependente da ativação de PKC [130]. Outra abservação interessante é o aumento dos níveis de mRNA de NHE-1, e aumento na atividade da proteína observada em linhagens de células tubulares renais em cultura em resposta a acidose crônica [56-58]. Além do aumento dos níveis de mensagem de NHE-1, foi observado também aumento da expressão de *c-fos* e *c-jun* associado a aumento da atividade de AP-1 em resposta a acidose.

A região promotora de NHE-3 de rato foi caracterizada por Cano [29]. O promotor de NHE-3 não tem TATA box ou CCAAT box antecendendo o sítio de início de transcrição. O mapeamento do ponto de início de transcrição revela um único ponto de início, tanto em rim como em estômago e colon ascendente. Foram identificados dois sítios para ligação do fator transcrição Sp-1 próximos ao ponto de início de transcrição, o que aparentemente pode facilitar a interação com o fator de transcrição IID (TFIID), na ausência de TATA box. Na região promotora de NHE-3 podem ser observados vários sítios para ligação de receptores para hormônio tireoideano e para glicocorticoides. Essa região promotora mostrou ser modulada, em células de túbulos proximais de opossum (OK cells), por glicocorticoides, por acidose e por hormônio tireoideano [29;30]. A modulação da atividade transcricional de NHE-3 na acidose crônica está relacionada a ativação de tirosina quinases da família src [132]. A modulação de NHE-3 por

glicocorticoides foi observada em vários laboratórios, mas NHE-1 e NHE-2 não se mostraram modulados por glicorticoides [12;32].

A região promotora de NHE-2 foi recentemente caracterizada em ratos e em seres humanos [74;85]. Como observado para NHE-3, o promotor de NHE-2 não tem TATA box, mas têm sequências consenso (GC box) para ligação de Sp-1 [10].

Análises funcionais em animais transgênicos ou com defeitos genéticos espontâneos

"Knockout" gênico é uma abordagem elegante para a obtenção de novos "insights" sobre o papel fisiológido das proteínas, embora a interpretação dos fenótipos obtidos possa ser difícil devido ao possível envolvimento das isoformas de NHE em passos críticos do processo de desenvolvimento embrionário e devido à presença de mecanismos compensatórios nos camundongos adultos. Schultheis e col [105] desenvolveram camundongos com inativação do gene *NHE-3*. Como era de se esperar, camundongos homozigotos para a inativação de NHE-3 mostravam redução na pressão arterial, eram levemente alcalóticos, e apresentavam defeitos absortivos tanto em rins como em intestinos.

Cox e col relataram a caracterização molecular do defeito genético apresentado por camundongos epiléticos e atáxicos – camundongos SWE [39]. Eles observaram que estes camundongos apresentam uma mutação pontual que induz um "stop codon" na sequência codificante de NHE-1, resultando na produção de uma proteína truncada, inativa. A inativação homozigótica do gene *NHE-1* induzida posteriormente em

camundongos [13] confirmou que a mutação não é letal, o que não é surpreendente devido à existência de outros mecanismos de regulação do pH_i. Estes camundongos não mostram alterações visíveis na homeostase ácido-base, nem na função renal ou intestinal. No entanto, eles mostram defeitos na função cerebral.

Kuro-o e col produziram camundongos transgênicos com hiper-exoressão de NHE-1[10]. Esses animais exibiam, com dieta hipersódica, redução na excreção urinária de sal e hipertensão arterial. Hiper-expressão de NHE-1 poderia, portanto, ser uma causa de hipertensão arterial dependente de ingestão de sal.

"Knockout" de NHE-2 em camundongos não resultou em modificações detectáveis na função intestinal, nem renal, mas resultou em uma mucosa gástrica alterada, com deficiente secreção de H^+ [104].

Passamos em seguida à apresentação e discussão de nossos trabalhos envolvendo o estudo de permutadores Na^+/H^+.

Iniciei meus estudos sobre a estrutura molecular dos permutadores Na^+/H^+, no final de 1989, trabalhando em um projeto cujo objetivo era clonar o permutador existente em células tubulares renais de coelhos. Visto que até então já haviam sido acumuladas muitas informações sobre a cinética, sensibilidade a drogas e modulação da atividade do transportador Na^+/H^+ presente nessas células, era importante conhecer sua estrutura molecular. Nessa ocasião, embora houvessem evidências que sugerissem a existência de diferentes isoformas dessa proteína, não havia ainda a confirmação experimental, pois o primeiro clone de um permutador Na^+/H^+ havia sido recentemente obtido por Sardet e col. [103]. Esse clone estava sendo utilizado nos laboratórios de Aronson e Igarashi para o "screening" de uma biblioteca genômica comercial feita a partir de DNA de hepatócitos de rato e construída em *EMBL3* (Clontech).

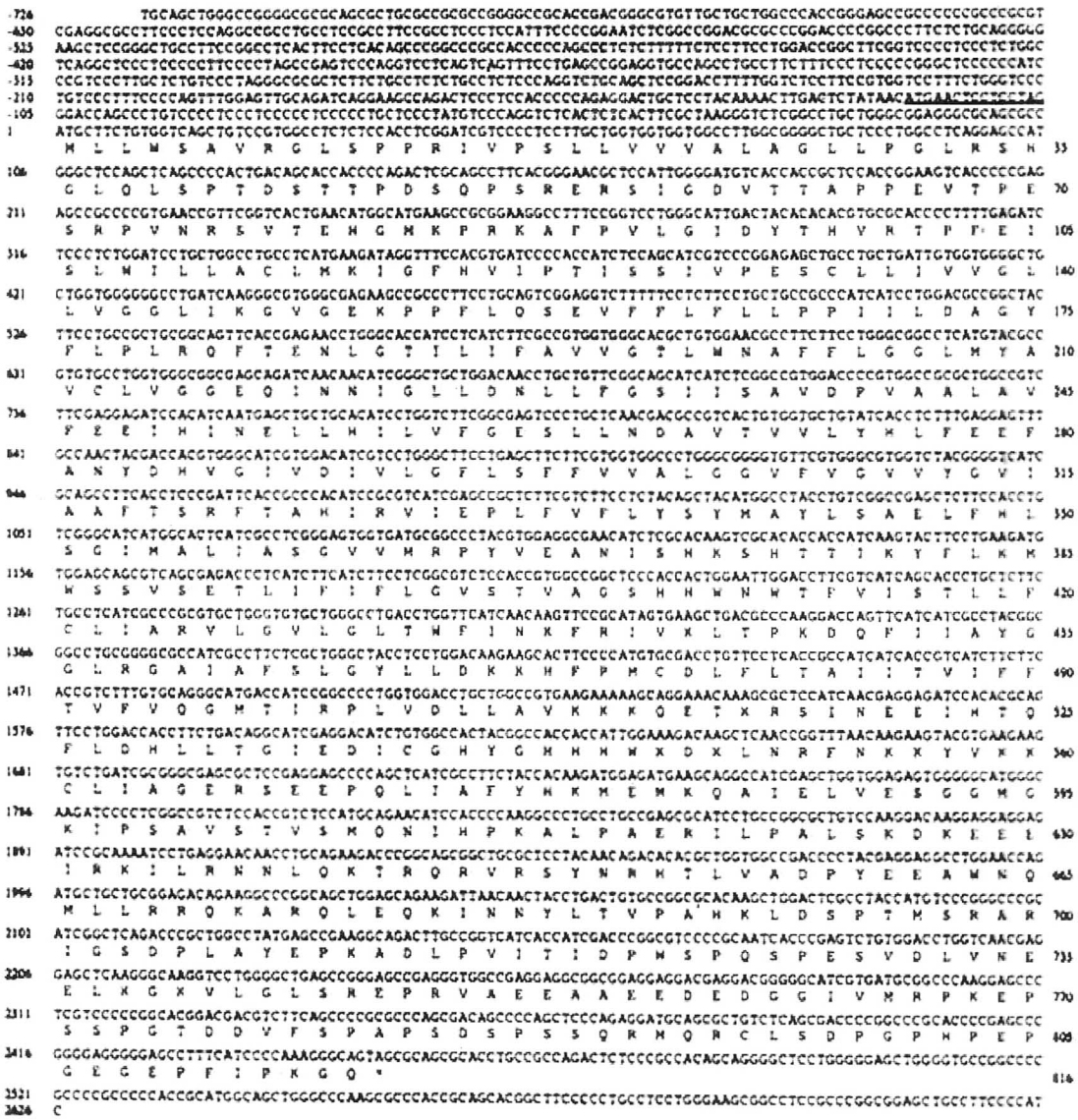
Ao chegar ao laboratório, em novembro de 1989, fui incluída no grupo de trabalho, juntamente com outro pós-doutorando vindo da Alemanha, Friedhelm Hildebrandt. Nosso objetivo era obter o cDNA completo do permutador cuja mensagem já havia sido detectada em tecido renal de coelho por Hildebrandt, através da hibridização de Northern blot que revelou uma mensagem de 4.700 pb (pares de base). Construimos uma biblioteca de cDNA de tecido renal de coelho no fago lambda gt11, cujo "screening" foi feito com uma sonda marcada com ^{32}P-dCTP a partir de um fragmento do cDNA do permutador Na^+/H^+ humano, obtido pela disgestão com a endonuclease *Bam HI*. O "screening" dessa biblioteca nos levou à obtenção de cDNA com 2.500 pb, cujos primeiros 1.087 pb podiam ser alinhados com o cDNA humano, correspodendo às posições –726 a + 351. Em seguida, identificamos uma sequência de nucleotídeos que demonstramos tratar-se de um segmento de intron, sugerindo que

durante a construção de nossa biblioteca de cDNA havia ocorrido transcrição reversa de mRNA não inteiramente processado.

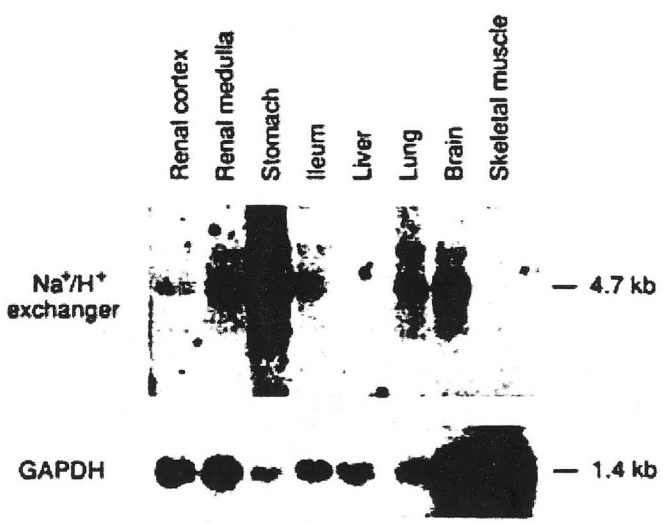
Para obtermos o restante do cDNA, construimos oligonucleotídeos para PCR iniciando nas posições $^{+}23$ (oligo sense), planejado com base na sequência já obtida para coelho, e $^{+}1453$ (oligo anti-sense), este último com base na sequência humana. Obtivemos por PCR um fragmento com 1.430 pb que foi clonado em pBlueScript e sequenciado. Simultaneamente, aquirimos uma biblioteca comercial de cDNA de rim de coelho (Stratagene) e fizemos seu "screening" com uma sonda construída a partir do fragmento obtido previamente por PCR. Através do screening desta biblioteca, obtivemos o fragmento de cDNA correspondente às posições 874 – 2626.

A sequência de nucleotídeos do cDNA clonado a partir de mRNA de tecido renal de coelhos mostrava 88% de identidade com o cDNA humano clonado por Sardet e col., apresentando 95% de identidade quando se comparava amino ácidos. Dos 38 amino ácidos divergentes, 17 eram substituições conservativas. As maiores divergências estavam a extremidade amino e na extremidade carboxílica.

cDNA do Permutador Na/H de coelho, sequenciado por nós, homólogo ao permutador humano clonado por Sardet e col.



Para verificar o nível de expressão da mensagem identificada, realizamos a hibridização de Northern blot contendo 25 ug de mRNA extraído de oito diferentes tecidos de coelho com o cDNA correspondente marcado com ^{32}P-dCTP. A expressão da mensagem clonada era mais significativa em estômago >> cérebro > medula renal = pulmão > ileum. A expressão era muito menor em córtex renal, fígado e músculo esquelético.



O fato de observarmos que a expressão desse permutador era menor em córtex renal que em medula já era uma sugestão de que o cDNA clonado não codificava a proteína responsável pela maior parte da secreção de H+ em túbulos proximais. Posteriormente, foi demonstrado no mesmo laboratório, com técnica de imunohistoquímica utilizando anticorpo contra a porção citoplasmática da proteína recombinante, que o transportador clonado se expressava em membrana basolateral de túbulos proximais e de outros segmentos tubulares [22]. Esse permutador foi denominado NHE-1. Na mesma ocasião já surgiam nos encontros científicos os

primeiros trabalhos referentes à clonagem de uma isoforma presente em membrana luminal da mucosa intestinal [114], posteriormente denominada tipo 3 (NHE-3).

Nas páginas seguintes estão anexadas as cópias do trabalho que realizamos em colaboração com Hildebrandt e outros colegas de laboratório, e a cópia de uma revisão feita sobre a Biologia Molecular dos Transportadores Na^+/H^+, da qual participamos nesse mesmo período.

- Hildebrandt F., Pizzonia J.H., Reilly R.F., **Rebouças N.A.**, Sardet C., Pouysségur J., Slayman C.W., Aronson P.S., Igarashi P. Cloning, sequence, and tissue distribution of a rabbit renal Na+/H+ exchanger transcript. *Biochimica et Biophysica Acta* (1991) 1129:105-108.

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Short Sequence-Paper

Cloning, sequence, and tissue distribution of a rabbit renal Na^+/H^+ exchanger transcript

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Rabbit kidney expresses a transcript that is similar to the human growth-factor-activatable Na^+/H^+ exchanger. PCR and library screening were used to clone overlapping 2.5 kb, 1.4 kb, and 1.8 kb cDNAs that together contain the entire coding region (2448 bp) and 5′ untranslated region (726 bp) and part of the 3′ untranslated region (128 bp) of a rabbit renal Na^+/H^+ exchanger transcript. The nucleotide and inferred amino acid sequences are highly conserved between rabbit and human (88% nucleotide identity, 95% amino acid identity). In rabbit, the transcript is expressed in both epithelial and non-epithelial tissues, with highest expression in stomach, brain, kidney, lung and ileum, and minimal expression in liver and skeletal muscle.

Na^+/H^+ exchangers are integral plasma membrane proteins that mediate the 1 : 1 exchange of extracellular Na^+ for intracellular H^+ and are involved in control of intracellular pH, regulation of cell volume, response to mitogens, and transepithelial flux of NaCl and $NaHCO_3$ [1,2]. A characteristic property is their sensitivity to inhibition by amiloride and its 5-amino substituted analogs. In mammalian kidney, Na^+/H^+ exchangers have been identified in mesangial cells [3] and tubule epithelial cells [2,4–6]. Recently, a cDNA encoding a human growth-factor-activatable Na^+/H^+ exchanger was cloned using genetic complementation [7,8]. Because the cDNA was cloned from a murine transformant, the tissue of origin in human is not known. Accordingly, the present studies were undertaken to determine whether a homologous transcript could be isolated from rabbit kidney.

Northern blot analysis indicated that rabbit kidney cortex expressed a 4.7 kb transcript that was related to the human growth-factor-activatable Na^+/H^+ exchanger. In order to clone this transcript we employed cDNA library screening and PCR. We obtained 2.5 kb, 1.4 kb, and 1.8 kb cDNAs containing the entire 5′ untranslated region (726 bp), coding region (2448 bp), and part of the 3′ untranslated region (178 bp) of a rabbit renal Na^+/H^+ exchanger transcript. Fig. 1 shows the composite nucleotide sequence of the cDNAs and inferred amino acid sequence of the rabbit renal Na^+/H^+ exchanger. The 3352 bp cDNA is predicted to encode a protein composed of 816 amino acids with a molecular mass of 90,847. The initiation codon resides in a consensus sequence for ribosomal attachment [9], and an in-frame stop codon is present further upstream. The 5′ untranslated sequence is full-length since transcript mapping indicates that the major "cap" site is at −726 (unpublished data).

The sequence of the rabbit renal Na^+/H^+ exchanger is similar to the human growth-factor-activatable Na^+/H^+ exchanger implying a high degree of evolutionary conservation of this transporter. Overall, there is 88% nucleotide identity between the two species, and the inferred amino acid sequences are 95% identical. Out of 38 divergent amino acids there

The sequence data in this paper have been submitted to the EMBL/Genbank Data Libraries under the accession number X61504.

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are 17 conservative changes. Greatest amino acid sequence divergence occurs at the amino terminus and within an acidic domain near the carboxyl terminus. The predicted secondary structures are identical consisting of an amino-terminal membrane-associated domain comprise of 10 membrane-spanning segments and a hydrophilic carboxyl-terminal domain that is presumably cytoplasmic. Amino acid sequences of both domains are conserved (95% and 96% identity, respectively). Also conserved are three putative sites for *N*-linked glycosylation (Asn-75, Asn-370, and Asn-410) and a serine-rich region near the carboxyl-terminus that may contain sites for phosphorylation. The high degree of amino acid identity did not permit identification of carboxyl or histidyl residues that might reside at the external transport site as predicted by kinetics studies [10,11], but three amino acids (Lys-116, His-120, and Glu-131) that have been predicted to form a charge relay mechanism for transfer of protons across the membrane bilayer [12] are conserved.

```
-726          TGCAGCTGGGCCGGGGCGCGCAGCGCTGCGCCGCGCCGGGGCCGCACCGACGGGCGTGTTGCTGCTGGCCCACCGGGAGCCGCCCCCCGCCCGCGT
-630  CGAGGCGCCTTCCCTCCAGGCCGCCTGCCTCCGCCTTCCGCCTCCCTCCATTTCCCCGGAATCTCGGCCGGACGCGCCCGGACCCCGGCCCTTCTCTGCAGGGGG
-525  AAGCTCCGGGCTGCCTTCCGGCCTCACTTCCTCACAGCCCGGCCCGCCACCCCCAGCCCTCTCTTTTTCTCCTTCCTGGACCGGCTTCGGTCCCCTCCCTCTGGC
-420  TCAGGCTCCCTCCCCCTTCCCCTAGCCGAGTCCCAGGTCCTCAGTCAGTTTCCTGAGCCGGAGGTGCCAGCCTGCCTTCTTTCCCTCCCCCGGGCTCCCCCCATC
-315  CCGTCCCTTGCTCTGTCCCTAGGGCGCGCTCTTCTGCCTCTCTGCCTCTCCCAGGTCTGCAGCTCCGGACCTTTTGGTCTCCTTCCGTGGTCCTTTCTGGGTCCC
-210  TGTCCCTTTCCCCAGTTTGGAGTTGCAGATCAGGAAGCCAGACTCCCTCCACCCCCAGAGGACTGCTCCTACAAAACTTGAGTCTATAACATGAACTCCTCCTAG
-105  GGACCAGCCCTGTCCCCTCCCTCCCCCTCCCCCTGCTCCCTATGTCCCAGGTCTCACTCTCACTTCGCTAAGGGTCTCGGCCTGCTGGGCGGAGGGGCGCAGCGCC
1    ATGCTTCTGTGGTCAGCTGTCCGTGGCCTCTCTCCACCTCGGATCGTCCCCTCCTTGCTGGTGGTGGTGGCCTTGGCGGGGCTGCTCCCTGGCCTCAGGAGCCAT
       M  L  L  W  S  A  V  R  G  L  S  P  P  R  I  V  P  S  L  L  V  V  V  A  L  A  G  L  L  P  G  L  R  S  H  35
106  GGGCTCCAGCTCAGCCCCACTGACAGCACCACCCCAGACTCGCAGCCTTCACGGGAACGCTCCATTGGGGATGTCACCACCGCTCCACCGGAAGTCACCCCCGAG
       G  L  Q  L  S  P  T  D  S  T  T  P  D  S  Q  P  S  R  E  R  S  I  G  D  V  T  T  A  P  P  E  V  T  P  E  70
211  AGCCGCCCCGTGAACCGTTCGGTCACTGAACATGGCATGAAGCCGCGGAAGGCCTTTCCGGTCCTGGGCATTGACTACACACACGTGCGCACCCCTTTTGAGATC
       S  R  P  V  N  R  S  V  T  E  H  G  M  K  P  R  K  A  F  P  V  L  G  I  D  Y  T  H  V  R  T  P  F  E  I  105
316  TCCCTCTGGATCCTGCTGGCCTGCCTCATGAAGATAGGTTTCCACGTGATCCCCACCATCTCCAGCATCGTCCCGGAGAGCTGCCTGCTGATTGTGGTGGGGCTG
       S  L  W  I  L  L  A  C  L  M  K  I  G  F  H  V  I  P  T  I  S  S  I  V  P  E  S  C  L  L  I  V  V  G  L  140
421  CTGGTGGGGGGCCTGATCAAGGGCGTGGGCGAGAAGCCGCCCTTCCTGCAGTCGGAGGTCTTTTTCCTCTTCCTGCTGCCGCCCATCATCCTGGACGCCGGCTAC
       L  V  G  G  L  I  K  G  V  G  E  K  P  P  F  L  Q  S  E  V  F  F  L  F  L  L  P  P  I  I  L  D  A  G  Y  175
526  TTCCTGCCGCTGCGGCAGTTCACCGAGAACCTGGGCACCATCCTCATCTTCGCCGTGGTGGGCACGCTGTGGAACGCCTTCTTCCTGGGCGGCCTCATGTACGCC
       F  L  P  L  R  Q  F  T  E  N  L  G  T  I  L  I  F  A  V  V  G  T  L  W  N  A  F  F  L  G  G  L  M  Y  A  210
631  GTGTGCCTGGTGGGCGGCGAGCAGATCAACAACATCGGGCTGCTGGACAACCTGCTGTTCGGCAGCATCATCTCGGCCGTGGACCCCGTGGCCGCGCTGGCCGTC
       V  C  L  V  G  G  E  Q  I  N  N  I  G  L  L  D  N  L  L  F  G  S  I  I  S  A  V  D  P  V  A  A  L  A  V  245
736  TTCGAGGAGATCCACATCAATGAGCTGCTGCACATCCTGGTCTTCGGCGAGTCCCTGCTCAACGACGCCGTCACTGTGGTGCTGTATCACCTCTTTGAGGAGTTT
       F  E  E  I  H  I  N  E  L  L  H  I  L  V  F  G  E  S  L  L  N  D  A  V  T  V  V  L  Y  H  L  F  E  E  F  280
841  GCCAACTACGACCACGTGGGCATCGTGGACATCGTCCTGGGCTTCCTGAGCTTCTTCGTGGTGGCCCTGGGCGGGGTGTTCGTGGGCGTGGTCTACGGGGTCATC
       A  N  Y  D  H  V  G  I  V  D  I  V  L  G  F  L  S  F  F  V  V  A  L  G  G  V  F  V  G  V  V  Y  G  V  I  315
946  GCAGCCTTCACCTCCCGATTCACCGCCCACATCCGCGTCATCGAGCCGCTCTTCGTCTTCCTCTACAGCTACATGGCCTACCTGTCGGCCGAGCTCTTCCACCTG
       A  A  F  T  S  R  F  T  A  H  I  R  V  I  E  P  L  F  V  F  L  Y  S  Y  M  A  Y  L  S  A  E  L  F  H  L  350
1051 TCGGGCATCATGGCACTCATCGCCTCGGGAGTGGTGATGCGGCCCTACGTGGAGGCGAACATCTCGCACAAGTCGCACACCACCATCAAGTACTTCCTGAAGATG
       S  G  I  M  A  L  I  A  S  G  V  V  M  R  P  Y  V  E  A  N  I  S  H  K  S  H  T  T  I  K  Y  F  L  K  M  385
1156 TGGAGCAGCGTCAGCGAGACCCTCATCTTCATCTTCCTCGGCGTCTCCACCGTGGCCGGCTCCCACCACTGGAATTGGACCTTCGTCATCAGCACCCTGCTCTTC
       W  S  S  V  S  E  T  L  I  F  I  F  L  G  V  S  T  V  A  G  S  H  H  W  N  W  T  F  V  I  S  T  L  L  F  420
1261 TGCCTCATCGCCCGCGTGCTGGGTGTGCTGGGCCTGACCTGGTTCATCAACAAGTTCCGCATAGTGAAGCTGACGCCCAAGGACCAGTTCATCATCGCCTACGGC
       C  L  I  A  R  V  L  G  V  L  G  L  T  W  F  I  N  K  F  R  I  V  K  L  T  P  K  D  Q  F  I  I  A  Y  G  455
1366 GGCCTGCGGGGCGCCATCGCCTTCTCGCTGGGCTACCTCCTGGACAAGAAGCACTTCCCCATGTGCGACCTGTTCCTCACCGCCATCATCACCGTCATCTTCTTC
       G  L  R  G  A  I  A  F  S  L  G  Y  L  L  D  K  K  H  F  P  M  C  D  L  F  L  T  A  I  I  T  V  I  F  F  490
1471 ACCGTCTTTGTGCAGGGCATGACCATCCGGCCCCTGGTGGACCTGCTGGCCGTGAAGAAAAAGCAGGAAACAAAGCGCTCCATCAACGAGGAGATCCACACGCAG
       T  V  F  V  Q  G  M  T  I  R  P  L  V  D  L  L  A  V  K  K  K  Q  E  T  K  R  S  I  N  E  E  I  H  T  Q  525
1576 TTCCTGGACCACCTTCTGACAGGCATCGAGGACATCTGTGGCCACTACGGCCACCACCATTGGAAAGACAAGCTCAACCGGTTTAACAAGAAGTACGTGAAGAAG
       F  L  D  H  L  L  T  G  I  E  D  I  C  G  H  Y  G  H  H  H  W  K  D  K  L  N  R  F  N  K  K  Y  V  K  K  560
1681 TGTCTGATCGCGGGCGAGCGCTCCGAGGAGCCCCAGCTCATCGCCTTCTACCACAAGATGGAGATGAAGCAGGCCATCGAGCTGGTGGAGAGTGGGGGCATGGGC
       C  L  I  A  G  E  R  S  E  E  P  Q  L  I  A  F  Y  H  K  M  E  M  K  Q  A  I  E  L  V  E  S  G  G  M  G  595
1786 AAGATCCCCTCGGCCGTCTCCACCGTCTCCATGCAGAACATCCACCCCAAGGCCCTGCCTGCCGAGCGCATCCTGCCGGGCGCTGTCCAAGGACAAGGAGGAGGAG
       K  I  P  S  A  V  S  T  V  S  M  Q  N  I  H  P  K  A  L  P  A  E  R  I  L  P  A  L  S  K  D  K  E  E  E  630
1891 ATCCGCAAAATCCTGAGGAACAACCTGCAGAAGACCCGGCAGCGGCTGCGCTCCTACAACAGACACACGCTGGTGGCCGACCCCTACGAGGAGGCCTGGAACCAG
       I  R  K  I  L  R  N  N  L  Q  K  T  R  Q  R  V  R  S  Y  N  R  H  T  L  V  A  D  P  Y  E  E  A  W  N  Q  665
1996 ATGCTGCTGCGGAGACAGAAGGCCCGGCAGCTGGAGCAGAAGATTAACAACTACCTGACTGTGCCGGCGCACAAGCTGGACTCGCCTACCATGTCCCGGGCCCGC
       M  L  L  R  R  Q  K  A  R  Q  L  E  Q  K  I  N  N  Y  L  T  V  P  A  H  K  L  D  S  P  T  M  S  R  A  R  700
2101 ATCGGCTCAGACCCGCTGGCCTATGAGCCGAAGGCAGACTTGCCGGTCATCACCATCGACCCGGCGTCCCCGCAATCACCCGAGTCTGTGGACCTGGTCAACGAG
       I  G  S  D  P  L  A  Y  E  P  K  A  D  L  P  V  I  T  I  D  P  W  S  P  Q  S  P  E  S  V  D  L  V  N  E  735
2206 GAGCTCAAGGGCAAGGTCCTGGGGCTGAGCCGGGAGCCGAGGGTGGCCGAGGAGGCGGCGGAGGAGGACGAGGACGGGGGCATCGTGATGCGGCCCAAGGAGCCC
       E  L  K  G  K  V  L  G  L  S  R  E  P  R  V  A  E  E  A  A  E  E  D  E  D  G  G  I  V  M  R  P  K  E  P  770
2311 TCGTCCCCCGGCACGGACGACGTCTTCAGCCCCGCGCCCAGCGACAGCCCCAGCTCCCAGAGGATGCAGCGCTGTCTCAGCGACCCCGGCCCGCACCCCGAGCCC
       S  S  P  G  T  D  D  V  F  S  P  A  P  S  D  S  P  S  S  Q  R  M  Q  R  C  L  S  D  P  G  P  H  P  E  P  805
2416 GGGGAGGGGGAGCCTTTCATCCCCAAAGGGCAGTAGCGCAGCGCACCTGCCGCCAGACTCTCCCGCCACAGCAGGGGCTCCTGGGGGAGCTGGGGTGCCGGCCCC
       G  E  G  E  P  F  I  P  K  G  Q  *                                                                  816
2521 GCCCCGCCCCCACCGCATGGCAGCTGGGCCCAAGCGCCCACCGCAGCACGGCTTCCCCCTGCCTCCTGGGAAGCGGCCTCCGCCCGGCGGAGCTGCCTTCCCCAT
2626 C
```

Fig. 1. Composite nucleotide sequence of the cDNAs and deduced amino acid sequence (below) of the rabbit renal Na^+/H^+ exchanger. Nucleotides −726 to 351 were contained in a 2.5 kb cDNA obtained by synthesizing a rabbit renal cortical cDNA library in λgt11 [22] and screening with a *Bam*HI fragment of the human Na^+/H^+ exchanger cDNA (remainder of the 2.5 kb cDNA contained part of the first intervening sequence). Nucleotides 23–1453 were obtained by PCR using first-strand rabbit renal cortical cDNA as template and primers based on the sequence of the 2.5 kb rabbit cDNA and human cDNA. Nucleotides 874–2626 were obtained by screening a commercial rabbit renal library (Stratagene) with the 1.4 kb PCR product. Methods for library screening, PCR, and DNA sequencing were similar to those described elsewhere [13,22]. Overlapping sequence was obtained from both strands of all three cDNAs. The in-frame 'mini-cistron' is shown underlined.

In addition to similarities in the coding regions, there is 75% nucleotide identity in the 5′ untranslated regions. One of four upstream start codons in the human cDNA that initiates a short in-frame sequence ('mini-cistron') and may play a role in translational regulation is conserved in rabbit. The 3′ terminus of the human cDNA is interrupted by the artifactual presence of a mouse B1 repeat. The rabbit 3′ untranslated region is shorter and incomplete, does not contain repeat elements, but retains a high degree of similarity with the human sequence (83% nucleotide identity) implying that it also may contain important regulatory sequences. When the rabbit renal sequence is compared to a Na^+/H^+ exchanger cDNA that we isolated from porcine renal epithelial cells (LLC-PK_1) nucleotide identity is 91% and amino acid identity is 95% [13].

Recently, a partial-length cDNA encoding the cytoplasmic domain and 3′ untranslated region of a Na^+/H^+ exchanger was cloned from rabbit heart [14]. In the region of overlap (nt. 1414-2626) the kidney and heart sequences are not completely identical. The kidney sequence has 10 base changes and two deletions which result in a non-conservative amino acid substitution (Lys-569 to Glu). It is not known whether these differences are due to sequencing errors, allelic or strain variations, or expression of different gene products in kidney and heart.

In rabbit, expression of the cloned Na^+/H^+ exchanger transcript is tissue-specific (Fig. 2). Highest expression is in stomach followed by brain, renal medulla, lung, ileum, and renal cortex; levels in liver and skeletal muscle are minimal. As a control, transcripts encoding GAPDH are present in all tissues with relative abundances comparable to that reported for rat [15]. The major Na^+/H^+ exchanger transcripts is 4.7 kb, but there is minor hybridization to an 8 kb transcript that may represent an incompletely processed RNA. We observed no hybridization to transcripts less than 4.7 kb as reported by Fliegel et al. [14].

Functionally distinct isoforms of plasma membrane Na^+/H^+ exchangers are expressed in different cell types. One form that is highly sensitive to inhibition by amiloride analogs is expressed in the basolateral membrane of epithelia in kidney and ileum [16,17] and the plasma membranes of non-epithelial cells including fibroblasts [18], lymphocytes [19], pneumocytes [20], and neurons [21]. Another form that is relatively amiloride-resistant is present in the apical membrane of epithelia in the renal proximal tubule and ileum [16,17]. In rabbit, the distribution of transcripts highly homologous to the human growth-factor-activatable Na^+/H^+ exchanger in both epithelial and non-epithelial tissues is consistent with the amiloride-sensitive Na^+/H^+ exchanger activity, and would not be expected for an isoform of the exchanger that is limited to the apical membrane of epithelia in kidney and ileum. Indeed, antibodies to the cloned Na^+/H^+ exchanger localize to the basolateral membrane of porcine renal epithelial cells (LLC-PK_1) where only the amiloride-sensitive transporter is present [13].

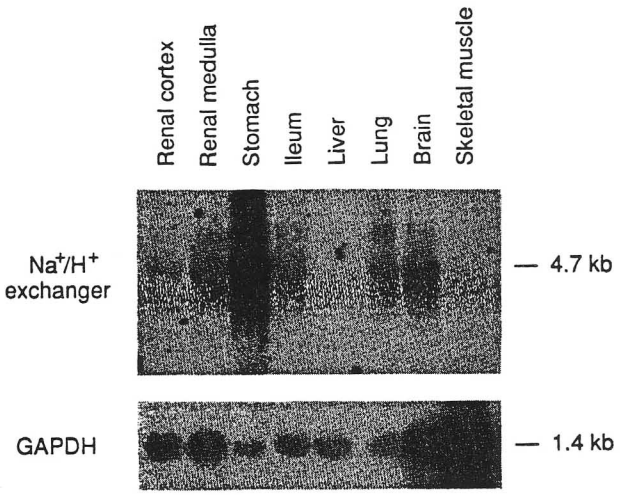

Fig. 2. Expression of the Na^+/H^+ exchanger transcript in rabbit tissues. Total RNA was extracted from 0.5–1 g of rabbit tissues using the method of Chomczynski and Sacchi [23], Puissant and Houdebine [24], and poly(A)$^+$ RNA was isolated as described by Hartmann et al. [25]. 25 μg of poly(A)$^+$ RNA was electrophoresed through 1% agarose-0.66 M formaldehyde gels, transferred to nylon membranes, and hybridized with the 1.4 kb rabbit Na^+/H^+ exchanger cDNA radiolabeled by random primer extension [26,27]. Filters were washed at room temperature in 2×SSC, than at 60 °C in 0.1×SSC containing 0.5% SDS. The autoradiogram was exposed for 6 days at −70 °C (upper panel). Northern blots were stripped and re-hybridized with a ^{32}P-labeled cDNA encoding rat GAPDH (lower panel). The stringent wash was at 60 °C in 1×SSC containing 0.5% SDS, and exposure was for 2 h at −70 °C.

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Molecular biology of renal Na^+–H^+ exchangers

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The Na^+–H^+ exchanger is an integral membrane protein that mediates 1:1 electroneutral exchange of extracellular Na^+ for intracellular H^+. Plasma membranes of most vertebrate cells contain an Na^+–H^+ exchanger that regulates intracellular pH and cell volume and responds to mitogenic stimuli. In epithelia, such as the mammalian renal proximal tubule, the Na^+–H^+ exchanger is responsible for transepithelial flux of Na^+, Cl^-, and HCO_3^- (reviewed in [1, 2]). Study of renal Na^+–H^+ exchangers is of potential clinical significance because inherited variations in activity of some forms may be causally associated with essential hypertension. Alterations in Na^+–H^+ exchange activity may also be involved in the pathogenesis or adaptation to metabolic acidosis, states of salt-wasting or edema formation, and abnormal growth and development.

Properties of plasma membrane Na^+–H^+ exchangers

Primarily using plasma membrane vesicles as the experimental model, much has been learned about the kinetic properties of the Na^+–H^+ exchanger including its stoichiometry, substrate specificity, and sensitivity to inhibitors, especially the pyrazine diuretic amiloride and its 5-amino-substituted congeners [3]. Studies using chemical modification by group-specific reagents have implicated critical carboxyl [4], histidyl [5], lysyl [6], and sulfhydryl [7] groups in the transport mechanism of Na^+–H^+ exchange. For example, amiloride or the transportable substrates Na^+ and Li^+ could protect against irreversible inhibition of the rabbit renal brush border Na^+–H^+ exchanger by the carboxyl-reagent, *N,N'*-dicyclohexylcarbodiimide [8]. More recently, covalent labeling [8–10] or affinity chromatography [11] have been used to identify proteins of molecular weights 100, 79, 72, 65, or 25 kDa that were candidates for the transport protein. However, none of these studies unambiguously identified the proteins mediating Na^+–H^+ exchange in the kidney.

Two types of Na^+–H^+ exchangers exist and are distinguished by their different sensitivities to inhibition by amiloride and its analogs [12–14]. One type is relatively sensitive to amiloride and its congeners [IC_{50} of 20–100 n*M* for 5-*N*-ethylisopropyl amiloride (EIPA) [15]]. This form of Na^+–H^+ exchanger is found in many cell types including fibroblasts, smooth muscle cells, lymphocytes, and mesangial cells, and will be referred to here as "NAH-1." In accordance with convention, the gene encoding the protein, NAH-1, will be designated in italics as "*NAH-1*." In epithelia, such as ileum and certain nephron segments including thin descending limb [16] and collecting tubule [17], NAH-1 is restricted to the basolateral membrane where it is involved in control of intracellular pH (an exception is the placenta where this form is present in the apical membrane [18]). A second type of Na^+–H^+ exchanger is relatively resistant to inhibition by amiloride and its congeners (IC_{50} of 2–10 μM for EIPA [19]). This form of Na^+–H^+ exchanger is restricted to the apical membrane of epithelia such as the renal proximal tubule and ileum where it participates in active $NaHCO_3$ and/or NaCl absorption and will be referred to here as "NAH-2." Importantly, ileal villus enterocytes and the "proximal tubule-like" porcine renal epithelial cell line, LLC-PK_1/Clone 4, contain in the same cell both types of Na^+–H^+ exchangers, an apical, amiloride-resistant form (NAH-2), and a basolateral, amiloride-sensitive form (NAH-1). In addition to differences in sensitivity to amiloride analogs, NAH-1 and NAH-2 also differ in sensitivity to sulfhydryl modification [20], regulation by protein kinase C [12], and inhibition by external K^+ [13]. The structural basis for the functional differences between NAH-1 and NAH-2 is unknown.

Regulation of renal Na^+–H^+ exchangers

Transport activities of plasma membrane Na^+–H^+ exchangers are regulated in the kidney (reviewed in [21]). Short-term regulation (seconds to minutes) of both NAH-1 and NAH-2 occurs following exposure to a number of growth factors and peptide hormones (e.g., angiotensin II, parathormone). These factors alter intracellular levels of calcium and cyclic nucleotides, and their effects on Na^+–H^+ exchange are probably mediated via protein phosphorylation. The transport protein itself may be a substrate for phosphorylation since Sardet et al. recently demonstrated that a human Na^+–H^+ exchanger is phosphorylated in response to epidermal growth factor and thrombin [22].

Both types of Na^+–H^+ exchangers are also subject to long-term regulation (hours to days). For example, stimulation of the renal apical Na^+–H^+ exchanger (NAH-2) has been observed in animals or cultured proximal-tubule cells following chronic administration of glucocorticoids [23, 24], thyroid hormone [25], partial renal ablation [26], chronic potassium depletion [27], or chronic metabolic acidosis [28–34]. Alterations in Na^+–H^+ exchange due to some of these factors may explain, for example, the Na^+ wasting that occurs in hypothyroidism [35]. Where examined, these factors increased V_{max} for trans-

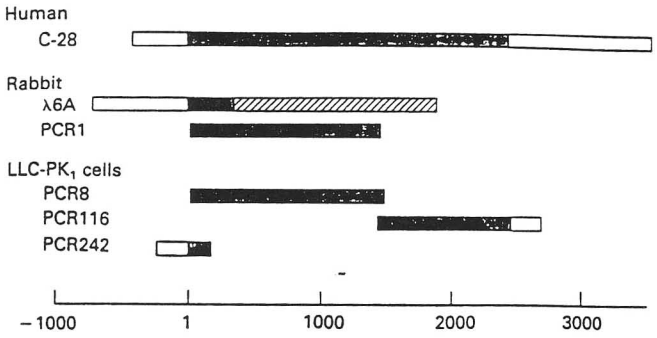

Fig. 1. *cDNAs encoding plasma membrane Na^+–H^+ exchangers.* The boxes depict cDNAs cloned from rabbit kidney (middle) and LLC-PK_1 cells (bottom) which are aligned with the human sequence (top). Open boxes on the left and right represent 5′ and 3′ untranslated regions, respectively. Shaded boxes denote coding sequences. The hatched box in λ6A represents an intervening sequence. The scale at the bottom indicates the distance in nucleotides from the initiation codon for the protein.

port with no change in apparent K_m for substrate consistent with increased synthesis of transport protein, perhaps due to increased gene transcription. Long-term regulation of NAH-1 occurs in metabolic acidosis. For example, chronic acid-loading stimulated the transport activity NAH-1 in rat mesangial cells consistent with its role in regulation of pH_i (Michael Ganz, personal communication). The mechanisms underlying changes in transport due to metabolic acidosis are unknown but may also involve altered gene transcription. Consistent with this, Krapf et al. observed increased NAH-1 transcript levels in the kidneys of rats with metabolic acidosis [36].

Cloning of a human Na^+–H^+ exchanger cDNA and gene

Recently, Sardet et al. used genetic complementation to clone a partial-length cDNA encoding a human Na^+–H^+ exchanger [37]. Human genomic DNA was transfected into murine fibroblasts that had been rendered deficient in the plasma membrane Na^+–H^+ exchanger. Restoration of transport activity indicated transfection of human DNA containing an Na^+–H^+ exchanger gene. A *PstI* restriction fragment of the transfected human DNA was then used to screen a cDNA library synthesized from a secondary transformant that overexpressed Na^+–H^+ exchanger activity. A 3977-bp cDNA was cloned, sequenced, and found to contain a 2451-bp open reading frame encoding a 90-kDa protein (see Fig. 1). Hydropathy plots of the deduced amino acid sequence suggested that the protein consisted of two domains: an amino-terminal membrane-associated domain with ten putative membrane spanning α-helices (499 amino acids), and a carboxyl-terminal cytoplasmic domain (316 amino acids). Transfection of this cDNA into mutant fibroblasts that were deficient in the Na^+–H^+ exchanger restored transport activity, indicating that it encoded the transport protein. Because the human cDNA was obtained from a murine transformant, the tissue of origin was unknown, and it was unclear whether the cDNA encoded an NAH-1 or an NAH-2.

Cloning of rabbit renal Na^+–H^+ exchanger cDNAs

To further understand the molecular mechanism of Na^+–H^+ exchange in the kidney, we used a molecular biological approach. Our first objective was to obtain a cDNA encoding a rabbit renal Na^+–H^+ exchanger, since previous physiological studies had been performed in this species. Such a cDNA could be useful for inferring the primary structure of the transport protein, functionally expressing the transport protein, studying expression of mRNA encoding the protein, and cloning the Na^+–H^+ exchanger gene. We reasoned that one of the rabbit renal Na^+–H^+ exchangers would be structurally similar to the human Na^+–H^+ exchanger. Indeed, using high-stringency Northern blot analysis we determined that a single 4.7-kb transcript structurally similar to the human Na^+–H^+ exchanger cDNA was expressed in the rabbit kidney. To clone this transcript we synthesized a rabbit renal cDNA library in *λgt11* and screened it by filter hybridization to the radiolabeled human Na^+–H^+ exchanger cDNA (generously provided by Drs. Jacques Pouysségur and Claude Sardet). We obtained a 2.4-kb cDNA called λ6A which is schematically depicted in Fig. 1. This cDNA was subcloned into a plasmid and sequenced using the dideoxynucleotide chain termination method. λ6A contained 725 bp of 5′ untranslated region and 351 bp of coding region followed by 1.4 kb of unrelated sequence that we hypothesized was an intervening sequence, presumably due to cloning of an incompletely processed transcript. We have verified this organization by comparison to a 17-kb clone of the corresponding rabbit gene that contains 6 kb of 5′ flanking sequence, the first exon, and a portion of the first intervening sequence (intron). The deduced amino acid sequence of the coding region of λ6A was 86% identical to the amino-terminal sequence of the human Na^+–H^+ exchanger. Interestingly, there was also 68% nucleotide sequence identity in the overlapping 5′ untranslated region, including conservation of two upstream "mini-cistrons" that may be involved in posttranscriptional control [38].

To obtain a cDNA encoding the remainder of the membrane-domain of the Na^+–H^+ exchanger, we employed mixed-oligonucleotide-primed amplification of cDNA [39], an application of the polymerase chain reaction (PCR) [40]. Based on the sequences of the 2.4-kb rabbit cDNA and the human cDNA, we synthesized two sets of degenerate oligonucleotides corresponding to the expected sequence of the transcript flanking the membrane-associated domain, the region that we expected to be most conserved between species. Poly(A)$^+$RNA was isolated from rabbit kidney, and first strand cDNA was synthesized using reverse transcriptase. The oligonucleotides were annealed, and PCR was performed by 45 repetitive cycles of heat denaturation, annealing of oligonucleotides, and elongation with *Taq* DNA polymerase. Only the mRNA that contained sequence flanked by the two oligonucleotides, and therefore encoded the membrane-associated domain, should be amplified by this procedure. We obtained a single 1430-bp cDNA (called PCR1, see Fig. 1) which was subcloned and sequenced. The sequence of PCR1 was also highly similar to the human cDNA (92% nucleotide identity and 94% amino acid identify, see Fig. 2) indicating that it encodes the membrane-associated domain of a rabbit renal Na^+–H^+ exchanger. Moreover, 328 bp of overlapping sequence between PCR1 and λ6A were completely

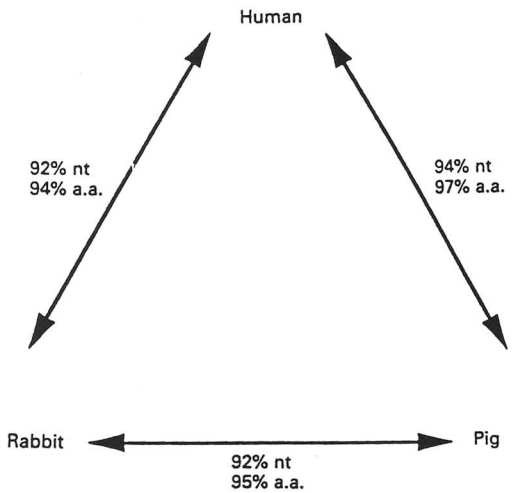

Fig. 2. *Similarities between sequences of cDNAs encoding Na^+–H^+ exchangers.* Percent identities of the nucleotide (nt) and deduced amino acid (a.a.) sequences are shown for the membrane-associated domains of the human, rabbit renal, and LLC-PK_1 Na^+–H^+ exchangers.

identical, and both clones hybridized to the same transcripts on Northern blots and shared bands on genomic Southern blots, indicating that they originated from the same transcript and gene.

Cloning of porcine renal Na^+–H^+ exchanger cDNAs

We next attempted to clone cDNAs encoding a Na^+–H^+ exchanger from the porcine renal epithelial cell line LLC-PK_1/Clone 4. As mentioned previously, these cells contain both NAH-1 and NAH-2 on their basolateral and apical membranes, respectively. By comparing the sequences of cDNAs from human, rabbit, and pig we hoped to identify conserved amino acids and domains that might be functionally important. Northern blot analysis showed that the human Na^+–H^+ exchanger cDNA hybridized to a single 5-kb transcript from LLC-PK_1 cells, indicating the presence of a structurally similar transcript. To clone this transcript we again utilized PCR. Using oligonucleotides flanking the membrane-associated domain of the human Na^+–H^+ exchanger we amplified, cloned, and sequenced a 1468-bp cDNA (called PCR8, see Fig. 1). The sequence was highly similar to the Na^+–H^+ exchangers from human (94% nucleotide identity and 97% amino acid identity) and rabbit (92% nucleotide identity and 95% amino acid identity, see Fig. 2). A second pair of oligonucleotides was used to amplify a 1256-bp cDNA corresponding to the cytoplasmic domain of the transporter (called PCR116, see Fig. 1) which was also highly similar to the human sequence (94% nucleotide identity and 98% amino acid identity) [41]. Using PCR we have also cloned 228 bp of 5′ untranslated sequence (PCR242, see Fig. 1) which were 76% identical to the human sequence.

These results indicate that we have obtained partial-length cDNAs encoding rabbit and porcine renal Na^+–H^+ exchangers. From the sequence of the cDNAs we inferred the primary structures of the transport proteins which are highly conserved between species. The high degree of similarity in amino acid sequences permitted only limited conclusions about possible functional correlates. Hydropathy plots suggested that the overall architecture was maintained and consisted of an amino-terminal membrane-associated domain with multiple hydrophobic α-helices, and a carboxyl terminal hydrophilic domain. Two putative glycosylation sites were present in sequences from the three species. A region that McDaniel et al. have hypothesized may contain a charge relay mechanism is also conserved [42]. More recently, cDNAs homologous to the human Na^+–H^+ exchanger have been cloned from rat kidney [43] and rabbit ileum [44]. The 890-bp rat renal cDNA encodes three putative membrane-spanning segments and part of the cytoplasmic domain and is greater than 90% identical to the human sequence. The 4-kb rabbit ileal cDNA contains 322 bp of 5′ untranslated region, 2451 bp of coding sequence, and 1163 bp of 3′ untranslated region with 70%, 91%, and 40% nucleotide identity to the human sequence, respectively.

Localization of expression of rabbit and porcine renal cDNAs

To determine whether the cDNAs from rabbit kidney and LLC-PK_1 cells encoded an apical, amiloride-resistant Na^+–H^+ exchanger (NAH-2) or a basolateral, amiloride-sensitive Na^+–H^+ exchanger (NAH-1), we wished to localize the proteins encoded by these cDNAs. Our strategy was based on the observation that NAH-2 and NAH-1 are restricted to the apical and basolateral membranes, respectively, in ileal villus enterocytes and confluent LLC-PK_1/Clone 4 cells. Oligopeptides were synthesized based on the deduced amino acid sequences of the cloned cDNAs and used to generate specific antisera. For example, we synthesized a 17-mer (called JK42) that was derived from a sequence which is identical in human, rabbit, and pig. The sequence of JK42 is near the amino-terminus of the protein between the first two putative transmembrane helices. Affinity-purified antisera were raised against this oligopeptide in guinea pigs and used for immunoblots and indirect immunofluorescence. Immunoblots of rabbit ileal plasma membrane proteins were probed with the affinity-purified antisera, and a single protein of 105 kDa was recognized in the basolateral but not brush-border membrane. The size of the protein is approximately the same as reported by Sardet et al. for the Na^+–H^+ exchanger in Chinese hamster lung fibroblasts [22]. This basolateral localization was confirmed by indirect immunofluorescence of LLC-PK_1/Clone 4 cells and rabbit ileum. Antisera to JK42 and a monoclonal antibody against the Na^+–K^+–ATPase co-localized to the basolateral plasma membrane and not the brush-border membrane of confluent LLC-PK_1/Clone 4 cells. Studies in rabbit ileum verified the absence of localization to the brush-border membrane and were consistent with labeling of the basolateral membrane. Taken together, these results indicate that the protein containing the sequence of JK42 is present in the basolateral and not the brush-border membrane of LLC-PK_1/Clone 4 cells and ileal villus enterocytes. Therefore, the cDNAs that we have cloned from rabbit kidney and LLC-PK_1 cells encode the basolateral, amiloride-sensitive Na^+–H^+ exchanger, NAH-1, and not NAH-2.

Differences between NAH-1 and NAH-2

Since the protein that mediates Na^+–H^+ exchange in the apical membrane of epithelia, NAH-2, has not been identified and its cDNA has not been cloned, the structural features responsible for differing sensitivities to amiloride analogs remain unknown. However, our cloning and immunolocalization

results permit certain inferences. Antisera raised against JK42 recognize the basolateral, amiloride-sensitive Na^+–H^+ exchanger, NAH-1. Moreover, they are specific and do not detect the apical, amiloride-resistant Na^+–H^+ exchanger, NAH-2. Evidently, the primary structures of NAH-2 and NAH-1 differ, at least in the region of JK42. This suggests that the difference between NAH-1 and NAH-2 is probably not due simply to variations in glycosylation, phosphorylation, or lipid environment since the primary structures are different. More likely, NAH-2 is encoded by the same gene, *NAH-1*, but differs from NAH-1 due to alternative transcript splicing and/or promoter use, or NAH-2 is encoded by a different Na^+–H^+ exchanger gene, *NAH-2*, that is similar to *NAH-1* or structurally unrelated. Consistent with this hypothesis, Haggerty et al. have produced a mutant LLC-PK_1 cell line that overexpresses NAH-2 but not NAH-1 [14]. Another mutant is solely deficient in NAH-1 (unpublished observations). These results suggest that NAH-1 and NAH-2 are under specific genetic control.

Since the primary structures of NAH-1 and NAH-2 differ, the region of NAH-1 corresponding to JK42 is either absent in NAH-2 or structurally-dissimilar in NAH-2. If so, the cDNAs encoding this region should also be specific for NAH-1, and should not detect transcripts or genes encoding NAH-2 when hybridizations are performed at high stringency. Indeed, Northern blots of rabbit renal and LLC-PK_1 poly(A)$^+$ RNA showed only single transcripts when hybridized at moderately high stringency with cDNAs encoding JK42, the amino acid sequence that is specific for NAH-1. On Southern blots, these probes hybridized to single restriction fragments of rabbit and porcine genomic DNA. Moreover, the sizes of the restriction fragments from rabbit genomic DNA and a 17-kb rabbit *NAH-1* genomic clone were identical, consistent with a single rabbit Na^+–H^+ exchanger gene encoding this region. Taken together, these results suggest that if NAH-2 is encoded by another gene (*NAH-2*), it lacks sufficient sequence similarity in the region of JK42 to be detected by high-stringency Southern or Northern blot analysis. Alternatively, if NAH-2 is a product of the same gene, *NAH-1* (e.g., a splicing variant), it must lack this nucleotide and amino acid sequence. In either event, when used at high-stringency, the cDNAs encoding this region are specific for NAH-1 and not NAH-2.

Regulation of *NAH-1* during metabolic acidosis

In addition to providing details about the structure of the encoded protein, having cDNAs that were specific for NAH-1 permitted us to begin study of the *NAH-1* gene and its regulation. Transport activity of renal Na^+–H^+ exchangers is increased by metabolic acidosis, and the kinetics are consistent with increased protein synthesis. Therefore, we set out to examine whether metabolic acidosis stimulated expression of *NAH-1* in the kidney. The overall strategy was to measure NAH-1 transport activity and steady-state transcript levels under conditions simulating metabolic acidosis. Studies were performed in cultured renal-tubule cells to better control exposure conditions and to avoid confounding variables in intact animals such as increases in glomerular filtration which independently stimulate apical Na^+–H^+ exchanger activity [26].

LLC-PK_1/Clone 4 cells were grown on porous filters to permit independent access to both apical and basolateral membranes. After formation of a confluent monolayer, cells were exposed to medium that had been acidified by titration with HCl to simulate uncompensated hyperchloremic metabolic acidosis (pCO_2 was maintained at 40 torr). After pretreatment for 48 h with acidified medium (pH 6.9) or control medium (pH 7.4), transport activity of the basolateral membrane Na^+–H^+ exchanger (NAH-1) was measured as the initial rate of 15 m*M* $^{22}Na^+$ influx into cells that were acid-loaded using the NH_4^+ pre-pulse technique [14]. $^{22}Na^+$ influx was stimulated 80% in cells that had been pretreated with acidified medium.

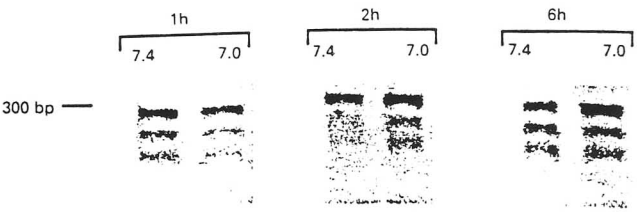

Fig. 3. *Steady-state levels of NAH-1 transcript levels in LLC-PK_1 cells following medium acidification.* LLC-PK_1/Clone 4 cells were exposed to control (pH 7.4) or acidified (pH 7.0) medium for 1, 2, or 6 h, and NAH-1 transcript levels were measured by S1 nuclease protection as described in the text. The expected position of a 300-bp protected band is indicated.

To determine whether increases in transport activity of NAH-1 were preceded by corresponding changes in transcript levels, monolayers of LLC-PK_1/Clone 4 cells were exposed to control or acidified medium, and after 1 to 6 h, steady-state levels of transcripts encoding NAH-1 were measured by S1 nuclease protection assays [45]. Preliminary studies indicated that mRNA encoding NAH-1 was of very low abundance and could not be detected in Northern blots of total RNA even with high specific activity cRNA probes. Accordingly, it was necessary to use a more sensitive solution-hybridization assay based on S1 nuclease protection. 50 μg of total cellular RNA were isolated from control and acid-treated cells by guanidinium isothiocyanate/CsCl ultracentrifugation [46] and hybridized at high stringency to an excess of an end-labeled restriction fragment of PCR8 that contained 300 bp of coding sequence and 29 bp of vector sequence. Digestion with S1 nuclease removed the vector sequence and any probe that had not hybridized to NAH-1 transcripts. The products were analyzed by denaturing polyacrylamide gel electrophoresis, and the autoradiogram is shown in Fig. 3. A protected band of 300 bp was present in both control and acid-treated cells (shadow bands are probably an artifact). The intensities of the 300-bp protected bands from cells treated for 2 and 6 h with medium acidification were increased two-fold over the respective controls, which corresponded to the observed stimulation in basolateral Na^+–H^+ exchanger transport rates. These results were confirmed by Northern blot analysis of poly(A)$^+$ RNA, which in addition showed no change in γ-actin transcript levels as an internal control. Taken together, these results demonstrate that metabolic acidosis coordinately increases the transport activity and steady-state transcript levels of NAH-1 in LLC-PK_1 cells. Effects of acid-base status on renal gene expression are not unprecedented. For example, metabolic acidosis increases levels of transcripts encoding phosphoenolpyruvate carboxykinase (PEPCK) [47] and glutaminase [48] in the kidney. Stimulation of expression of specific genes (including those encoding PEPCK,

glutaminase, and NAH-1) may comprise part of the renal response to metabolic acidosis, and common *cis*-acting elements or transcription factors may be involved. Since adaptation to metabolic acidosis is a uniquely renal responsibility, defining its mechanisms, especially genetic mechanisms that may be common to other genes, would be of particular interest to nephrological study in general.

Conclusion

cDNAs encoding Na^+–H^+ exchangers have been cloned from rabbit kidney and porcine renal cells (LLC-PK_1). The encoded proteins are very similar in primary structure, suggesting a high degree of evolutionary conservation. The cloned cDNAs encode a renal basolateral Na^+–H^+ exchanger, NAH-1, and not a renal apical Na^+–H^+ exchanger, NAH-2. Transport activity and steady-state transcript levels of NAH-1 are increased coordinately in LLC-PK_1 cells under conditions simulating metabolic acidosis. Future studies will be directed at defining structure-function correlates for renal NAH-1, establishing the structural relationship between NAH-1 and NAH-2, and elucidating the mechanisms responsible for regulation of NAH-1 during metabolic acidosis.

Summary

Na^+–H^+ exchangers are plasma membrane proteins that are responsible for Cl^- and HCO_3^- reabsorption and regulation of intracellular pH in several nephron segments. Recently, a cDNA encoding a human Na^+–H^+ exchanger of unknown tissue origin was cloned, and Northern blot analysis revealed that structurally similar transcripts were expressed in rabbit kidney and porcine renal epithelial cells (LLC-PK_1). To clone these renal transcripts we employed cDNA library screening and the polymerase chain reaction (PCR). We obtained 2.5 kb and 1.4 kb cDNAs corresponding to the 5′ untranslated region and the membrane-associated domain of a rabbit renal Na^+–H^+ exchanger. From LLC-PK_1 cells we obtained 1.5 kb and 1.3 kb cDNAs encoding the membrane-associated and cytoplasmic domains. The sequences of cDNAs from these three species were very similar and suggested a high degree of evolutionary conservation. Immunolocalization of synthetic oligopeptides derived from the deduced amino acid sequences indicated that the cloned cDNAs encoded the amiloride-sensitive form of the Na^+–H^+ exchanger present in basolateral membranes of epithelia. cDNAs were also used to study regulation of Na^+–H^+ exchanger gene expression in the kidney, and we found that metabolic acidosis stimulated both the transport rate and steady-state transcript levels of the basolateral Na^+–H^+ exchanger in LLC-PK_1 cells.

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Durante o segundo ano do estágio de pós-doutorado me dediquei ao estudo da região promotora do gene que havíamos clonado a partir de cDNA de tecido renal de coelho (gene *NHE-1*).

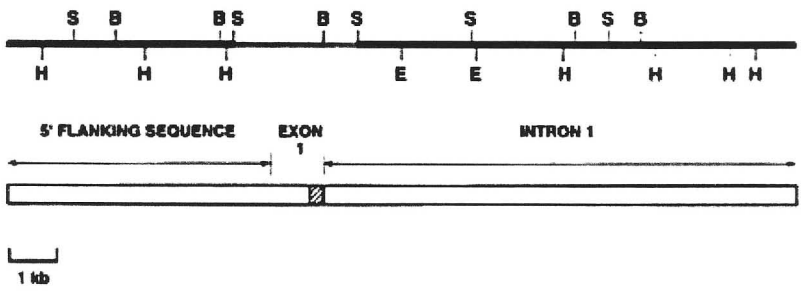
Madelaine Blaurock, ao fazer o "screening" da biblioteca genômica de rato, à qual já nos referimos previamente, utilizando como sonda a extremidade 5' do cDNA que havíamos clonado a partir de mRNA de rim de coelho, havia identificado dois clones que se mostraram idêntidos, denominados λRNH2.

Os diversos fragmentos deste clone de 17.000 pb, obtidos com enzima de restrição, foram hibridizados isoladamente com a sonda correspondente à extremidade 5' de nosso cDNA. Escolhemos um fragmento de 2.700 pb obtido com a enzima *Sac I* por ter este se hibridizado com nossa sonda, e o sequenciamos. Os primeiros 1077 nucleotídeos de nosso cDNA estavam presentes também nesse fragmento, correspondendo aos 726 pb da região 5' não codificante e a 351 pb da sequencia codificante. No fragmento total de 17.000 nucleotídeos nenhum outro segmento se hibridizava com sequências do nosso cDNA.

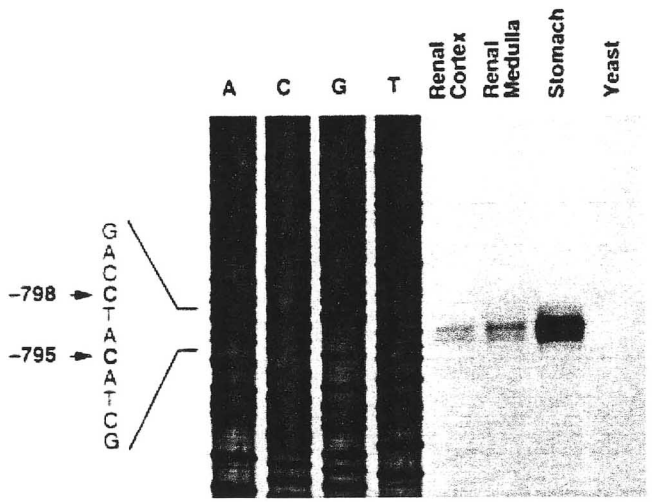
A figura abaixo mostra o mapa de restrição do clone λRNH2 e a posição de nosso cDNA (exon1) em relação ao mesmo.

Enzimas de restrição: *Sac I* (S), *Bgl II* (B), *Hind III* (H), *Eco RI* (E).

Segmento em cinza foi a porção sequenciada. A porção achuriada corresponde à sequência codificante de 351 pb.



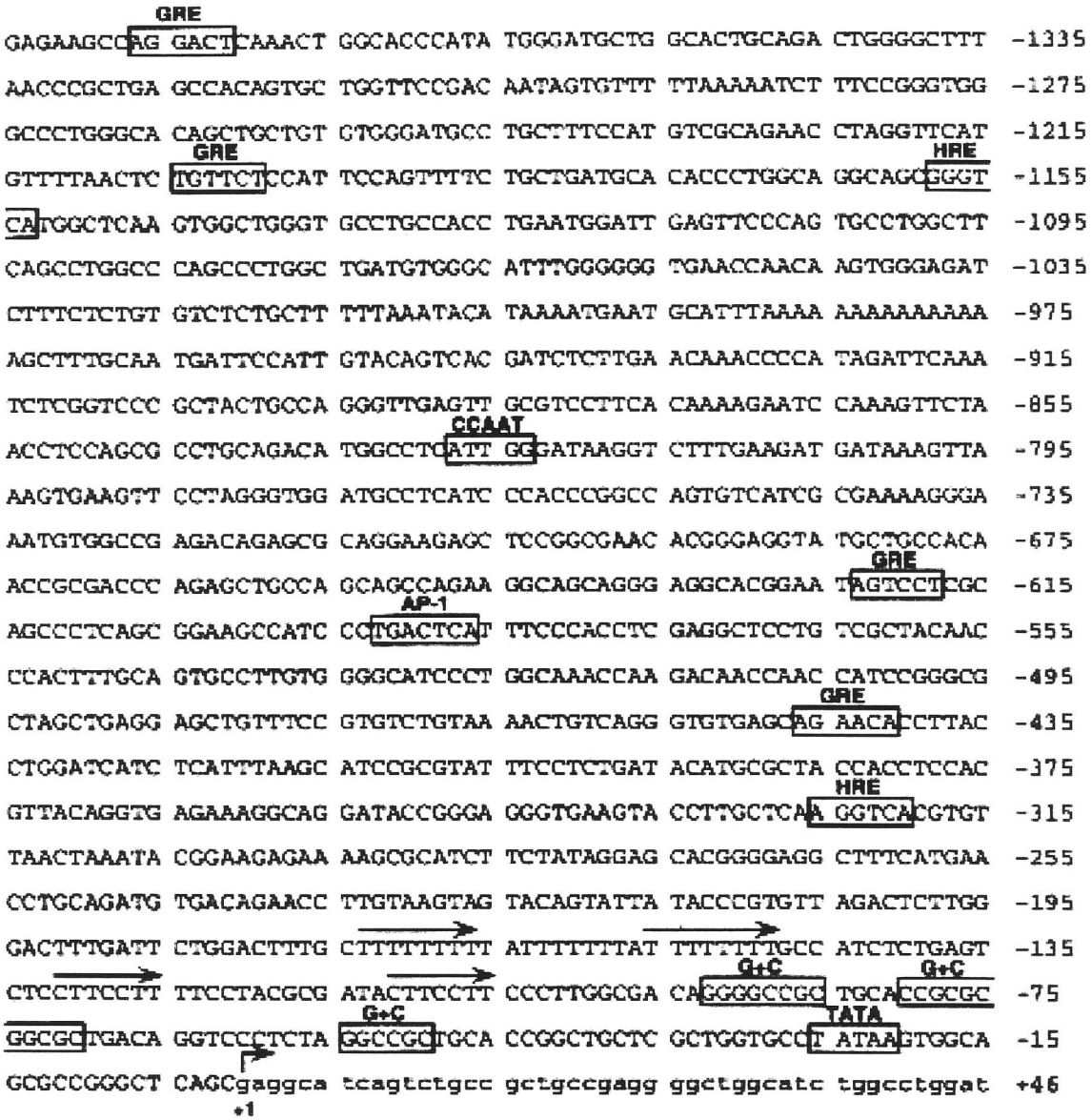
Identificamos o ponto de início de transcrição do RNA através de "RNase Protection Assay". Um segmento de restrição com 745 pb obtido por digestão do clone λRNH2 com a enzima *Taq I* (nucleotídeos –1.375 a –631 a partir do ATG inicial) foi clonado em pBlueScript e uma sonda de RNA antisense marcada com ^{32}P-UTP foi transcrita usando a T7 RNA-polimerase. A sonda de RNA complementar foi hibridizada com 100 ug de RNA provenientes de córtex e medula renais e de estômago de coelho, ou 50 ug de tRNA de levedura. Após disgestão com RNase A e T1, os produtos protegidos da digestão enzimática (duplas fitas de mRNA/RNA complementar) foram analisados através de eletroforese em gel de poliacrilamida. Identificamos um ponto mais importante de início de transcrição na posição –798, a partir do ATG inicial. Esse mesmo início de transcrição foi confirmado através de "5' primer extension".



As faixas marcadas com A, C, G e T correspondem a uma reação de sequenciamento de DNA que foi utilizada para indicar o tamanho das bandas protegidas. As flechas indicam os pontos de início de transcrição.

Na região que flanqueia a extremidade 5' do gene do permutador Na^+/H^+ encontramos elementos promotores eucarióticos típicos, como a TATA box na posição –25 e 3 GC boxes (GGGCCC), que são sítios de ligação para Sp-1, como comentado na intradução. Observamos, à montante, alguns elementos reguladores que incluem 4 hemi-sítios para ligação de receptores para hormônios esteróides (GRE) e dois hemi-sítios para ligação de receptores nucleares para hormônios não esteróides (HRE). Um possível sítio para ligação de AP-1 foi encontrado na posição –592 (Sequência mostrada na página seguinte).

Sequência de nucleotídeos correspondente à região que flanqueia a extremidade 5' do gene *NHE1* em coelhos:



Fragmentos de restrição com 4.700 pb e 1.100 pb contendo 4.300 e 708 pb, respectivamente, da região flanqueadora 5' imediatamente à montante do sítio de início de transcrição foram ligados em vetor contendo o gene repórter luciferase, sem promotor, e este foi expresso de forma transiente em células LLC-PK1, uma linhagem de células de túbulos proximais de rim de porco. Observamos que ambos os fragmentos usados como promotores para o gene da luciferase aumentaram significativamente a expressão da proteína, quando comparado ao efeito do promotor da região precoce de SV40 (SV40 early region promoter), observado quando as células eram transfectadas com o vetor pSV2-LUC. A indução do gene da luciferase foi mais evidente com o fragmento de 1.100 pb, evidenciando algum elemento inibidor à montante da posição –708.

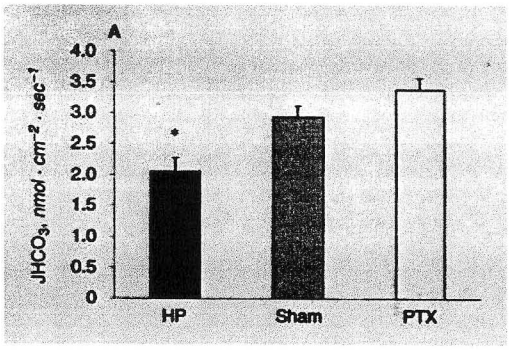
A cópia do artigo com os dados acima relatados está anexada nas páginas seguintes.

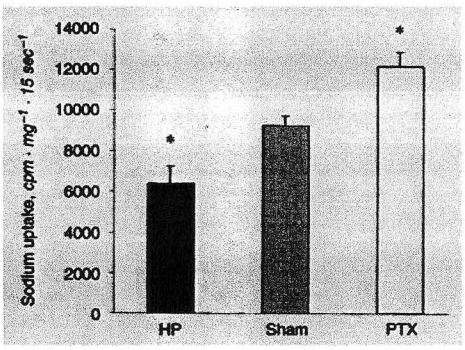
- Blaurock M.C., **Rebouças N.A.**, Kusnezov J.L., Igarashi P. Phylogenetically conserved sequences in the promoter of the rabbit sodium-hydrogen exchanger isoform 1 gene (*NHE1/SLC9A1*). *Biochimica et Biophysica Acta* 1262 (1995) 159-163.

em nosso laboratório, utilizando como molde cDNA sintetizado a partir de mRNA de tecido renal de ratos. Esse fragmento de cDNA estava clonado no plasmídeo BlueScript, que tem o promotor da T7 RNA polimerase, enzima utilizada para síntese do RNA complementar marcado.

Os animais com excesso de PTH, apresentaram redução significativa da taxa de reaborção de HCO_3^-, avaliada através da técnica de micropunção tubular com medida contínua de pH em gota estacionária, e significativa redução na captação de $^{22}Na^+$ sensível a amiloride, em vesículas de bordo em escova de túbulos proximais. Os animais paratireidectomizados, por sua vez, apresentaram aumento significativo desses parâmetros indicativos da atividade do permutador Na^+/H^+.

Logo abaixo reproduzimos as figuras que mostram os valores observados de fluxos de HCO_3^- e captação de ^{22}Na.

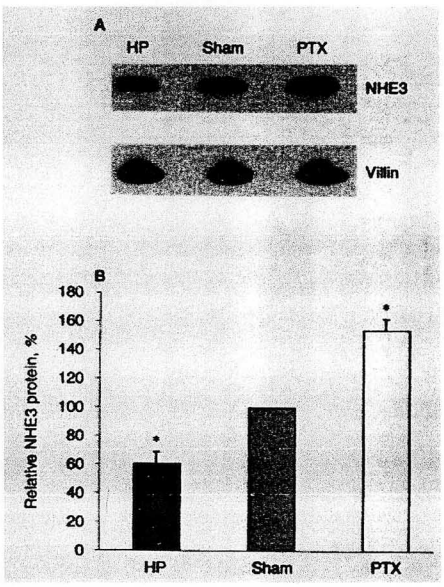


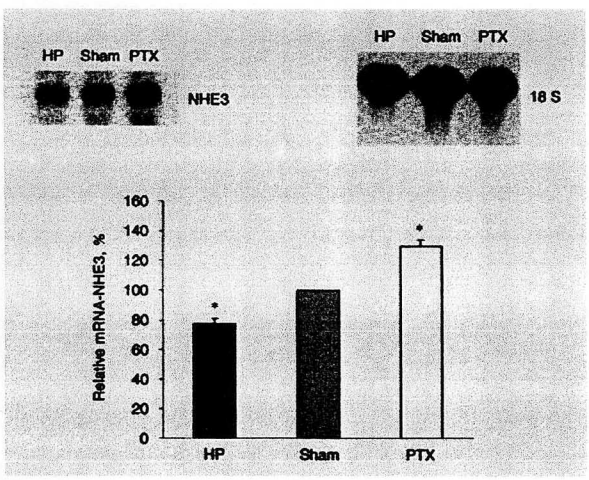


A quantidade da proteína NHE-3 em preparações de "brush border", assim como os níveis de mRNA específico para essa proteína em amostras de RNA total de rins, se mostraram significativamente reduzidos nos animais com excesso de PTH e aumentados nos animais paratireidectomizados. Isso mostra que a alteração funcional observada se deve a alteração nos níveis da proteína, e que a modificação nos níveis da proteína se

devem a alteração na taxa de transcrição do gene *NHE-3* ou na estabilidade de seu mRNA específico.

As figuras que respresentam as quantificações da proteína em vesícula de bordo em escova através da utilização da técnica de Western blot, e as quantificação de mRNA em amostras de RNA total extraído de rins inteiros através da utilização da técnica de Northern blot, estão reproduzidas abaixo. Vilina e 18 S foram utilizados como controles internos.





As análises histológicas com técnica imunohistoquímica mostram redução na quantidade de NHE-3 em membrana apical de túbulos proximais de ratos hiperparatireoideos, e aumento na quantidade da proteína nos animais hipoparatireoideos.

Neste trabalho discutimos o significado fisiológico dessa modulação por PTH da secreção de H^+ em túbulos proximais. O hiperparatireoidismo usualmente não está associado a acidose metabólica, uma vez que a secreção de H^+ em nefro distal está aumentada nesta condição [107]. Levantamos a possibilidade de que a inibição da atividade de NHE-3 em túbulos proximais por PTH pudesse ser um mecanismo adicional para a manutenção da homeostase do Ca^{2+}, a principal função do PTH no organismo. Foi observado em outros laboratórios que a reabsorção transcelular de Ca^{2+} em túbulos distais é estimulada por bicarbonato luminal [108]. Além disso, a condutância de canais para Ca^{2+} presentes em membrana apical poderia ser modulada pelo pH luminal. Assim, o aumento da concentração luminal de HCO_3^-, induzido pelo PTH, poderia ser um mecanismo adicional de controle da reborção renal de Ca^{2+}. Trata-se apenas de uma especulação que precisa ser testada experimentalmente.

Nas páginas seguintes está anexado um exemplar original do trabalho comentado acima.

- Girardi A.C.C., Titan S.M.O., Malnic G., and **Rebouças N.A.** Chronic effect of parathyroid hormone on NHE3 expression in rat renal proximal tubules. *Kidney Int.* 58:1623-1631, 2000.

Kidney International, Vol. 58 (2000), pp. 1623–1631

ION CHANNELS – MEMBRANE TRANSPORT – INTEGRATIVE PHYSIOLOGY

Chronic effect of parathyroid hormone on NHE3 expression in rat renal proximal tubules

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Chronic effect of parathyroid hormone on NHE3 expression in rat renal proximal tubules.

Background. The most abundant Na^+/H^+ exchanger in the apical membrane of proximal tubules is the type 3 isoform (NHE3), and its activity is acutely inhibited by parathyroid hormone (PTH). In the present study, we investigate whether changes in protein abundance as well as in mRNA levels play a significant role in the long-term modulation of NHE3 by PTH.

Methods. Three groups of animals were compared: (*1*) HP: animals submitted to hyperparathyroidism by subcutaneous implantation of PTH pellets, providing threefold basal levels of this hormone (2.1 $U \cdot h^{-1}$); (*2*) control: sham-operated rats in which placebo pellets were implanted; (*3*) PTX: animals submitted to hypoparathyroidism by thyroparathyroidectomy followed by subcutaneous implantation of thyroxin pellets, which provided basal levels of thyroid hormone. After eight days, we measured bicarbonate reabsorption in renal proximal tubules by in vivo microperfusion. NHE3 activity was also measured in brush border membrane (BBM) vesicles by proton dependent uptake of ^{22}Na. NHE3 expression was evaluated by Northern blot, Western blot and immunohistochemistry.

Results. Bicarbonate reabsorption in renal proximal tubules was significantly decreased in HP rats. Na^+/H^+ exchange activity in isolated BBM vesicles was 6400 ± 840, 9225 ± 505, and 12205 ± 690 $cpm \cdot mg^{-1} \cdot 15\ s^{-1}$ in HP, sham, and PTX groups, respectively. BBM NHE3 protein abundance decreased 39.3 ± 8.2% in HP rats and increased 54.6 ± 7.8% in PTX rats. Immunohistochemistry showed that expression of NHE3 protein in apical BBM was decreased in HP rats and was increased in PTX rats. Northern blot analysis of total kidney RNA showed that the abundance of NHE3 mRNA was 20.3 ± 1.3% decreased in HP rats and 27.7 ± 2.1% increased in PTX.

Conclusions. Our results indicate that the chronic inhibitory effect of PTH on the renal proximal tubule NHE3 is associated with changes in the expression of NHE3 mRNA levels and protein abundance.

The multigene family of Na^+/H^+ exchangers (NHEs) in mammalian cells mediates the electroneutral, amiloride-sensitive exchange of intracellular protons for extracellular sodium, thereby playing an important role in the regulation of cell volume and intracellular pH. In polarized epithelia, NHEs are also involved in transepithelial $NaHCO_3$ and NaCl transport [1]. Using molecular biological approaches, six different NHE isoforms have been identified in mammalian tissues [2–7]. The various isoforms are functionally distinguished by their specific pharmacological properties, distinctive tissue distributions, and subcellular localization patterns. Four isoforms (NHE1 through NHE4) are present in plasma membrane of renal tubular cells [8]. Of these, NHE3, the least sensitive to amiloride and the most abundant isoform in renal tissue, is confined to the apical membranes of proximal tubule and thick ascending limb [9–11]. A variety of studies has documented that NHE3 is a major target for modulation of proximal tubular reabsorption of bicarbonate [11–14]. Its transport function is tightly regulated in response to acute as well as chronic perturbations.

It has long been known that parathyroid hormone (PTH) acts on kidney to alter urinary electrolyte and fluid excretion, leading to phosphaturia and, less consistently, bicarbonaturia [15–21]. In 1979, Iino and Burg reported inhibition of bicarbonate and fluid reabsorption by PTH (in vitro) in perfused rabbit proximal tubules [21]. Studies pursuing the PTH effect on Na^+-dependent H^+ secretion by renal epithelial cells have been performed in brush-border membrane (BBM) vesicles [22] and in various renal cell lines such as opossum kidney (OK cells) [22–25], LLC-PK1/PKE_{20} cells [26], and murine [27] and rabbit [28] proximal tubule cells. PTH inhibits the Na^+-dependent and amiloride-sensitive H^+ secretion present at the luminal membrane of these cells, where NHE3 is particularly abundant. The acute inhibitory effect of PTH on the luminal NHE has been attributed to activation of both protein kinase A (PKA) and protein kinase C (PKC) signaling cascades [24, 29]. PKA activation has an inhibitory effect on NHE3 that is attributed to Ser^{605} phosphorylation [30], in addition to an indirect mechanism involving recently identified inter-

Key words: Na^+/H^+ exchange, renal proximal tubules, sodium/hydrogen exchangers, cell volume regulation, intracellular pH, bicarbonate modulation.

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mediary proteins [31–33]. Inhibitory effects of PKC activation on NHE3 have also been shown [34–36], although this effect does not appear to be due to changes of the phosphorylation state of NHE3 [37].

In the present study, we demonstrated that long-term PTH modulation of sodium bicarbonate reabsorption at renal proximal tubules is correlated with significant changes in the amount of NHE3 protein abundance at the luminal membranes of proximal tubule and NHE3-specific message in total RNA of renal tissue. These results indicate that in addition to the acute inhibitory effect on functional luminal NHE activity, PTH also alters NHE3 gene expression, reducing NHE3 protein levels and thereby contributing to changes in $NaHCO_3$ reabsorption observed in chronic parathyroid dysfunctions.

METHODS

Experimental animals

Experiments were performed on male Wistar rats (180 to 220 g) with free access to water and standard laboratory chow until the moment of kidney removal. Three groups of animals were used: (*1*) in the HP group, 12 animals received interscapular subcutaneous (SC) implantation of slow-release PTH pellets, providing threefold basal levels of this hormone (2.1 $U \cdot h^{-1}$); (*2*) the sham group consisted of 44 animals that were submitted to a mock-surgery followed by SC implantation of placebo pellets; (3) in the PTX group, 26 animals were submitted to thyroparathyroidectomy under ether anesthesia, immediately followed by SC implantation of slow-release thyroxin pellets (1 μg/100 g body weight/day) in order to obtain physiological levels of T3 and T4. Eight days after these procedures, the animals were anesthetized with ether; the left carotid artery was cannulated for blood collection and they were sacrificed by heart incision. Kidneys from each animal were immediately removed for RNA extraction or BBM preparation.

Successful hyperparathyroidism or hypoparathyroidism in the HP or PTX groups was indicated by a significant increase in plasma Ca and a fall in plasma Pi levels or a fall in plasma Ca and an increase in plasma Pi levels, respectively, relative to values observed in control animals. In PTX animals, the success of thyroid hormone replacement was evaluated by testing T3 and T4 levels compared with those of the sham group.

Microperfusion experiments

In vivo microperfusion experiments were carried out essentially as described previously [36], including anesthesia and surgical preparation for microperfusion. Proximal tubules were perfused by means of double-barreled micropipettes: One barrel was filled with Sudan black-colored castor oil and the other with the luminal solution. Luminal perfusion solution had the following composition: 90 mmol/L NaCl, 25 mmol/L $NaHCO_3$, 1 mmol/L $MgSO_4$, 1 mmol/L $CaCl_2$, 4 mmol/L KCl, pH 7.8, plus raffinose to isosmolality. The rate of tubular acidification was measured by injecting a fluid droplet at a pH of 7.8 between the oil columns and following the pH changes toward the steady-state level (stationary perfusion). pH was measured by means of double-barreled microelectrodes, one barrel being filled with H^+-sensitive resin (Fluka) and the other with 1 mol/L KCl. pH changes were recorded with a Beckman model RP dynograph and digitized by a Dell 333D microcomputer equipped with an analog-to-digital conversion board (Lynx, São Paulo, Brazil) for data acquisition and processing. The rate of tubular acidification was evaluated by means of half-life of the injected bicarbonate. The net bicarbonate reabsorption (J_{HCO3}) was calculated from this equation:

$$J_{HCO3} = K\,[(HCO_3^-)_t - (HCO_3^-)_s]\,r/2$$

where K is the rate constant calculated from the exponential fitting of luminal bicarbonate decay. The subscripts t and s, appended to the bicarbonate concentration, indicate instantaneous and steady state concentrations, and r is the tubular radius. The bicarbonate concentrations were calculated from pH at time t and s and systemic PCO_2.

^{22}Na uptake studies

Uptake of ^{22}Na into the membrane vesicles was assayed at room temperature using a rapid filtration technique [38]. Briefly, BBM vesicles were washed and equilibrated for one hour at room temperature in 254 mmol/L mannitol, 35 mmol/L KOH, 68 mmol/L HEPES, and 50 mmol/L Mes, pH 6.0. The vesicles were then centrifuged and resuspended in the same medium at a final protein concentration of 10 μg/μL. Uptake experiments were then performed in triplicate by the addition of 10 μL of membrane suspension to 90 μL of experimental solution containing 4×10^5 cpm ^{22}Na, 300 mmol/L mannitol, 42 mmol/L KOH, and 80 mmol/L HEPES, pH 7.5. After incubation for 15 seconds at room temperature, the reaction was terminated by the rapid addition of 3.0 mL of an iced "stop solution" consisting of 300 mmol/L mannitol, 42 mmol/L KOH, and 80 mmol/L HEPES, pH 7.5. The mixture was immediately poured on a 0.65 μm Millipore filter and washed with an additional 9.0 mL of "stop solution." Filters were then placed in vials containing 3.0 mL Ready Solv HP (Beckman, Palo Alto, CA, USA) and counted by scintillation spectroscopy. Values for the nonspecific retention of ^{22}Na by the filters were subtracted from the values for the incubated samples. Some experiments were performed in the presence of 100 μmol/L 5-N-ethyl-N-isopropyl-amiloride (EIPA) to inhibit NHE3 activity.

Brush-border membrane preparation and Western blots

Immediately after kidney removal, cortexes were separated at 4°C and homogenized in HEPES buffer with the protease inhibitors pepstatin A, leupeptin, phenylmethylsulfonyl fluoride (PMSF) and K_2EDTA at 1 mmol/L each. Pools of BBM obtained from eight rats (equivalent amount from each animal) in each one of the described groups were prepared using a Mg^{2+} precipitation method [39]. Fifty micrograms of BBM proteins were solubilized in Laemmli sample buffer, and proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 7.5% polyacrylamide gels according to Laemmli [40]. For immunoblotting, proteins were transferred to polyvinylidene difluoride (PVDF; Immobilon-P; Millipore, Bedford, MA, USA) from polyacrylamide gels at 300 mA for 6 to 10 hours at 4°C with a Transphor transfer electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA) and stained with Ponceau S in 0.5% trichloroacetic acid. Entire sheets of PVDF membranes containing transferred protein were incubated first in Blotto [5% nonfat dry milk and 0.1% Tween 20 in phosphate-buffered saline (PBS), pH 7.4] for one to three hours to block nonspecific binding of antibody, followed by overnight incubation in primary antibody diluted 1:1.000 in Blotto (Monoclonal NHE3 antibody-2B9; a gift from D. Biemesderfer and P. Aronson, Yale University, Saybrook, CT, USA [11]; villin antibody was obtained from Immunotech, Narseilla, France). The membranes were then washed five times in Blotto and incubated for one hour with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (γ chain specific) from Zymed (San Francisco, CA, USA). Bound antibody was detected with enhanced chemiluminescence (ECL; Amersham, Arlington Heights, CA, USA) according to manufacturer's protocols. Hyperfilm-MP (Amersham) exposed to membranes for one to three minutes was developed in an x-ray film processor (100 Plus; All Pro Imaging Corp., Hicksville, NY, USA). The visualized bands were digitized using a scanner (Storm; Molecular Dynamics, Sunnyvale, CA, USA) and were quantitated by the ImageQuant program (Molecular Dynamics).

Tissue preparation for immunohistochemistry

Eight days after the initial surgery and/or pellet implantation, three rats from each group were anesthetized with chloral hydrate. After inserting a cannula into the descending aorta distal to renal arteries, the kidneys were fixed by retrograde perfusion, with PBS, pH 7.4 at 37°C, to remove blood, followed by PLP fixative containing 2% paraformaldehyde, 75 mmol/L lysine, and 10 mmol/L sodium periodate in phosphate buffer, pH 7.4 [41].

After initial fixation, kidneys were cut into 2 to 4 mm cubes and postfixed in PLP for four to six hours. For ultrathin cryosections, blocks of fixed tissues were sequentially cut from the cortex, and representative sections of this region were selected, preserving the orientation of the blocks. Blocks of tissue were cryoprotected by incubation for one hour in 2.3 mol/L sucrose in phosphate buffer (pH 7.2) with 50% polyvinylpyrrolidone, mounted on aluminum nails, frozen, and stored in liquid N_2 [42, 43]. Sections of 0.5 μm were cut using a Reichert Ultracut E ultramicrotome fitted with a FC-4E cryoattachment and mounted on gelatin-coated slides.

For staining, sections were washed sequentially, for 10 minutes each in PBS, 50 mmol/L NH_4Cl in PBS, and blocking buffer [1% bovine serum albumin (BSA) in PBS] to reduce background, followed by incubation for one hour with primary antibody diluted in 10% goat serum in PBS. After washing in PBS, sections were incubated for one hour with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Zymed) diluted in PBS containing 50% goat serum. Slices were then washed and mounted in 75% glycerol in PBS containing 0.1% p-phenylenediamine to inhibit photobleaching. Photomicrographs were taken with a Zeiss Axiophot microscope using either Tri-X (ASA 400) or T-Max (ASA 100) films.

Northern blots

Whole kidneys were homogenized with a Polytron in a denaturing solution of 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol. Sequentially, 0.1 volume of 2 mol/L sodium acetate, pH 4.0, 1 volume of water-saturated phenol, and 0.2 volumes of chloroform-isoamyl alcohol mixture (24:1) were added to the homogenate. Total RNA was isolated as previously described by Puissant and Houdebine [44] and resuspended in water treated with diethylpyrocarbonate. Absorbances at 260 and 280 nm were obtained to quantitate RNA and to assess its purity. The 260/280 ratios were 1.7 to 1.8.

Pools of 20 μg of total RNA from eight rats (2.5 μg from each) in the three groups, denatured in a sample buffer containing formaldehyde, were size fractionated in 1% agarose gel with 0.66 mol/L formaldehyde. After electrophoresis, gels were stained with SYBR®Green II (Molecular Probes, Eugene, OR, USA) and were laser scanned (STORM 840; Molecular Dynamics) in order to assess RNA integrity and to quantitate it by the ImageQuant program. Sequentially, gels were washed in 10 $\times$ standard saline citrate (SSC; 1 $\times$ SSC is 0.15 mol/L NaCl and 0.015 trisodium citrate, pH 7.0) and transferred to nylon membrane (Hybond N; Amersham) by capillarity, for 16 hours, in the same buffer. The RNA was immobilized by irradiation with ultraviolet light (UV-crosslinker; Amersham). At this point, the membranes were stained with methylene blue to verify the transfer

efficiency and to label the rRNA positions (28S and 18S). Prehybridization (4 hours at 65°C) and hybridization (18 hours at 65°C) of the RNA blots were performed with a buffer consisting of 5 × SSC, 25 mmol/L K_2PO_4, 5 × Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, and 0.1% BSA-fraction V), 50 μg/mL denatured salmon sperm DNA, and 50% deionized formamide containing 10% dextran sulfate. For cRNA probe synthesis, we used a partial-length cDNA of rat NHE3, obtained in our laboratory by polymerase chain reaction (PCR), using oligonucleotides for the cytoplasmic domain coding region, which is least conserved between the different isoforms of Na/H exchangers (sense, 5′-GTCATCTGGACATGGAACACA-3′; antisense, 5′-CCTTGTCCTGCTTCTCATCCT-3′). The obtained fragment was cloned in pBlueScript (KS+) and sequenced and linearized with *Hin*dIII endonuclease. pTRI 18 S rRNA from Ambion (Austin, TX, USA) was used as template for the 18S-RNA probe. We used T7 RNA polymerase from Ambion (StripEZ T7/T3 Kit) to synthesize cRNA probes labeled with ^{32}P-UTP (NEN-DuPont, Bad Homburg, Germany). After hybridization, the blots were washed twice for 30 minutes in 2 × SSC + 0.1% SDS at 65°C and twice for 15 minutes in 0.1 × SSC + 0.1% SDS at 65°C. The membranes were exposed to phosphor screen (Molecular Dynamics) for 24 hours and scanned using a STORM 840 (Molecular Dynamics). NHE3 mRNA levels and 18 S rRNA levels were quantitated using the ImageQuant program (Molecular Dynamics).

Materials

Chemicals used were purchased from Sigma (St. Louis, MO, USA). Reagents were from Life Technologies (Grand Island, NY, USA), unless otherwise specified. Slow-releasing T4 and PTH pellets were purchased from Innovative Research of America (Toledo, OH, USA).

Statistics

Data are expressed as mean ± SE unless otherwise specified. Statistical tests were performed using the unpaired Student's *t*-test or analysis of variance with the Tukey post-test if more than two groups were compared. $P < 0.05$ was considered statistically significant.

RESULTS

Animal selection for studies

We have analyzed 82 rats, 12 implanted with slow release PTH pellets, 44 sham-operated, and 26 PTX implanted with slow release T4 pellets. In all of them, T3 and T4 levels were measured by radioimmunoassay; rats were considered to possess normal thyroid function if serum T3 levels were 30.1 to 60.0 ng/dL (mean ± SD) and T4 levels were 0.88 to 2.38 mg/dL (mean ± SD). As shown in Table 1, T3 and T4 levels for all groups were well within these ranges and did not vary statistically between groups. Total calcium and phosphate serum levels were measured from blood collected from these three groups of rats. Serum levels of PTH were not evaluated, but animals were considered to exhibit low levels of PTH if serum calcium was below 1.97 mmol/L (mean − SD) and serum Pi was above 2.29 mmol/L (mean + SD). Animals were considered to have high levels of PTH if serum Ca was above 2.27 mmol/L (mean + SD) and serum Pi was below 1.77 mmol/L (mean − SD). As shown in Table 1, serum Ca and Pi levels in HP and PTX rats were significantly different from the control group. HP rats satisfied the criteria for hyperparathyroidism, and PTX rats met the criteria for hypoparathyroidism.

Table 1. Serum biochemical parameters evaluated in the experimental animals

	HP (N = 12)	Sham (N = 44)	PTX (N = 26)
T3 *ng/dL*	40.5 ± 3.0	45.1 ± 15.0	42.0 ± 11.0
T4 *mg/dL*	1.65 ± 0.54	1.63 ± 0.75	1.53 ± 0.46
Calcium *mmol/L*	2.47 ± 0.19[a]	2.12 ± 0.15	1.78 ± 0.26[a]
Phosphorus *mmol/L*	1.52 ± 0.15[a]	2.03 ± 0.26	3.08 ± 0.79[a]

T3 and T4 serum levels were measured to check the adequacy of thyroid hormone replacement in PTX rats. Calcium and phosphorus serum levels were measured in order to verify if HP and PTX animals met the criteria for hyperparathyroidism and hypoparathyroidism, respectively. Data are expressed as mean ± SD. N is the number of animals in each group.

[a] Significantly different when compared with sham operated group

Analysis of bicarbonate reabsorption and NHE3 activity

The chronic effect of PTH on bicarbonate reabsorption (J_{HCO_3}) in rat renal proximal tubules was evaluated by stationary microperfusion and continuous measurement of luminal pH. In vivo microperfusion experiments were conducted in individual proximal convoluted tubules (S2 segments) from HP (N = 9), sham (N = 8), and PTX (N = 9) rats. As indicated in Figure 1, J_{HCO3} was clearly inhibited in HP rats (2.06 ± 0.21 nmol · cm^2 · s^{-1}) when compared with sham rats (2.93 ± 0.17 nmol · cm^2 · s^{-1}). Stationary pH was also significantly higher in HP (6.97 ± 0.05) compared with Sham-operated rats (6.72 ± 0.08). J_{HCO3} (3.37 ± 0.19 nmol · cm^2 + s^{-1}) was increased and stationary pH (6.60 ± 0.05) was decreased in PTX rats, but these changes were not statistically significant (P = 0.055 and P = 0.051, respectively).

We next evaluated the NHE3 activity by measuring the proton-dependent uptake of ^{22}Na in BBM vesicles from HP (N = 5) rats, sham (N = 9), and PTX (N = 5). The results of these experiments are shown in Figure 2. Compared with the sham-operated group (9225 ± 505 cpm/mg), NHE3 activity was significantly decreased in HP rats (6400 ± 840 cpm/mg) and increased in PTX rats (12205 ± 690 cpm/mg, $P < 0.05$). There was no significant

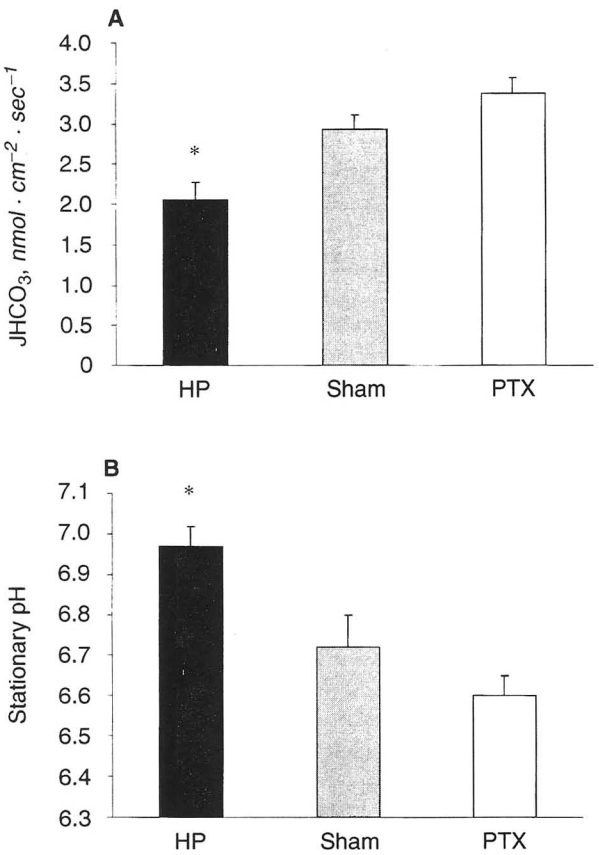

Fig. 1. Bicarbonate reabsorption and stationary pH in proximal tubules of hyperparathyroid (HP), control (sham), and parathyroidectomized (PTX) rats. The chronic effect of parathyroid hormone (PTH) on bicarbonate reabsorption in renal proximal tubules was evaluated by stationary microperfusion and continuous measurement of luminal pH. (*A*) Bicarbonate reabsorption (J_{HCO_3}). (*B*) Stationary pH. Arterial blood pH was not different between these groups (data not shown). Data are expressed as means ± SE. *Significantly different ($P < 0.05$) compared with sham-operated group.

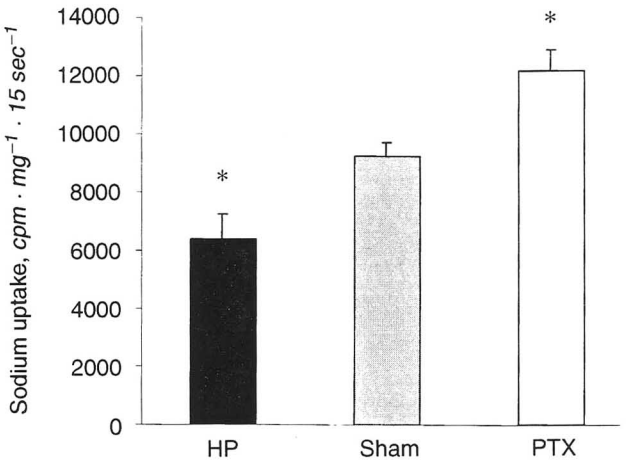

Fig. 2. Type 3 isoform of the Na^+/H^+ exchanger (NHE3) activity (cpm/mg) in brush border membrane (BBM) vesicles from HP, sham, and PTX rats. Na^+/H^+ exchange activity is the proton-dependent uptake of ^{22}Na after 15 seconds of incubation at room temperature. Data are expressed as means ± SE. *Significantly different ($P < 0.05$) compared with the sham-operated group.

difference among the three groups when BBM vesicles were assayed in the presence of 100 μmol/L EIPA.

Analysis of NHE3 protein expression

The monoclonal antibody to NHE3 (2B9) has been previously evaluated relative to its specificity for NHE3-protein in renal tissue [11]. This antibody was used in Western blots of BBM preparations from eight animals in each of the groups. As illustrated in Figure 3, the NHE3 monoclonal antibody recognized one band of approximately 80 kD in the BBM fraction from each group. Densitometric analyses, corrected to villin expression used as internal control, revealed a decrease of 39.3 ± 8.2% in NHE3 protein expression in HP animals and an increase of 54.6 ± 7.8% in PTX animals compared with the sham-operated group.

To analyze further the influence of these experimental maneuvers on NHE3 protein expression, another NHE3 monoclonal antibody (19F5), for which specificity for NHE3 has been previously demonstrated [11], was used for immunohistochemistry. Overviews of sections of the kidney cortex revealed NHE3 immunostaining at the BBM. Compared with control rats, HP rats showed a decrease, and PTX rats showed an increase in NHE3-specific staining (Fig. 4).

Analysis of NHE3 mRNA levels

Northern blot analyses of pooled RNA from each group (2.5 μg of total RNA per animal/8 animals per group) are shown in Figure 5. The same results were obtained when experiments were performed using RNA from each rat individually (20 μg per rat/lane). NHE3 mRNA signal corresponds to a unique band slightly above the 28S signal (at 5.6 kbp). In HP rats, we observed a reduction of 19% in a blot obtained from a pool of RNA and 20.3 ± 1.3% ($N = 8$) in blots from individual samples. The NHE3-specific band was stronger in the PTX group; an increase of 28% was observed in a blot obtained from a pool of RNA ($N = 8$) and 27.7 ± 2.1% ($N = 8$) in a blot obtained from individual samples. Both differences were significant compared with control rats (sham). When the blot was stripped and rehybridized with the 18S-cRNA probe, the signals were similar in all three groups, indicating minimal differences in RNA loading.

DISCUSSION

The acute inhibition of NHE type 3 isoform (NHE3) activity by PTH has been consistently reported by several

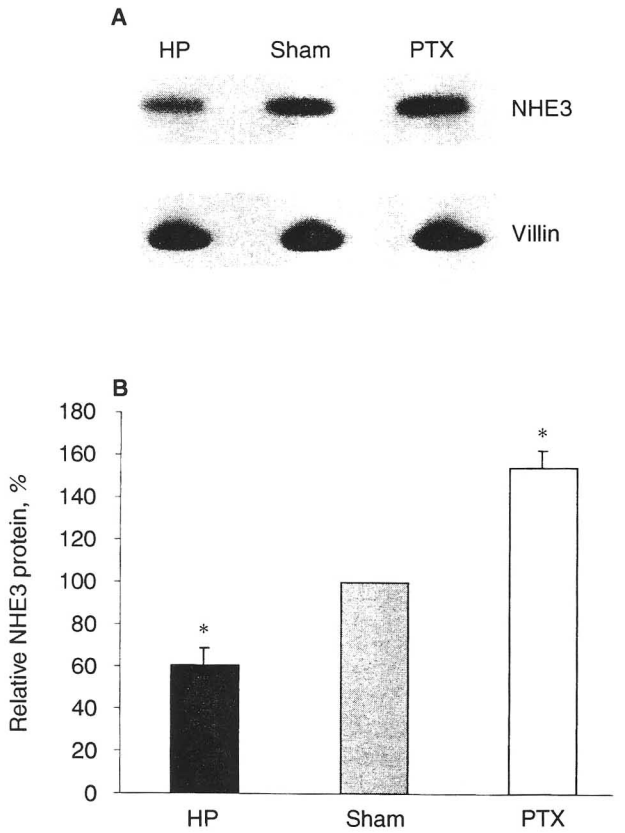

Fig. 3. Abundance of NHE3 and villin protein in BBM from HP, sham, and PTX rats. Western blot analyses were performed as described in the **Methods** section. The NHE3 monoclonal antibody recognized one band of 80 kD. Analyses of villin expression were used as an internal control. Blots were quantitated by densitometry, and the combined data from eight animals in each group are represented as columns in a bar graph. Data are expressed as means ± SE. *Significantly different ($P < 0.05$) compared with the sham-operated group.

laboratories in experiments using isolated BBMs [22, 23] and various cultured cell lines [23, 26–28]. However, long-term modulation of NHE3 by PTH has not been rigorously evaluated. The present study examined the chronic effect of PTH on NHE3 activity, protein expression, and specific mRNA abundance in rat renal proximal tubule. We found that PTH induced a decrease and parathyroidectomy induced an increase in NHE3 activity, NHE3 protein, and NHE3-mRNA levels.

A major fraction of proximal bicarbonate reabsorption is mediated by proton secretion through NHE3 [10–14]. By in vivo microperfusion analyses, we were able to demonstrate the chronic inhibitory effect of PTH on H^+ secretion at the apical membrane of renal proximal tubules. Proximal tubule bicarbonate reabsorption (J_{HCO3}) was significantly lower in rats that received high levels of PTH over eight days (HP) than in sham-operated rats. J_{HCO3} was increased in functionally PTX rats, although this increment was not statistically significant ($P = 0.055$). In vivo microperfusion analysis evaluates proximal tubule proton secretion function in a most physiological condition. However, it does not represent the whole population of proximal apical membranes as well as a BBM preparation. Amiloride-sensitive ^{22}Na uptake studies performed in renal BBM vesicles from our three experimental groups confirmed that NHE3 activity was decreased in HP and increased in PTX when compared with sham rats. The results obtained from both experiments indicate that PTH chronically inhibits NHE3 activity in rat renal proximal tubules.

Studies by Cano, Preisig, and Alpern have shown that chronic increases in cAMP levels for six hours led to the stimulation of luminal Na^+/H^+ exchange in opossum kidney (OK) cells when measured 16 to 20 hours after cAMP removal [45]. The authors suggested that hormones such as PTH, which acts on NHE3 at least in part by increasing cAMP levels, acutely inhibits NHE3, but chronically stimulates the activity of this transporter. The discrepancy between those observations and ours can be partially explained by the sustained modification in cAMP levels expected in our experiments as well as by other different experimental approaches used in each study. Consistent with our findings, recent studies from Fan et al showed that parathyroidectomized rats have increased apical NHE3 activity [46].

Western blot and immunohistochemical studies have suggested that changes in NHE3 protein abundance in renal proximal apical membrane play a significant role in long-term inhibition of NHE3 by PTH. Recent studies have been performed in PTX rats that received an intravenous bolus of PTH in order to examine the mechanisms by which PTH acutely inhibits NHE3. Both increase in NHE3 phosphorylation and redistribution of NHE3 transporter from BBM to endosomal stores seem to be involved. The authors did not observe changes in NHE3 protein abundance in apical membrane in response to acute PTH. However, a statistically nonsignificant decrease occurred 20 hours after the PTH bolus administration [46]. Our data strongly suggest that not only does PTH have acute effects on NHE3 activity, but also chronic effects on inhibiting renal proximal apical membrane Na^+/H^+ exchange via down-regulation of NHE3 protein expression.

Northern blot analysis of renal tissue total RNA made it evident that changes in protein expression occur concurrently with changes in NHE3 mRNA levels. These results suggest that the chronic effect of PTH on this NHE isoform is, at least in part, due to regulation of transcription or mRNA lifetime. Other modulators of NHE3 expression have also been found to work via regulation at the mRNA level. For example, chronic acidosis leads to an increase in NHE activity, which is associated with increased NHE3 mRNA levels in the thick as-

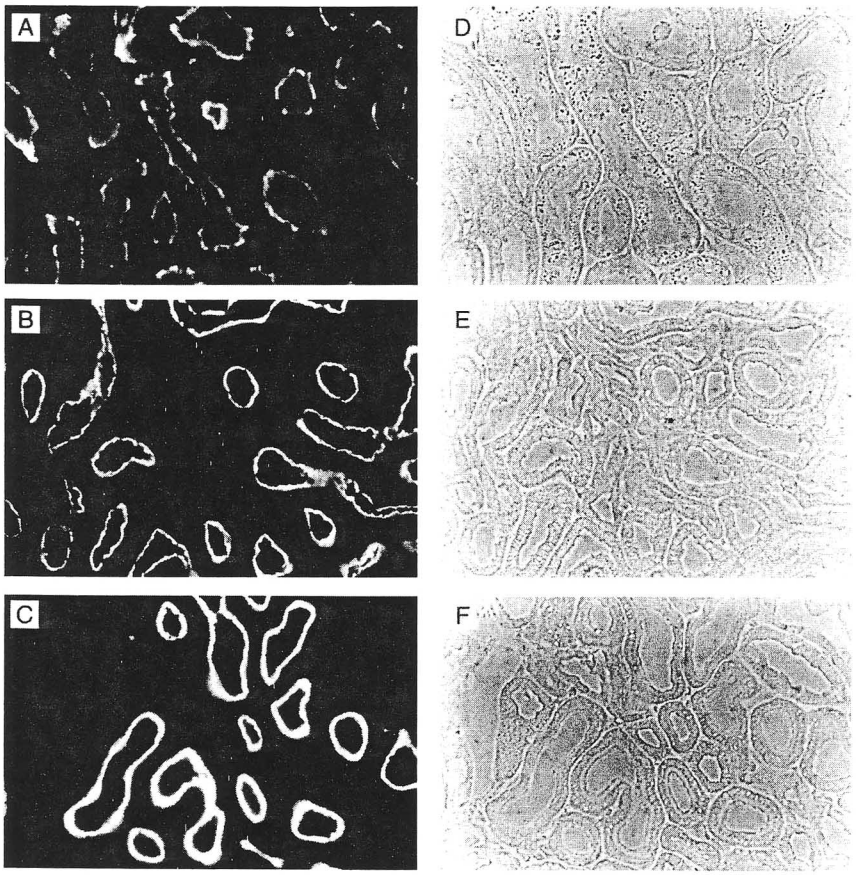

Fig. 4. Specific NHE3 protein immunofluorescence of renal proximal tubule from HP (*A*), sham (*B*), and PTX (*C*) rats. Semithin cryosections of rat kidney were labeled with mAb 19F5 (anti-NHE3). (*D*) Phase contrast image of A. (*E*) Phase contrast image of B. (*F*) Phase contrast image of C. Magnification ×400.

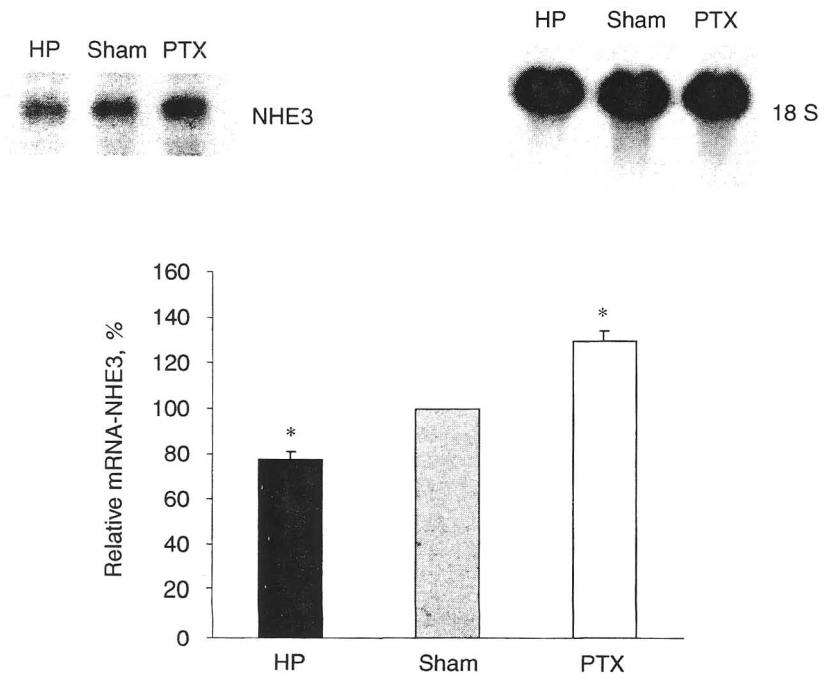

Fig. 5. Abundance of NHE3-mRNA and 18S rRNA in kidney tissue from HP rats, sham rats, and PTX rats. Northern blot analyses were performed as described in the **Methods** section. The NHE3 mRNA signal corresponds to a unique band slightly above the 28S signal at 5.6 kb. When the blot was stripped and rehybridized with 18S-cRNA probe, the signal was similar in all three groups, indicating minimal differences in RNA loading. The abundance of NHE3 mRNA relative to 18S RNA was quantitated by densitometry. Combined data from eight animals in each group are represented as columns in a bar graph. Data are expressed as means ± SE. *Significantly different ($P < 0.05$) compared with sham-operated group.

cending limb in rat [47] and in OKP cells, a renal proximal tubule cell line [48]. Glucocorticoids chronically up-regulate proximal tubule apical Na^+/H^+ exchange activity. This up-regulation is associated with an increase in the steady-state NHE3 mRNA abundance that has been shown to be due to transcriptional activation [49]. To elucidate the mechanisms underlying the chronic effects of PTH on NHE3 mRNA level demonstrated in this study, further examination of the influence of our experimental maneuvers on NHE3 mRNA half-life and transcriptional rates will be necessary, in addition to the use of promoter-reporter gene constructs.

The inhibition of bicarbonate reabsorption by PTH was one of the first pieces of evidence of the hormonal regulation of ionic transport in renal proximal tubules that was reported. However, the physiological significance of this control is not completely clear. Hyperparathyroidism is not usually associated with metabolic acidosis, since H^+ secretion in the distal nephron is increased in this condition [50]. Whether PTH is involved in a systemic acid-base balance as a consequence of regulation of H^+ and HCO_3^- transport in gut and bone cells remains to be clarified. The inhibitory effect of PTH on luminal NHE in proximal tubule cells could be an additional mechanism by which PTH maintains calcium homeostasis. Previous studies have reported that transcellular calcium reabsorption in distal tubule is stimulated by luminal bicarbonate [51, 52]. Calcium channel conductance could also be sensitive to the changes in luminal pH induced by PTH. So far, the mechanisms of calcium transport at the luminal membrane of proximal tubules are not well known and still need to be further elucidated.

In summary, our present studies show that parathyroidectomy or high levels of PTH in rats over eight days induces significant changes in the amount of NHE type 3 at the luminal membrane of proximal tubule cells. The parallel changes observed in NHE3-specific mRNA suggest control at the transcriptional level or on mechanisms that define the stability of this message. These results strongly suggest that changes in NHE3 protein abundance may have a relevant role on the inhibition of proximal tubule bicarbonate reabsorption by PTH.

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Passamos em seguida à apresentação e discussão dos resultados parciais dos projetos relacionados a permutadores Na^+/H^+ que estão sendo executados em nosso laboratório no momento (abril/2001).

Clonagem e identificação do permutador Na/H existente em guelras posteriores de *Casmagnathus granulata.*

Douglas Vasconcelos Cancherini* e Nancy Amaral Rebouças

(* - aluno de Iniciação Científica)

Introdução:

A capacidade de vários crustáceos de habitar meios aquáticos com salinidades bastante variadas (há espécies que sobrevivem tanto no mar quanto em água doce) certamente depende de sua habilidade de, quando em meios pouco concentrados, evitar a diluição do meio interno pela entrada de água ou perda de íons. Essa habilidade deriva de características tais como um tegumento pouco permeável à água e a íons, glândulas especiais nas antenas para eliminar o excesso de água, produção de urina diluída (hipoosmolar) e captação ativa de íons do meio externo [94], este o processo que aqui nos interessa. Em muitas espécies, ele é realizado principalmente num epitélio especializado presente nas guelras posteriores do animal, com abundantes microvilosidades na face voltada para o meio externo (mucosa) e invaginações da membrana basolateral, entre as quais há abundantes mitocôndrias. Aspecto este, característico de todo epitélio cuja função primeira seja o transporte de íons.

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Promoter Paper

Phylogenetically conserved sequences in the promoter of the rabbit sodium-hydrogen exchanger isoform 1 gene (*NHE1/SLC9A1*) ☆

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Abstract

NHE1 (gene symbol *SLC9A1*) encodes an isoform of the amiloride-sensitive Na^+-H^+ exchanger that is present in many cells and the basolateral membrane of renal epithelia. Expression of *NHE1* is modulated in response to chronic metabolic acidosis, growth factors, and phorbol esters. To begin examining the molecular basis for this regulation, a rabbit genomic clone that contained 6 kb of 5′ flanking region, the first exon, and a portion of the first intervening sequence of *NHE1* was obtained. The principal transcription start site in native rabbit tissues was located 798 bp 5′ to the initiation codon. The sequence of the proximal 5′ flanking region of rabbit *NHE1* was similar to the human sequence and contained a TATA-box, (G + C)-boxes, homopyrimidine direct repeats, and a putative AP-1 site. When ligated to luciferase and transfected into porcine renal epithelial cells (LLC-PK_1), 708 bp of proximal 5′ flanking region exhibited orientation-dependent promoter activity.

Keywords: Sodium-hydrogen antiporter; Carrier protein; Promoter; (LLC-PK_1 cell)

Na^+/H^+ exchangers are integral plasma membrane proteins that mediate the electroneutral antiport of Na^+ and H^+ and are important for the control of intracellular pH, cell volume, response to mitogenic stimuli, and transepithelial flux of Na^+ and HCO_3^- [1,2]. To date, five mammalian genes that encode isoforms of Na^+-H^+ exchangers with distinct tissue distributions and kinetic properties have been identified (designated *NHE1-NHE5*) [3,4]. *NHE1* (*SLC9A1*) encodes the growth factor-activatable Na^+-H^+ exchanger that was originally cloned from human genomic DNA [5]. *NHE1* is expressed in many cell types, although the levels of expression vary in different tissues. In the rabbit, *NHE1* transcripts are most abundant in stomach, with lower levels in brain, renal medulla, lung, ileum, and renal cortex [6]. In the rabbit kidney, NHE1 protein has been immunolocalized exclusively to the basolateral membrane of tubule epithelial cells [7]. Expression of the *NHE1* gene is stimulated by mitogenic growth factors [8], phorbol esters [9,10], retinoic acid [11], and chronic metabolic acidosis [12–15]. However, the mechanisms involved in regulation of *NHE1* gene expression remain poorly understood. The present study was undertaken to clone the rabbit *NHE1* gene promoter and identify phylogenetically conserved sequences that may have functional significance

Cloning of the rabbit NHE1 gene. A rabbit genomic library was screened with a restriction fragment containing the 5′ end of the rabbit renal *NHE1* cDNA [6], and five positive clones were obtained. Two clones contained an identical 17-kb insert (designated λRNH2) and were selected for further study. Fig. 1 shows the restriction map of λRNH2. Southern blot analysis revealed that the lengths of *Hin*dIII, *Pvu*II, and *Bgl*II restriction fragments that hybridized to the *NHE1* cDNA were identical in λRNH2 and rabbit genomic DNA, verifying the authenticity of the clone (not shown). A 2.7-kb *Sac*I restriction fragment of λRNH2 that hybridized to the cDNA probe was subcloned and found to contain a sequence identical to the 5′ end of the rabbit renal *NHE1* cDNA (nucleotides 1–1077) [6]. Beyond nucleotide 1077 (nucleotide 351 of the coding region), the sequence of λRNH2 diverged from the cDNA and contained a consensus splice donor site (5′-

☆ The nucleotide sequence data reported in this paper have been submitted to the EMBL/GenBank database under the accession number U21015.

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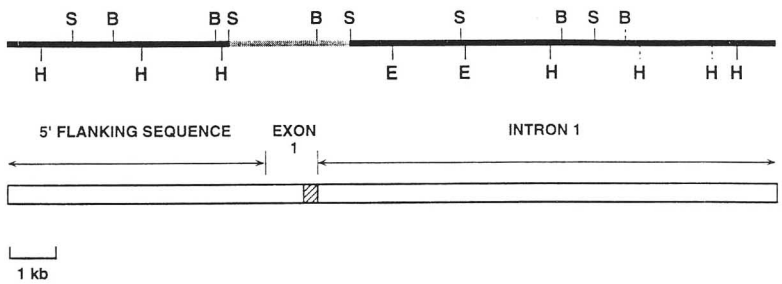

Fig. 1. Partial restriction map and structural organization of genomic clone λRNH2. λRNH2 was isolated by screening a rabbit genomic library in *EMBL3* (Clontech, Palo Alto, CA) with a ^{32}P-labeled, 1-kb *Bgl*II restriction fragment derived from the 5′ end of the rabbit renal *NHE1* cDNA [6]. Hybridization was performed at 68° C in Church-Gilbert medium [21] containing 100 μg/ml denatured salmon sperm DNA and radiolabeled probe (10^6 cpm/ml; $4 \cdot 10^9$ cpm/μg). The final wash was for 1 h at 68° C in 1 × SSC containing 0.5% SDS. (Upper) Restriction map of λRNH2 generated by digestion with various restriction endonucleases. Vertical bars indicate positions of *Sac*I (S), *Bgl*II (B), *Hin*dIII (H), and *Eco*RI (E) restriction sites. Dashed bars indicate positions for which the order was not determined unambiguously. Gray line indicates the *Sac*I restriction fragment that contained sequences identical to the 5′ end of the *NHE1* cDNA. (Lower) Arrows indicate the extent of the 5′ flanking sequence, first exon, and portion of the first intron. Hatched box depicts the coding region within the first exon.

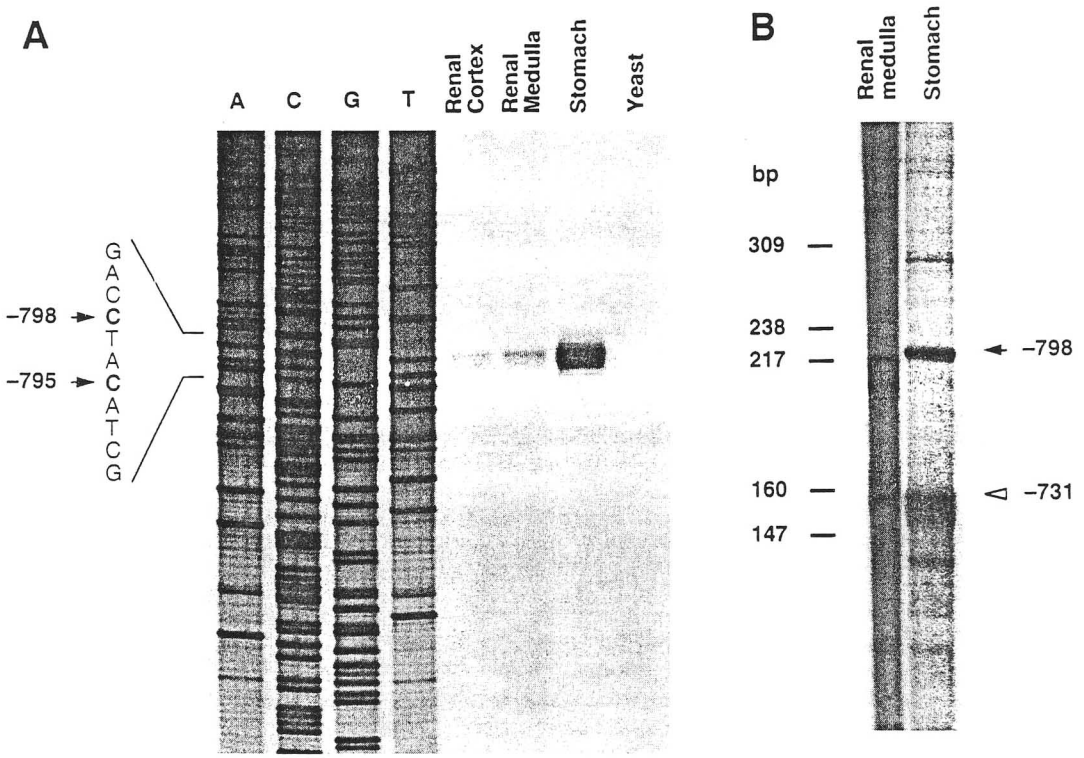

Fig. 2. (A) Mapping of *NHE1* transcription initiation sites by ribonuclease protection assay. A 745-bp *Taq*I restriction fragment of λRNH2 (nucleotides −1375 to −631 with respect to the initiation codon) was subcloned into pBluescript II, and an antisense riboprobe was transcribed using T7 RNA polymerase and [α-^{32}P]UTP [22]. Gel-purified riboprobe ($5 \cdot 10^5$ cpm) was annealed to 100 μg RNA from rabbit renal cortex, renal medulla, or stomach, or 50 μg yeast tRNA as a negative control. Total RNA was extracted from rabbit tissues using acid guanidinium thiocyanate-phenol-chloroform extraction [23]. Following digestion with RNase A (40 μg/ml) and RNase T1 (2.2 μg/ml), products were resolved on 6% polyacrylamide sequencing gels. An autoradiogram of a representative gel is shown. Lanes labeled A, C, G, T contain sequencing ladders for size calibration. Arrows depict the positions of protected fragments and the corresponding start sites with respect to the initiation codon. (B) Mapping of *NHE1* transcription initiation sites by primer extension. An antisense oligonucleotide complementary to the 5′ untranslated region of the rabbit renal *NHE1* cDNA (nucleotides 134–161) was end-labeled with [γ-^{32}P]ATP ($9 \cdot 10^7$ cpm/μg), annealed to 100 μg RNA from rabbit stomach or renal medulla, and elongated with 200 units RNase H-free M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD). Products were analyzed on 6% polyacrylamide sequencing gels, and a representative autoradiogram is shown. Positions of molecular weight standards are indicated on the left. Arrows denote the major products and corresponding start sites.

TAG/GTAAGT-3′). Moreover, the full-length *NHE1* cDNA did not hybridize to any restriction fragments of λRNH2 that were 3′ to nucleotide 1077. This analysis demonstrated that λRNH2 contained at least 726 bp of 5′ untranslated region, 351 bp of coding region, and a portion of the first intervening sequence, which was > 10 kb (Fig. 1).

Mapping of transcription initiation sites. To identify the transcription initiation site, ribonuclease protection assays were performed. Two protected fragments were detected in samples containing RNA from rabbit tissues but were absent from the negative control containing yeast tRNA (Fig. 2A). Importantly, the autoradiographic densities of the protected fragments corresponded to the known relative abundance of the *NHE1* transcripts in stomach, renal medulla, and renal cortex [6]. The lengths of the protected fragments indicated that transcription initiation sites were located 798 bp and 795 bp 5′ to the translation initiation codon. To confirm these results using an independent method, primer extension analysis was performed. As shown in Fig. 2B, the abundance of primer extension product from stomach was greater than renal medulla, consistent with the relative expression of *NHE1* in the two tissues. The lengths of the products also corresponded to a transcription start site at −798. The resolution of the assay was insufficient to exclude the presence of the second transcription start site at −795. The band corresponding to a start site at −731 was not observed in the ribonuclease protection assays and likely represented premature termination of reverse transcription in a (G + C)-rich region. Taken together, these studies agreed that the principal transcription initiation site was located 798 bp 5′ to the translation initiation codon and was identical in kidney and stomach. Therefore, genomic clone λRNH2 contained approx. 6 kb of 5′ flanking region (Fig. 1).

Sequence of the proximal 5′ flanking region of the rabbit NHE1 gene. The sequence of λRNH2 upstream from the transcription initiation site was obtained (Fig. 3). The proximal upstream sequence was (G + C)-rich and contained 14 CpG dinucleotides indicating that the 5′ end of the gene was located in a CpG island. Typical eukaryotic promoter elements were identified including a TATA

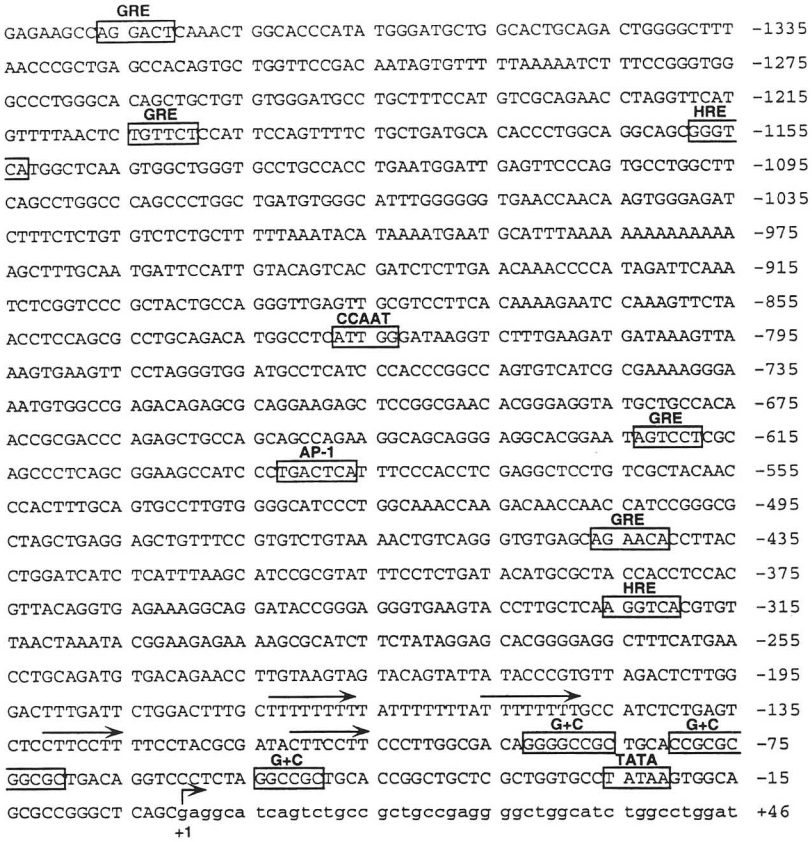

Fig. 3. Sequence of the proximal 5′ flanking region of the rabbit *NHE1* gene. Restriction fragments of λRNH2 were subcloned into pBluescript II and sequenced using the dideoxynucleotide chain termination method as described previously [6]. Nucleotide positions are numbered on the right. The major transcription initiation site is indicated by the bent arrow at +1, and lower case letters represent transcribed sequences. Labeled boxes enclose the putative TATA box; AP-1 site; half-sites for receptors for glucocorticoids or progesterone (GRE); half-sites for receptors for thyroid hormone, vitamin D, or retinoids (HRE); CCAAT box; and (G + C)-boxes. Arrows indicate homopyrimidine direct repeats that could form triple helical DNA H.

tor half-sites, and a cAMP response element that were identified in the human sequence were absent in the rabbit. Recently, four elements that interacted with nuclear extracts from rat liver and were required for transcription in several cell types were identified in the 5′ flanking region of the human *NHE1* gene [20]. Comparison between the human and rabbit sequences revealed that elements A (−3 to −25), B (−59 to −102), and C (−105 to −118) were 96%, 80%, and 86% identical, respectively. Element D (−220 to −243), which contained a consensus C/EBP binding site in the human gene and appeared to be essential for transcription in HepG2 and VSM A7r2 cells, was less highly conserved in the rabbit (67% identity) and lacked the C/EBP site.

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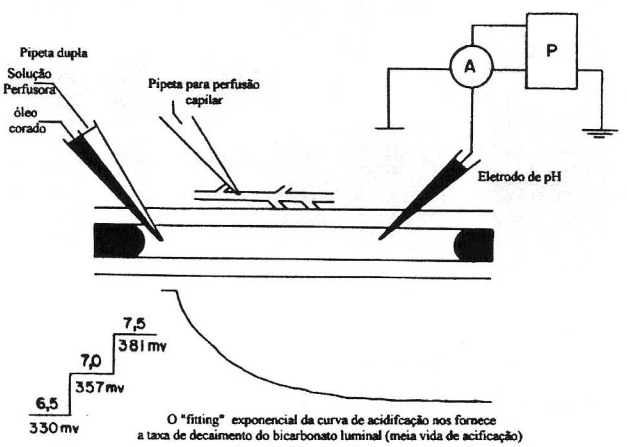
Trabalhos desenvolvidos no ICB-USP, após o estágio de pós-doutorado.

Regulação do Permutador Na^+/H^+ por Proteína Quinase C em Túbulos Proximais Ratos.

Nosso propósito ao iniciar esse estudo era avaliar o efeito que ativadores (PKC) teriam sobre a taxa de secreção de H^+ dependente de Na^+ em túbulos proximais de ratos, *in vivo*. Os dados disponíveis na literatura relativos ao efeito da ativação de PKC sobre a reabsorção de fluido em túbulos proximais eram muito contraditórios [11;72;126]. Embora os primeiros estudos de expressão da proteína recombinante NHE-3 em alguns sistemas heterólogos já evidenciasse que essa proteína tinha sua atividade inibida quando da ativação de PKC, os efeitos dessa ativação ou inibição sobre a secreção de H^+ em túbulos proximais *in vivo* não era clara.

Nesse trabalho, empregamos a técnica de medida contínua de pH intratubular com eletrodo sensível a íons H, em gota de fluido isolada com óleo (microperfusão estacionária), para avaliarmos o fluxo de H^+ e a meia vida de acidificação em túbulos proximais de ratos.

Um esquema ilustrando a técnica utilizada é reproduzido abaixo:



A partir dos valores de meia vida de acidificação (K), tendo a valor medido do raio tubular (r) e os valores de concentração luminal de HCO_3^- no momento inicial, t, e no estado estacionário, s, calculamos o fluxo de HCO_3^-, utilizando a seguinte equação:

$$J_{HCO3}^- = K[(HCO_3^-)_t - (HCO_3^-)_s]\ r/2$$

A concentração de HCO_3^- era calculada com base na equação de Henderson Hasselbalch, tendo os valores medidos de pCO_2 no sangue arterial, e o valor do pH luminal nos momentos acima referidos.

O ativador de PKC, o ester de forbol "phorbol-12-myristate-13-acetate" (PMA) ou o 1,2-dioctanoyl-sn-glycerol (DOG), foi adicionado tanto à luz tubular perfundida com solução tamponada com bicabonato, como em capilares peritubulares, em situação nominalmente livre de bicarbonato, quando a luz tubular e os capilares eram perfundidos com solução tamponada com fosfato (situação em a concentração de fosfato substituia a de HCO_3^- na equação acima, e calculávamos fluxo de H^+, ao invés de fluxo de HCO_3^-). O inibidor de fosfatases 1 e 2A, ácido ocadáico, e os inibidores de PKC, H7, e PKA, H89, utilizados em associação com PMA, foram adicionados apenas no lado luminal. HMA, 5-(N,N-hexamethylene)-amiloride, em concentração suficiente (1 mM) para inibir a forma de permutador Na^+/H^+ mais resistente a amiloride, NHE-3, foi utilizado no fluido luminal para avaliar o efeito da ativação de PKC sobre a secreção de H^+ não dependente de Na^+.

Original Paper

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Regulation of Rat Proximal Tubule Na/H Exchange by Protein Kinase C

Key Words
Protein kinase C
Bicarbonate
Proximal tubule
Phorbol esters
Na^+/H^+ exchange

Abstract
The effect of protein kinase C (PKC) activation on fluid and bicarbonate transport in renal tubules has been discussed controversially. Stimulation and inhibition have been shown to depend on factors such as experimental model and exposure time to the mediator of enzyme activation. We studied the role of PKC activation by phorbol-12-myristate-13-acetate (PMA) and by 1,2-dioctanoyl glycerol (DOG) in proximal bicarbonate reabsorption ($J_{HCO\bar{3}}$) by 'in vivo' stationary microperfusion and ion-exchange resin microelectrode determination of luminal pH. Both PMA (10^{-8} mol/l) and DOG (10^{-3} mol/l) added to lumen or to peritubular capillaries reduced the net $J_{HCO\bar{3}}$ significantly. When added to lumen, the inhibition was 44 and 32%, respectively. This reduction did not involve changes in lumen stationary pH, but was mediated by a marked increase in the halftime of luminal bicarbonate disappearance; from 4.22±0.23 to 6.27±0.51 s with PMA and from 3.90±0.25 to 6.33±0.48 s with DOG. This effect was intensified by 10^{-6} mol/l okadaic acid, a phosphatase inhibitor (inhibition of $J_{HCO\bar{3}}$ increased to 61%), and reduced by 30% by 10^{-6} mol/l H7, an inhibitor of PKC. H89, a protein kinase A inhibitor, did not affect the inhibitory action of PMA. Our data suggest that PKC activation reduces the rate of H ion secretion (bicarbonate reabsorption) in convoluted segments of rat renal proximal tubules and that phosphorylation of the Na^-/H^+ exchanger by this kinase is the cause of the reduction in net secretion of H ions.

Introduction

Most of the proximal bicarbonate reabsorption occurs by H^+ secretion through Na^+/H^+ exchanger activity, present in the luminal membrane [1]. The transport rate of the exchanger can be modified under several physiological or pathological conditions, such as changes in acid-base status and extracellular volume, hormone stimulation, in an adaptive process, or as consequence of an endocrine or hydroelectrolyte disturbance [2, 3]. The intracellular mechanisms responsible for modification of the exchanger transport rate involve several pathways of cellular signaling, including kinase and phosphatase activation, and changes in intracellular calcium levels. Both activation and inhibition of Na^+ reabsorption and H^+ secretion in proximal renal tubules by protein kinase C (PKC) activation have been described [4–8].

Molecular biology techniques identified a family of Na^+/H^+ exchangers (NHE) [9]. NHE-2, NHE-3, and NHE-4 are typical of epithelial tissues. NHE-3 is the most abundant in brush border membrane of rabbit proximal tubules [10]. Amino acid sequences in the COOH-terminal portion of the exchangers were recognized as putative consensus sequences for tyrosine kinase, calmodulin kinase II, PKC, and

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cyclic adenosine monophosphate dependent phosphorylation, suggesting that these protein kinases might play a role in modulating the activity of the exchangers [see ref. 3 for a review]. Levine et al. [11] have shown, in fibroblasts devoid of Na^+/H^+ exchanger activity and transfected with NHE-3 cDNA (PS120/NHE3), a decrease in Na^+/H^+ exchanger activity in the presence of phorbol esters. The same cells transfected with NHE-1 and NHE-2 cDNA (PS120/NHE-1 and PS120/NHE-2) showed stimulation of Na^+/H^+ exchanger activity by these agents as compared with untreated control cells. In this cell line, Levine et al. [11] did not observe a modification of any Na^+/H^+ exchanger activity in response to protein kinase A (PKA) activation.

Inhibition of Na^+/H^+ exchanger activity by PKA and PKC, on the luminal and basolateral side, has been observed in different cell lines [12–15]. On the other hand, Mrkic et al. [16] using a rabbit proximal tubule cell line (RKPC-2), observed that phorbol esters caused significant stimulation of both luminal (identified as NHE-2) and basolateral (NHE-1) exchangers, and both were also inhibited by 8-Br-cyclic adenosine monophosphate. These studies raise the possibility that in addition to the nature of the NHE isoforms, NHEs may be associated with other NHE-specific kinase(s) or regulatory protein(s) which add to the diversity in the regulation of Na^+/H^+ exchangers in different cell types and even in different cellular domains in the same cells.

In the present paper, our purpose was to analyze the integrated response of the Na^+/H^+ exchange system 'in vivo' to PKC activation by phorbol esters, measuring acidification kinetics of rat proximal tubules. PKC stimulation resulted in reduced rates of H^+ secretion, and this effect was potentiated by phosphatase inhibition. Furthermore, we present evidence that the PKC effect is a direct one and not mediated by PKA due to a 'cross-talk' between signaling pathways.

Materials and Methods

Male Wistar rats weighing 200–250 g with free access to water until the time of experiments and to standard laboratory chow (Nuvital) until 12–16 h before experiments were anesthetized with inactin (100 mg/kg i.p.) and prepared according to standard micropuncture techniques [17]. During the experiments, all rats received an infusion of 3% mannitol in 0.9% NaCl at a rate of 0.05 ml/min via a jugular vein. The carotid artery was cannulated for blood collection.

Proximal tubules, mainly S2 segments, were perfused by means of double-barreled micropipettes: one barrel was filled with Sudan (Black-)colored castor oil and the other with the control solution of the specific condition. Another single-barreled micropipette was used to perfuse the tubule with the experimental solution. Luminal perfusion solutions had a composition geared to avoid volume and concentration changes during perfusion, i.e., they contained equilibrium concentrations of the main ions: 90 mmol/l NaCl, 25 mmol/l $NaHCO_3$, 1 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$, 4 mmol/l KCl, pH 7.8, plus raffinose, a solute to which the proximal tubule epithelium presents low permeability, to isosmolality [18]. To this solution different drugs were added, specified for each experimental condition: phorbol-12-myristate-13-acetate (PMA; also referred as 12-o-tetradecanoylphorbol-13-acetate – TPA) from Pharmacia; okadaic acid from Gibco-BRL; 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H7) and N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) from Seikagaku and 1,2-dioctanoyl-sn-glycerol (C8:0; DOG) and 5-(N,N-hexamethylene)-amiloride (HMA) from Sigma. All drugs were dissolved in DMSO diluted to $< 0.01\%$. In some experiments in which we have used HMA, Na^+ was removed from luminal solution (90 mmol/l N-methyl-*D*-glucamine, 25 mmol/l $KHCO_3$, 1 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, raffinose to isosmolality).

When specified, peritubular capillary perfusion was performed with a single-barreled micropipette filled with a solution containing 125 mmol/l NaCl, 10 mmol/l Na_2HPO_4/NaH_2PO_4, 4 mmol/l KCl, 1.25 mmol/l $CaCl_2$, 0.5 mmol/l Na_2SO_4, 0.5 mmol/l $MgCl_2$, and 5 mmol/l glucose, pH 7.4. In those experiments in which capillaries were perfused, luminal perfusions were also made without bicarbonate (125 mmol/l/l NaCl, 10 mmol/l Na_2HPO_4, 4 mmol/l KCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgSO_4$, 80 mmol/l raffinose, pH 7.8).

The rate of tubular acidification was measured by injecting a fluid droplet at pH 7.8 between the oil columns and following the pH changes toward the steady state level (stationary perfusion). pH was measured by means of double-barreled microelectrodes, one barrel being filled with H^+-sensitive resin (Fluka) and the other with 1 mol/l KCl. pH changes were recorded with a Beckman model RP dynograph and digitized by means of a Dell 333D microcomputer equipped with an analog-to-digital conversion board (DT 2801) for data acquisition and processing.

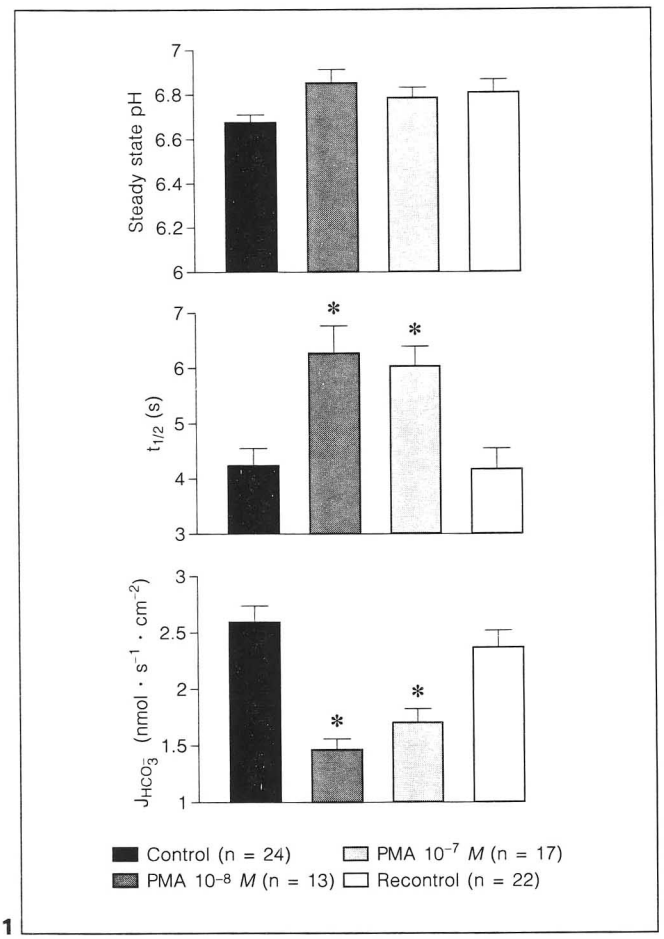
The rate of tubular acidification was evaluated by means of the half-life of injected bicarbonate. The net bicarbonate reabsorption ($J_{HCO_3^-}$) was calculated from the equation [17]:

$$J_{HCO_3^-} = K\,[(HCO_3^-)_t - (HCO_3^-)_s]\,r/2 \qquad (1)$$

where K is the rate constant calculated from the exponential fitting of luminal bicarbonate concentration decay; the subscripts t and s, appended to the bicarbonate concentration, indicate instantaneous and steady state concentrations; r is the tubular radius. The bicarbonate concentrations were calculated from pH at time t and s and systemic PCO_2. Data from our laboratory have shown that the renal cortical PCO_2 in our rats is equal to the systemic arterial PCO_2 [19]. $J_{HCO_3^-}$ given in the tables was calculated from K ($K = \ln 2/t_{1/2}$, where $t_{1/2}$ is the half-life of luminal bicarbonate); $[HCO_3^-]_t$ = initial (25 mmol/l) and $[HCO_3^-]_s$ = stationary bicarbonate concentrations.

Arterial blood pH and PCO_2 were measured approximately every hour by means of an IL model 113 pH/blood gas analyzer.

Averages of different experimental groups were compared using t test for unpaired data and analysis of variance with the Bonferroni contrast test when more than two groups were compared. Data are presented as mean ± SEM.



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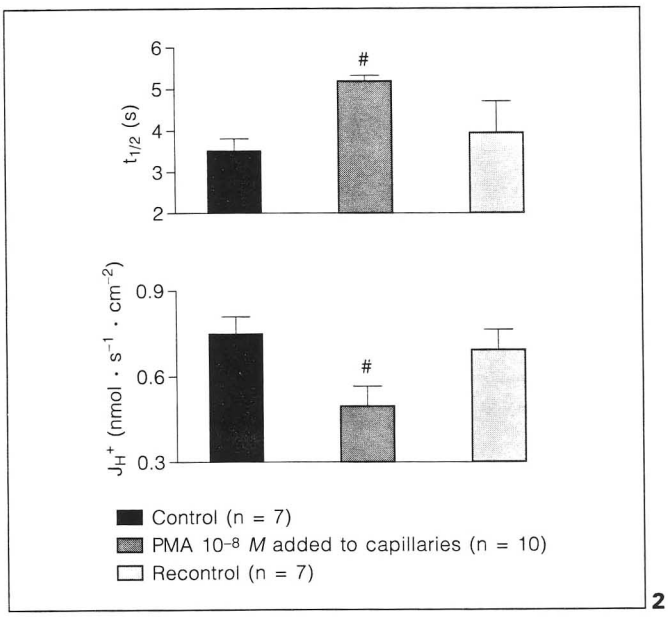

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Fig. 1. Effects of PMA, 10^{-7} and 10^{-8} mol/l, added to lumen on steady state pH, acidification halftime ($t_{1/2}$), and bicarbonate reabsorption ($J_{HCO_3^-}$) during luminal perfusion with bicarbonate Ringer solution. *$p<0.001$ as compared with control.

Fig. 2. Effects of PMA 10^{-8} mol/l added to peritubular capillaries on acidification halftime ($t_{1/2}$) and secretory hydrogen flux (J_H+) during luminal and capillary perfusion with phosphate Ringer solution. #$p<0.05$ as compared with controls.

Results

Microperfusion experiments were conducted in 68 proximal convoluted tubules (S2 segments) of 25 rats. The mean arterial blood acid-base values were pH 7.34 ± 0.01, PCO_2 38.63±1.28 mm Hg, and HCO_3^- 20.27±0.66 mmol/l. Each tubule was perfused first with control solution, then with the experimental solution (drugs added) and finally with the control solution again.

The addition of PMA to luminal perfusate induced a significant increase in the halftime of acidification, without change in steady state pH, with a reduction in the calculated net bicarbonate flux. The full inhibitory effect of PMA was attained within <1 min (fig. 1). The inhibitory effect of PMA on H^+ secretion was greater at a concentration of 10^{-8} than at 10^{-7} mol/l. A similar decrease in the inhibitory effect on H ion secretion, with a higher concentration of PMA, was previously reported [20]. In all the following experiments we have used PMA at a concentration of 10^{-8} mol/l.

The same inhibitory effect on H ion secretion was observed when PMA was applied to the basolateral surface, through capillary perfusion. The results are shown in figure 2. Here, a nominally bicarbonate-free perfusion system was used, and J_H was calculated substituting $[Na_2HPO_4]_t$ and $[Na_2HPO_4]_s$ for the bicarbonate concentrations of equation 1. The smaller value of J_H as compared with previous results of $J_{HCO_3^-}$ (fig. 1) is due to the lower buffering capacity of the perfusion solution.

The following results were obtained with drug application to the luminal compartment, since this represents a considerable technical simplification. Okadaic acid potentiates the effect of PMA. The results are shown in figure 3. PMA alone induces a 44% reduction in bicarbonate reabsorption. Its inhibitory effect is increased to 61% with simultaneous inhibition of cell phosphatases with okadaic acid. These results further support the view that protein phosphorylation by PKC, stimulated by PMA, is the event leading to a reduction in H ion secretion.

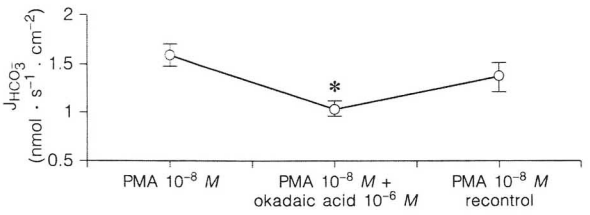

Fig. 3. Effect of okadaic acid 10^{-6} mol/l on PMA (10^{-8} mol/l) induced inhibition of bicarbonate reabsorption in proximal tubules. Both agents were added to lumen during perfusion with bicarbonate Ringer solution. *p<0.001 as compared with PMA 10^{-8} mol/l.

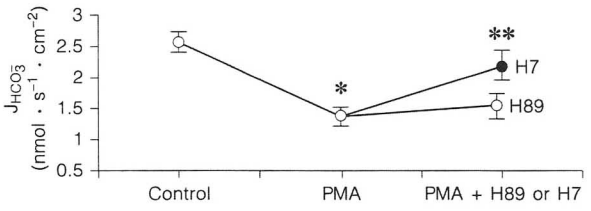

Fig. 5. Effect of H89 (10^{-8} mol/l) and H7 (10^{-6} mol/l) on PMA (10^{-8} mol/l) induced inhibition of bicarbonate reabsorption in proximal tubules, both added to lumen during perfusion with bicarbonate Ringer solution. *p<0.001 as compared with controls; **p<0.01 as compared with PMA 10^{-8} mol/l.

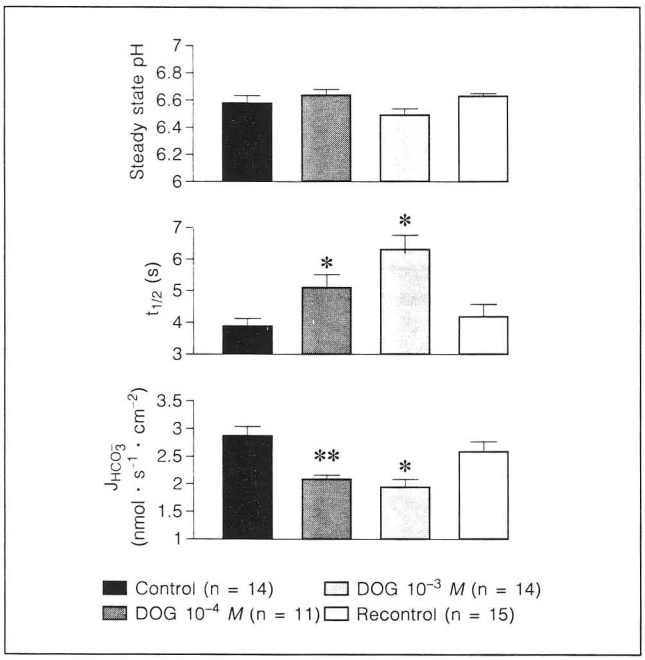

Fig. 4. Effects of DOG 10^{-4} and 10^{-3} mol/l added to lumen on steady state pH, acidification halftime ($t_{1/2}$), and bicarbonate reabsorption ($J_{HCO_3^-}$) during luminal perfusion with bicarbonate Ringer solution. **p<0.01; *p<0.001 as compared with controls.

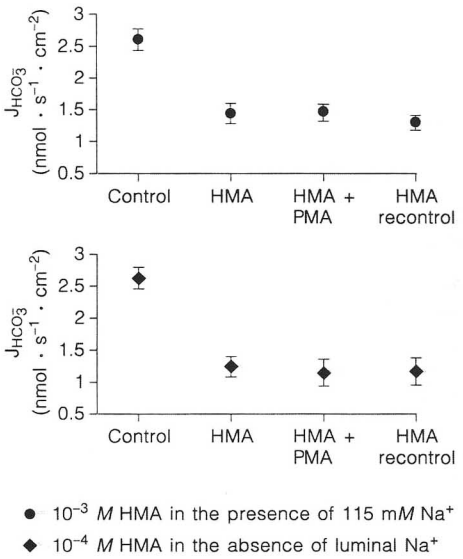

Fig. 6. Effect of HMA 10^{-3} and 10^{-4} mol/l on PMA (10^{-8} mol/l) induced inhibition of bicarbonate reabsorption in proximal tubules during luminal perfusion in the presence (115 mmol/l) and absence of Na^+.

Figure 4 shows the effect of DOG added to luminal perfusate. Stimulation of PKC by DOG (10^{-4} and 10^{-3} mol/l) also resulted in an increase in the halftime of acidification, without modification of steady state pH. The calculated bicarbonate net reabsorption was reduced by 27% (10^{-4} mol/l) and 32% (10^{-3} mol/l).

Effects of PKA and PKC Inhibitors on PMA Effect on Proximal Tubules

Most of the isoforms of Na^+/H^+ exchangers are inhibited by PKA activation. It is known that phorbol esters can activate some forms of adenylate cyclase [21, 22], an action that might result in an increase of cyclic adenosine monophosphate and PKA activation in proximal tubular cells treated

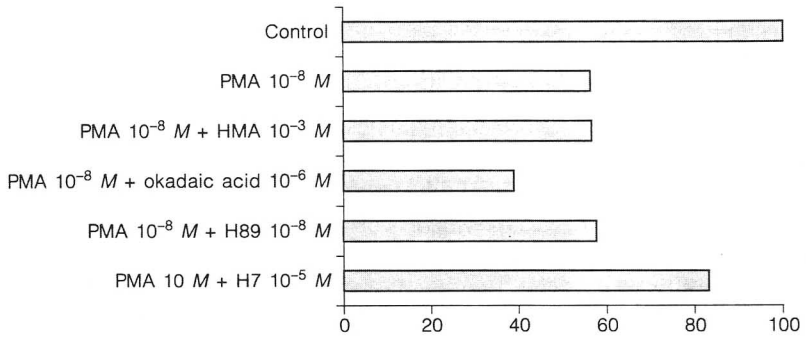

Fig. 7. Summary of observed effects on bicarbonate reabsorption in proximal tubules ($J_{HCO_3^-}$ as percentage of controls).

with PMA. We have explored this possibility using H89, a potent inhibitor of PKA, in combination with PMA. It is shown in figure 5 that addition of H89 had no effect on the inhibition of bicarbonate reabsorption induced by PMA. Addition of H7, an inhibitor of PKC, on the other hand, resulted in 30% reduction of the inhibitory effect of PMA on H^+ secretion in proximal tubules (fig. 5).

Effect of HMA on PMA-Induced Inhibition of H^+ Secretion

In order to investigate whether the Na^+/H^+ exchanger is the target mechanism for PKC action, we have used HMA as an inhibitor of the sodium and hydrogen exchange mechanism. As shown in figure 6, 10^{-3} mol/l HMA added to the luminal solution containing 115 mmol/l Na^+ or 10^{-4} mol/l HMA added to a luminal solution without Na^+ resulted in 53% and 47% inhibition of bicarbonate reabsorption, respectively, and this inhibition was not further increased by PMA (10^{-8} mol/l) addition. These experiments show that the inhibitory effect of PMA is completely blocked in a condition in which the remaining H^+ secretion is not through Na^+/H^+ antiport, suggesting that the inhibitory effect previously observed was on this transporter.

In figure 7 a summary of the effects we have observed on bicarbonate reabsorption in different conditions is shown, expressed as percentage of the calculated bicarbonate net flux in control conditions.

Discussion

The cascade of events leading to PKC activation exerts a great influence on processes involved in modulation of renal tubular transporting systems. Parathormone and angiotensin II are examples of hormones the action of which on renal proximal tubules involves the PKC activation pathway [4, 12, 13, 15, 23, 24]. Renal adaptation to metabolic acidosis is another condition in which PKC activation is important for tubular functional adaptation [25, 26].

Renal proximal tubules perfused in vivo with a solution containing PMA, either at the luminal or at the basolateral side, have shown a significant increase in acidification half-time and a 44% reduction in the calculated HCO_3^- reabsorption, without change in steady state pH. The major mechanism for bicarbonate reabsorption in S1 and S2 segments of proximal tubules is the Na/H exchanger which led us to suggest that PMA reduces hydrogen secretion by inhibiting this transporter. The inhibition is due to a reduction in the V_{max} of the transporter, without modifying its affinity for protons, since steady state pH is not modified. Concerning mechanisms, these results indicate that the phosphorylation of the antiporter by PKC either reduces its turnover rate or inactivates it, reducing the number of functioning sites. A similar kinetic effect of PKC activation by phorbol ester was observed with the NHE-3 isoform, expressed in fibroblasts [25, 26]. Considering immunolocalization studies in rabbit renal tissue [10] and our observations on kinetic parameters, NHE-3 seems to be the isoform of the exchanger most abundant in luminal membrane of rat proximal tubules.

The transport rate of luminal Na^+/H^+ exchanger obviously depends also on Na^+/K^+-ATPase activity at the basolateral membrane. Bertorello and Aperia [27] have shown inhibition of the Na^+/K^+-ATPase activity by PKC activation. However, these effects were observed only 10 minutes after the addition of phorbol esters, the maximum effect being observed with 10^{-6} mol/l PMA, and PMA 10^{-8} mol/l had no effect on the enzyme activity. Therefore, we believe it is unlikely that the reduction of H^+ secretion should be due to inhibition of Na^+/K^+-ATPase; certainly, any drug that activates PKC, a major element in cellular signaling, could re-

sult in phosphorylation of more than just one kind of transporter, and we cannot exclude the participation of other mechanisms.

The experiments with simultaneous application of PMA and okadaic acid further support our hypothesis of an inhibitory modulation of the Na^+/H^+ exchanger by PKC phosphorylation. Okadaic acid is an inhibitor of phosphatases 1 and 2A, widely employed in experiments with intact cells to explore the functional regulation of protein by phosphorylation/dephosphorylation. Our results demonstrate about 35% potentiation of the inhibitory effect of PKC activation when dephosphorylation is prevented by okadaic acid.

Phorbol esters interact with the regulatory domain of PKC with high specificity, and it is assumed that PKC activation by diacylglycerols and phorbol esters is equivalent. However, Slater et al. [28] have found evidence for the existence of two distinct activator-binding sites for phorbol esters and diacylglycerols that could result in distinct active conformation of the enzyme. The possibility of a nonphysiological activation of PKC by PMA was tested in the experiments in which we have used DOG as an activator of PKC. The same inhibitory effects on H ion secretion were observed with DOG and with PMA, indicating that the same PKC isoforms were activated by both. Furthermore, these results support the view that physiological activation of PKC may be a pathway for regulation of H ion secretion in proximal tubules.

A very important aspect of cellular physiology that has been intensively studied is the 'cross-talk' between the major signaling pathways. Many studies have indicated that cyclic adenosine monophosphate production within cells is altered upon PKC stimulation. Some members of the PKC family can phosphorylate members of the adenylate cyclase family [22, 29], and this phosphorylation can result in significant activation of adenylate cyclase, with a consequent increase in intracellular cyclic adenosine monophosphate levels and PKA activation. Na^+/H^+ antiporters so far described are either insensitive to or inhibited by the cyclic adenosine monophosphate signaling pathway. An exception is the Na^+/H^+ exchanger of trout red cells, which is activated when phosphorylated by PKA [30]. This isoform has two consensus sites for phosphorylation by PKA that are missing in other isoforms so far cloned. On the basis of these evidences, we have considered the hypothesis that the inhibitory effect of PKC activation on bicarbonate reabsorption could be ultimately due to PKA activation. This hypothesis, however, is refuted by the experiments in which bicarbonate reabsorption is reduced by PKC stimulation in the presence of H89, a relatively selective inhibitor of PKA.

H ion secretion in proximal tubules is carried out by two parallel mechanisms in the apical membrane of the cells: the Na/H exchanger and the electrogenic proton pump [31, 32]. In our experiments, the reduction of bicarbonate reabsorption by HMA was of the order of 53%. HMA is a highly specific inhibitor of Na/H exchangers. Although NHE-3 is much less sensitive to this inhibitor than NHE-1, we have used it at concentrations at which NHE-3 should be inhibited [33]. This finding is compatible with data obtained by Preisig et al. [34] who found an inhibition of proximal bicarbonate reabsorption of 46–47% using amiloride and t-butylamiloride and suggests that HMA is a more potent analogue than t-butylamiloride. The remaining portion of bicarbonate reabsorption is mostly due to an apical H^+-ATPase which has been shown to be responsible for 20–50% of the proximal bicarbonate reabsorption [35, 36]. In principle, the inhibitory effect of PKC activation on H ion secretion could be due to a modulatory effect on both of these mechanisms. However, our evidence indicates a major effect on the exchanger, since in those experimental conditions in which the exchanger is inhibited, the remaining proton secretion is not affected by PKC activation.

In summary, we have observed that PKC activation reduces the rate of H ion secretion in convoluted segments of proximal tubules. Furthermore, we present evidence strongly suggesting that phosphorylation of the Na/H exchanger by PKC is the cause of the reduction in the net secretion of H ions.

Acknowledgements

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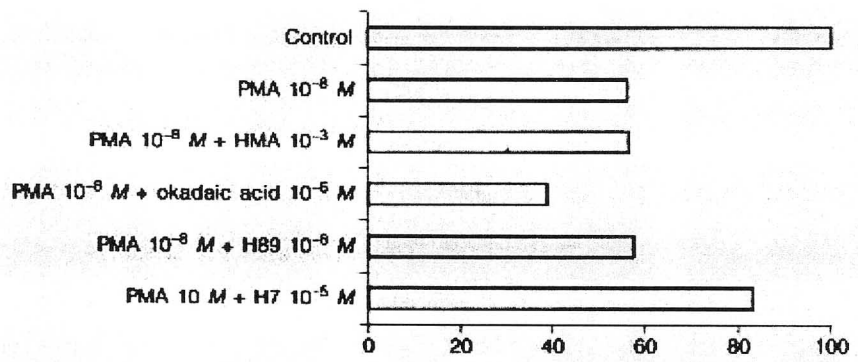
Observamos que em menos de 1 minuto PMA (10^{-8} M), tanto adicionado na luz tubular como em capilar, levava a redução da meia vida de acidificação, sem alterar o pH estacionário atingido na luz do túbulo. A adição de ácido ocadáico em associação com PMA levava a intensificação do efeito inibitório sobre a secreção de H^+ induzida por PMA. O inibidor de PKC, mas não o inibidor de PKA, utilizado em associação com PMA, praticamente eliminava o efeito de PMA sobre a secreção de H^+. PMA não mostrou qualquer efeito sobre a acificação tubular residual na presença de hexametileno-amiloride. Esses resultados estão sintetizados na figura abaixo, na qual as barras indicam fluxo calculado de HCO_3^- ($nmol.s^{-1}.cm^{-2}$).



Nas páginas seguintes está anexada a cópia da publicação referente a este trabalho.

- **Rebouças N.A.** and Malnic G. Regulation of Rat Proximal Tubule Na/H Exchange by Protein Kinase C. *Kidney and Blood Press Res.* 19:87-93, 1996.

Efeito crônico do hormônio da paratireóide sobre a expressão de NHE-3 em túbulos proximais de ratos.

O propósito deste trabalho, desenvolvido pela minha primeira aluna de pós-graduação, Adriana Castelo Costa Girardi, como projeto de tese de mestrado, era verificar se o efeito inibitório do hormônio da paratireóide (PTH) sobre a reabsorção de HCO_3^- em túbulos proximais era acompanhada de redução na expressão de NHE-3, a proteína responsável pela maior parte da reaborção de HCO_3^- neste segmento tubular. Esse foi um trabalho inédito, sendo a única publicação existente na literatura sobre o efeito crônico do PTH sobre a expressão de um permutador Na^+/H^+. Participou também na execução desse projeto a aluna de iniciação científica (hoje médica) Silvia Maria de Oliveira Titan.

Uma das primeiras evidências de que o transporte de eletrólitos e água em túbulos proximais também era modulado pela ação de alguns hormônios, e não apenas o transporte em nefro distal, foi a observação de que PTH inibe o transporte de bicarbonato nesse segmento tubular. Isso foi observado em túbulos proximais isolados e perfundidos [59] e em vesículas de bordo em escova de túbulos proximais [60]. Posteriormente, estudos utilizando linhagens de células epiteliais em cultura (células OK, LLC-PK1/PKE20, e outras) confirmaram que o permutador Na^+/H^+ era agudamente inibido por PTH, envolvendo as vias de sinalização de PKC e PKA [9;31;54;83;84;95].

Com a finalidade de avaliar o efeito crônico do PTH, utilizamos um grupo ratos (PTX) nos quais as paratireóides haviam sido removidas juntamente com a tiréoide, 8 dias antes da realização dos experimentos. Esses ratos eram implantados com "pellets" subcutâneos de liberação lenta do hormônio tireoideano tiroxina, de modo a fazer uma

reposição fisiológica deste hormônio. Outro grupo de animais (HP) tiveram "pellets" de PTH implantados, de modo a receberem por 8 dias uma dose suprafisiológica de PTH. Um terceiro grupo, controle, era constituído de animais de recebiam "pellets" placebo. Todos os "pellets" foram adquiridos da *Innovative Research of America*.

O sucesso da indução de hiperparatireoidismo (HP) ou hipoparatireoidismo (PTX) era avaliado, indiretamente, através dos significantes aumento do cálcio e redução do fósforo plasmáticos, ou redução do cálcio e elevação do fósforo plasmáticos, respectivamente. A eficácia da reposição do hormônio tireoideano era avaliada através da medida dos níveis plasmáticos de T3 e T4.

A atividade de NHE-3 foi avaliada através de medidas da cinética de acidificação em túbulos proximais, nos três grupos de ratos, utilizando o método de microperfusão estacionária descrito no trabalho anterior, e através da medida da captação de $^{22}Na^{+}$ em vesículas de bordo em escova de túbulos proximais, isoladas de rins de ratos provenientes dos três grupos.

A quantidade da proteína NHE-3 presente em túbulos proximais foi avaliada através Western blot, nos quais utilizamos anticorpos específicos para NHE-3 desenvolvidos por Biemesderfer e col., no laboratório do Prof. Aronson (Yale University), e através de imuhistoquímica em secções de córtex renal, preparadas em lâminas, tratadas com anticorpos específicos para NHE-3, da mesma procedência, que eram localizados através de anticorpos secundários conjugados a fluoresceína e analisadas em microscópio de fluorescência.

A quantidade de mRNA específico para NHE-3 para avaliada por Northern blot, utilizando uma sonda de RNA complementar marcada com ^{32}P-UTP, sintetizada a partir do fragmento de cDNA que codifica a porção citoplasmática de NHE-3, obtido por PCR

Parece bem estabelecido que nas guelras posteriores destes crustáceos existe um influxo ativo de íons Cl^- e Na^+, o qual aumenta bastante quando o animal está em meio diluído e pode ser facilmente medido com o uso dos isótopos $^{22}Na^+$, $^{24}Na^+$ e $^{36}Cl^-$, seja no epitélio intacto, seja somente com vesículas de membranas do epitélio. Estudos mostram que a permeabilidade da via paracelular ao Cl^- e especialmente ao Na^+ é pequena, donde o transporte de NaCl deveria ser predominantemente transcelular. A substituição seletiva de certos íons do meio e o uso de inibidores com razoável especificidade para certos trocadores de íons como o trocador Na^+/H^+, o trocador Cl^-/HCO_3^- e a Na^+/H^+ ATPase permitiu que se concluísse pela presença dos dois primeiros na membrana apical das células do epitélio, e da terceira na membrana basolateral. Também há evidências da presença de canais de K^+ e Cl^- na membrana basolateral [94].

Para o caso do trocador Na^+/H^+, as evidências de sua existência na membrana apical são as seguintes: o influxo de Na^+ é diminuído quando se faz a aplicação de íons H^+ ou de amiloride na face mucosa do epitélio, ou ainda por inibidores da anidrase carbônica (acetazolamida). Também em vesículas de membranas isoladas, temos que o influxo de H^+ aumenta gradativamente com a concentração extravesicular de Na^+, para um mesmo tempo de experimento. Finalmente, em bancos de dados de biologia molecular, como o GenBank, está disponível a sequência do cDNA para um trocador Na^+/H^+ presente nas guelras do caranguejo *Carcinus Maenas*, que está incluído na categoria dos crustáceos capazes de sobreviver em baixas salinidades.

A maioria dos resultados acima foi obtida em um número reduzido de espécies de crustáceos, não incluindo o caranguejo com que trabalhamos, *Chasmagnatus granulata*. Estamos admitindo que esses resultados, ao menos em parte, valem para o mesmo. O único resultado para *C. granulata* de que dispomos foi produzido no nosso próprio

laboratório: as células do epitélio das suas guelras posteriores têm a capacidade de reverter uma sobrecarga ácida, mas esta diminui muito quando não há sódio no meio externo ou na presença de elevadas concentrações de amiloride. Ou seja, tal capacidade resultaria de um efluxo de íons H^+ através de um trocador Na^+/H^+.

O controle do transporte de Na^+ e Cl^- através das guelras posteriores isoladas e perfundidas do caranguejo *Eriocheir sinensis* mostrou ser modulada por N6-2'-O-dibutiril AMPc (um análogo do AMPc que é capaz de atravessar membranas celulares), 3-isobutil-1-metilxantina (um inibidor da AMPc fosfodiesterase) ou forskolina (um ativador da adenilato ciclase). Assim, um dos controles da atividade do transporte seriam os níveis de AMPc na célula [17].

Objetivos:

Como nosso laboratório tem trabalhado principalmente com estudos de expressão e regulação da atividade do trocador Na^+/H^+, houve interesse em obter o cDNA deste trocador em crustáceos para, após a obtenção de sua expressão em células (células de mamífero deficientes em trocador Na^+/H^+), iniciar estudos da regulação de sua atividade por outras proteínas intracelulares, bem como introduzir mutações no trocador para descobrir sítios de ligação de íons ou proteínas.

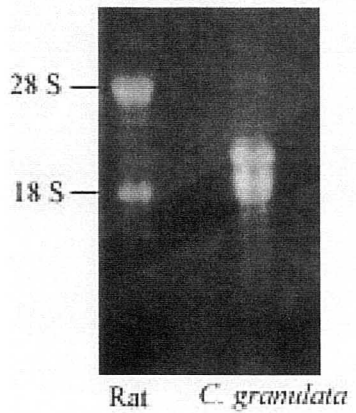
Métodos e Resultados:

Caranguejos da espécie *Chasmagnathus granulata* foram coletados em região de estuário, no Rio Grande do Sul (Coleta feita sob coordenação do Prof. Adalto Bianchini, da Universidade do Rio Grande). Os animais foram mantidos em laboratório em tanques

com água do mar diluída 2‰, de modo que no momento da coleta das guelras os animais já estavam perfeitamente adaptados à baixa salinidade.

Foram feitas coletas de guelras anteriores e posteriores, separadamente, de 12 animais, em um primeiro experimento, e depois de 25 animais. As guelras foram imediatamente colacadas em solução de guanidinum isotiocianato e homogeinizaddas em um homogeinizador Brikman, por 10 a 15 segundos (15.000 rpm). A extração de RNA total foi feita de acordo com protocolo descrito por Puissant e Houdebine [98].

RNA total (5 ug) de guelras posteriores foram submetidos a eletroforese em gel de agarose, contendo 1,7 % de formaldeído. As bandas correspondentes aos RNAs ribossômicos (3 bandas) podiam ser vistos após a coloração com brometo de etídeo. Na figura abaixo mostramos o gel de eletroforese do RNA total extraído de guelras de *C. granulata* ao lado de RNA total extraído de rins de ratos para comparação dos tamanhos dos RNAs ribossomais.



Este RNA foi transferido para membrana de nylon (Northern blot) e em seguida foi hibridizado com cDNA de beta-actina de rato marcado com ^{32}P-dCTP, já que não tínhamos uma sonda específica para *C. granulata*. Mesmo com hibridização bastante

estringente (42°C, 50% formamida), seguida de lavagem também estringente (0,1 X SSC- 0,1% SDS - 65 oC, por 10 a 20 minutos) pudemos observar uma banda única, correspondente a RNA de beta-actina, com aproximadamente 1,3 kb.

Em seguida, RNAs totais de guelras anteriores e posteriores foram aplicados em coluna de oligo-dT celulose, para purificação de mRNA. A eletroforese em gel de agarose de 1,2 ug de cada uma destas amostras de mRNA foi seguida de transferência para membrana de nylon. A hibridização destas membranas com beta-actina mostrou uma banda única, de forte intensidade, confirmando a integridade do mRNA purificado.

Por fim, utilizando 3 ug de mRNA (e hexanucleotídeos aleatórios como primers para a transcrição reversa), fizemos a síntese de cDNA dupla fita. Foi usado o kit da Amershan e o protocolo sugerido pelo fabricante.

Simultaneamente, foi feita uma pesquisa em banco de dados (através dos programas GOPHER e RETRIEVE) de modo a obter-se o maior número possível de sequências de aminoácidos de trocadores Na^+/H^+. Com o auxílio do programa CLUSTAL, foi feita a comparação entre as várias sequências, buscando-se as regiões mais conservadas. Selecionaram-se as sequências assemelhadas ao trocador crustáceo já clonado, o que incluiu todas as sequências disponíveis de trocadores de eucariotos, mas nenhuma de procariotos. Entre as espécies eucariotas estavam: *C. elegans*, *Didelphis virginiana* (um marsupial), truta, rato, hamster, porco, coelho e homem.

Para obtenção do cDNA específico, fizemos PCR com oligonucleotídeos degenerados correspondentes a duas regiões pequenas, mas bastante conservadas entre os trocadores Na^+/H^+.

- oligonucleotídeo sense:

aminoácidos: A V D P VA V

nucleotídeos: 5' GGC/T/A GTG GAT/C CCC/T/A GTG GCI GT 3'

- oligonucleotídeo anti-sense:

aminoácidos: E T I I F M F L

nucleotídeos (sense): 5'GAA/G ACI ATA/T/C ATA/T/C TTC ATG TTT/C CT 3'

Conseguimos amplificar um segmento de ~520 bp, que foi clonado em pMOS e sequenciado. Fazendo comparação de seqüências usando o programa BLAST, verificamos que o segmento clonado pertencia a um segmento do gene de uma proteína pertencente à família dos permutadores Na^+/H^+, sendo maior a identidade com o tracador Na^+/H^+ identificado em guelras de *C. maenas*.

Abaixo, apresentamos a seqüência de nucleotídeo do fragmento obtido por PCR:

1 Asp Pro Val Ala Val Leu Ala Val Phe Glu Glu Ile 12

1 G GAT CCA GTG GCG GTG TTG GCC GTG TTC GAG GAG ATA 37

13 Gln Val Glu Glu Val Leu Tyr Ile Leu Val Phe Gly Glu 25

38 CAG GTG GAG GAG GTG CTG TAT ATC CTG GTC TTC GGC GAG 76

26 Ser Leu Leu Asn Asp Gly Val Thr Val Val Arg Tyr His 38

77 TCC CTC CTC AAC GAT GGT GTG ACG GTG GTC CGT TAC CAT 115

39 Leu Phe Glu Gly Phe Ser Glu Leu Gly Glu Asp Asn Ile 51

116 CTG TTC GAG GGC TTC AGC GAG CTC GGT GAG GAC AAC ATC 154

52 Lys Ala Val Asp Ile Ala Ser Xxx Val Ala Ser Phe Leu 64

155 AAG GCC GTT GAC ATC GCC AGC NGG GTC GCC TCC TTC CTT 193

65 Leu Val Ala Leu Gly Gly Thr Ala Ile Gly Ile Ile Trp 77

194 CTC GTG GCC CTC GGC GGC ACG GCC ATC GGC ATC ATC TGG 232

78 Gly Phe Leu Thr Ala Phe Ile Thr Arg Leu Thr Ser Gln 90

233 GGC TTC CTC ACC GCC TTC ATC ACC AGG TTA ACG AGT CAG 271

91 Val Pro Arg Asp Arg Ala Ile Phe Val Phe Val Met Ala 103

272 GTN CCG CGT GAT CGA GCC ATC TTC GTG TTC GTG ATG GCC 310

104 Tyr Leu Asn Ala Glu Met Leu Ala Ser Ser Ser Glu Thr 116

311 TAC CTC AAT GCG GAG ATG CTG GCC TCG TCC TCG GAG ACC 349

117 Ile Ile 118

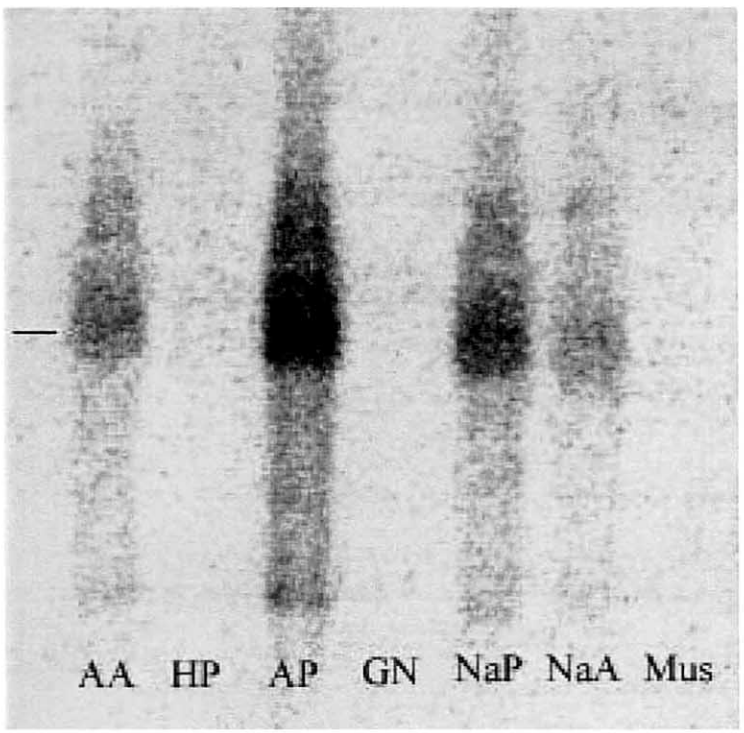
350 ATC ATC T 356

Em vermelho aparecem as duas regiões à primeira vista não apresentavam identidade com o trocador Na^+/H^+ de *C. maenas*. Posteriormente, verificamos que, na verdade, elas também apresentavam homologia, pois conpressões no seqüenciamento haviam alterado a matriz de leitura do DNA nestas regiões. Em verde aparecem os aminoácidos que, estando contidos numa região em que há alta similaridade com o

trocador Na^+/H^+ de *C. maenas*, entretanto diferem dos aminoácidos presentes neste último. Os demais aminoácidos (em negro) são idênticos nas duas proteínas. Os nucleotídeos de número 1 e 356 correspondem, respectivamente aos nucletídeos de número 1093 e 1581 do trocador de *C. maenas*.

Dispondo já de um fragmento do cDNA, tornou-se natural desejarmos avaliar seu nível de expressão nos vários tecidos do animal. Para tanto, realizamos nova extração de RNA total, desta vez a partir de vários tecidos de *C. granulata*. Usamos dois tipos de animais: alguns que haviam sido mantidos em água do mar a 42 % (animais não aclimatados à baixa salinidade), e outros mantidos em água do mar a 0,2 % (animais aclimatados à baixa salinidade). O RNA obtido foi usado numa eletroforese em gel de agarose, seguida de transferência para membrana de nylon e hibridização com sonda marcada com nucleotídeos radioativos, preparada usando como molde o fragmento de cDNA já clonado. A membrana foi lavada com elevada estringência (0,1 X SSC, 0,1 % SDS, a 65°C, por três vezes). A análise da distribuição da radioatividade na membrana através do sistema Storm 840, da Molecular Dynamics, forneceu a seguinte relação de intensidade de expressão: guelras posteriores de animais aclimatados > guelras posteriores de animais não aclimatados > guelras anteriores de animais aclimatados > guelras anteriores de animais não aclimatados > hepatopâncreas de animais aclimatados (note-se que não há qualquer significância estatística nestes dados, pois foi feito uma única hibridização de "Northern blot"). A expressão do RNA de interesse em gânglio nervoso e músculo não era detectável.

Na figura seguinte mostramos a imagem desse Northern blot obtida no analisador de imagens - STORM.



AA – guelras anteriores aclimatas a baixa salinidade;

HP - hepatopâncreas

AP – guelras posteriores aclimatadas;

GN – gânglio neural

NaP – guelras posteriores não aclimatadas;

NaA – guelras anteriores não aclimatadas

Mus – músculo esquelético

Esses resultados são bastantes informativos. A isoforma de permutador Na+/H+ por nós identificada é uma isoforma primordialmente epitelial e tem sua expressão modulada por

alteração na salinidade do meio. Este é um dado inédito, que não havia sido investigado no crustáceo *C. maenas*.

Tendo já um segmento do cDNA do permutador Na^+/H^+ de guelras posteriores *de C. granulata*, construimos uma biblioteca de cDNA deste tecido, em lambda ZAP (Stratagene). Para construção do cDNA usamos como "primers" para a transcriptase reversa oligonucleotídeos poli-dT + um sítio de restrição para a enzima Xho I, o que nos permitiu realizar uma clonagem direcional; na outra extremidade do cDNA foram ligados adaptadores que correspondem às extremidades coesivas obtidas após a clivagem de sítios para EcoRI. A integridade do cDNA foi verificada em três momentos de sua síntese através de marcação radioativa de uma alíquota depois usada numa eletroforese em gel de agarose; o objetivo era observar se continuava a haver na amostra moléculas de alta massa molecular. Este cDNA foi ligado ao vetor Uni-ZAP XR, derivado do fago lambda.

Após duas tentativas mal sucedidas de "screening" da biblioeca por hibridização com o fragmento de cDNA obtido previamente por PCR marcado com ^{32}P-dCTP, decidimos fazer o "screening" por PCR. Para isso foram sintetizados os seguintes oligonucleotídeos:

- oligonucleotídeo 1 para PCR (sense): 5' GTG GAG GAG GTG CTG TAT ATC 3'
- oligonucleotídeo 2 para PCR (anti-sense): 5' GTA GGC CAT CAC GAA CAC GAA 3'

Essa abordagem nos levou à identificação de um clone, com inserto de 1500 pb, que apresentava similaridade com os trocadores Na^+/H^+. Como já ocorrera com o trecho previamente clonado, a similaridade mais extensa era com o trocador Na^+/H^+ do crustáceo *Carcinus maenas* seguido dos trocadores Na^+/H^+ de mamíferos (obviamente não por serem estes mais similares aos dos crustáceos, mas porque em poucos filos e classes de animais foi feita a clonagem dos seus trocadores Na^+/H^+). Pode ser vista

abaixo a seqüência de nucleotídeos do trecho recém-clonado, e a dos aminoácidos correspondentes:

```
TTNAACTTGGGGNAANGCCTTTTTCGGTTTTCCTTTTTTTTTTTTAAATTAGACACCTTT
ATTTACAAACGTTATATATATATATATATATATCAAAATACGGTCCACAGTGCAATGGGGG
AAGGCATATCAAACCACTGAAGGGGAGGAGGGGGAG GAG GAT GAC AAA CCA
GGT GTT
                                      L   I   V   F   W   T   N

CCA GCC GTG CTT GCT TTG GAT GGT GGN NAC GCC GAG GAA CAT GAA
  W   G   H   K   S   Q   I   T   X   X   G   L   F   M   F

GAT GAC GGT CTC CGA GGA CGA GGC CAG CAT CTT CAT GGC GTA CTT
 I   V   T   E   S   S   S   A   L   M   K   M   A   Y   K

GAT GGT CGT GTG GGA CTT GGT CGA GAT GTT CTG CTC CAC GTA ATT
 I   T   T   H   S   K   T   S   I   N   Q   E   V   Y   N

CTT CAT GGT GAA GCC GCA GAA AGT GAT GGA TAG AAT GCC GGA CAA
 K   M   T   F   G   C   F   T   I   S   L   I   G   S   L

GTG GAA GAT CTC CGC ATT GAG GTA GGC GAG GTA GGC CAT CAC GAA
 H   F   I   E   A   N   L   Y   A   L   Y   A   M   V   F
```

CAC GAA GAT GGG CTC GAT CAC G

V F I P E I V

Representamos em azul os nucleotídeos e/ou aminoácidos que coincidem nas seqüências dos trocadores de *Chasmagnathus granulata* e de *Carcinus maenas*; em vermelho, os que em princípio diferem; em negro ficaram aqueles que é ainda impossível avaliar por não ter sido encontrada similaridade, porém possivelmente por problemas na matriz de leitura. Notar que a seqüência acima está anotada invertida, caminhando da extremidade 3' para a 5' do cDNA, e por isso todos os códons também o estão. Os nucleotídeos correspondentes ao primeiro aminoácido da seqüência (leucina) alinham-se, ao analisarmos a similaridade dos nucleotídeos, aos pares de bases de número 1636 a 1634 do trocador de *C. maenas*; os nucleotídeos correspondentes ao último aminoácido (valina) alinham-se com os pares de bases 1364 a 1366; não há neste intervalo praticamente nenhuma seqüência faltando que não a dos dois nucleotídeos marcados como N, os quais alinham-se a apenas dois nucleotídeos da seqüência do trocador *de C. maenas*.

Reunindo as informações dadas pelas duas seqüências guiados pela seqüência de aminoácidos, que é bastante homóloga à do trocador de *C. maenas* em todos os trechos em que se conseguiu fazer o alinhamento das seqüências de nucleotídeos, obtemos as seguintes seqüências de aminácidos e nucleotídeos:

1 Asp Pro Val Ala Val Leu Ala Val Phe Glu Glu Ile 12

1 G GAT CCA GTG GCG GTG TTG GCC GTG TTC GAG GAG ATA 37

13 Gln Val Glu Glu Val Leu Tyr Ile Leu Val Phe Gly Glu 25

38 CAG GTG GAG GAG GTG CTG TAT ATC CTG GTC TTC GGC GAG 76

26 Ser Leu Leu Asn Asp Gly Val Thr Val Val Arg Tyr His 38

77 TCC CTC CTC AAC GAT GGT GTG ACG GTG GTC CGT TAC CAT 115

39 Leu Phe Glu Gly Phe Ser Glu Leu Gly Glu Asp Asn Ile 51

116 CTG TTC GAG GGC TTC AGC GAG CTC GGT GAG GAC AAC ATC 154

52 Lys Ala Val Asp Ile Ala Ser Xxx Val Ala Ser Phe Leu 64

155 AAG GCC GTT GAC ATC GCC AGC NGG GTC GCC TCC TTC CTT 193

65 Leu Val Ala Leu Gly Gly Thr Ala Ile Gly Ile Ile Trp 77

194 CTC GTG GCC CTC GGC GGC ACG GCC ATC GGC ATC ATC TGG 232

78 Gly Phe Leu Thr Ala Phe Ile Thr Arg Leu Thr Ser Gln 90

233 GGC TTC CTC ACC GCC TTC ATC ACC AGG TTA ACG AGT CAG 271

91 Val Xxx Val Ile Glu Pro Ile Phe Val Phe Val Met Ala Tyr 104

272 GTN NNC GTG ATC GAG CCC CTA TTC GTG TTC GTG ATG GCC TAC 313

105 Leu Ala Tyr Leu Asn Ala Glu Ile Phe His Leu Ser Gly Ile 118

314 CTC GCC TAC CTC AAT GCG GAG ATC TTC CAC TTG TCC GGC ATT 355

119 Leu Ser Ile Thr Phe Cys Gly Phe Thr Met Lys Asn Tyr Val 132

356 CTA TCC ATC ACT TTC TGC GGC TTC ACC ATG AAG AAT CAT GTG 397

133 Glu Gln Asn Ile Ser Thr Lys Ser His Thr Thr Ile Lys Tyr 146

398 GAG CAG AAC ATC TCG ACC AAG TCC CAC ACG ACC ATC AAG TAC 439

147 Ala Met Lys Met Leu Ala Ser Ser Ser Glu Thr Ile Ile Phe 160

440 GCC ATG AAG ATG CTG GCC TCG TCC TCG GAG ACC ATC ATC TTC 481

151 Met Phe Leu Gly Xxx Xxx Thr Ile Gln Ser Lys His Gly Trp 174

482 ATG TTC CTC GGC GTN NCC ACC ATC CAA AGC AAG CAC GGC TGG 523

165 Asn Thr Trp Phe Val Ile Leu 181

524 AAC ACC TGG TTT GTC ATC CTC 544

Em verde, novamente os aminoácidos que diferem dos presentes no cDNA de *C. maenas*. São apenas 12 dentre os 177 aminoácidos já identificados. E, mais importante que isso, não se encontrou nenhum trecho ainda em que diminuísse ou aumentasse o número de aminoácidos presentes no trecho correspondente do outro trocador, o que poderia implicar em alteações conformacionais ou funcionais maiores.

Para a obtenção do cDNA completo, optamos pela técnica de PCR, com utilização do método descrito como RACE: "Rapid Amplification of cDNA Ends" . Para isso, decidimos utilizar ou *Marathon cDNA amplification kit*, da Clontech, que é capaz de fazer a amplificação tanto da extremidade 3' quanto da 5' do cDNA, pois ainda não

temos nenhuma delas. No caso deste kit, faz-se a transcrição reversa usando uma mistura de primers com a seqüência abaixo:

5' TTCTAGAATTCAGCGGCCGC$(T)_{30}N_{-1}N$ 3'

Aqui, $(T)_{30}$ é uma região com 30 timinas que irá anelar-se à cauda póli-A do RNAm; N_{-1} é G, A ou C; N é G, A, C ou T. Esta degeneração do primer garante que seu anelamento se fará logo no início da cauda póli-A, de modo que todas as moléculas de um RNAm gerem moléculas de cDNA do mesmo tamanho, o que torna muito mais nítidas as bandas na reação de 3'-RACE.

As sequências dos oligonucleotídeos específicos para o cDNA de interesse podem ser vistas abaixo (estão em vermelho na sequência apresentada acima):

- primer para a 5′-RACE (anti-sense, sendo batizado dou5race1): cctgtatctcctcgaacacggccaaca;

- primer para a 3′-RACE (sense, sendo batizado dou3race1): ccatccaaagcaagcacggctggaaca.

Foram escolhidos com o cuidado de serem próximos às extremidades da região conhecida (e portanto algo mais próximos das extremidades do cDNA), com elevado ponto de fusão (27 nucleotídeos, com 55% de G ou C), sem complementaridade excessiva internamente ou com o primer AP1 do kit, e com extremidade 3' pobre em G/C, mas com último nucleotídeo diverso de T.

As reações já foram feitas e algumas bandas com tamanho adequado foram obtidas. Estamos realizando a clonagem e sequenciamento destas bandas de cDNA amplificado.

Uma vez que esses segmentos que estamos buscando tenham sido obtidos, os diversos segmentos clonados serão ligados em seqüência, de modo a obter o cDNA completo, que deverá ser usado para expressão da proteína em linhagem celular desprovida de trocador Na^+/H^+ (PS120), para estudos da cinética de transporte e da sensibilidade a inibidores farmacológicos específicos.

Análise da expressão da isoforma 3 do trocador Na/H (NHE-3) em tecido renal remanescente 4 e 24 h após nefrectomia unilateral.

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Introdução:

O epitélio tubular renal constitui um bom modelo para a investigação dos eventos que desencadeiam o crescimento celular "in vivo". Essas células são normalmente quiescentes, com raras células em fase mitótica, mas a proliferação pode ser induzida por lesão tubular aguda, causada por drogas ou isquemia tecidual, ocorrendo, então, hiperplasia regenerativa. A ablação parcial de massa renal, por sua vez, induz alterações que resultam em ***hipertrofia compensatória***, que se caracteriza por aumento do tamanho e do conteúdo protéico celular, além de proliferação celular; esta última, embora existente, é menor que a observada após lesão tubular aguda, e é tanto mais evidente quanto mais jovem é o animal.

A atividade do trocador Na^+/H^+ presente em membrana luminal de túbulos proximais mostra-se aumentada (aumento de $V_{max.}$) em vesículas de bordo em escova de túbulos proximais de cães, ratos e coelhos submetidos a estímulo hipertrófico por ablação de massa renal, observação feita já na primeira hora após nefrectomia parcial e também após 3 a 5 semanas [33;52;88]. É interessante a observação de que o aumento de $V_{max.}$ do trocador Na^+/H^+ não se acompanha de alteração similar para outros

cotransportes dependentes de Na^+, como Na^+/glicose e Na^+/alanina. A atividade da bomba de Na^+/K^+ também está aumentada, e este aumento parece ser decorrente do maior transporte de Na^+ em membrana luminal, através do trocador Na^+/H^+, visto que não é observada na presença de amiloride no lado luminal [46].

Até o momento, seis isoformas de trocador Na^+/H^+ foram clonadas e identificadas em mamíferos. NHE-1 é a isoforma relacionada com a manutenção da homeostase celular ("house keeping"), e está presente em praticamente todas as células eucarióticas. Entre as células onde sua presença foi investigada, está ausente apenas em túbulo proximal de rim de opossum, em segmentos S1 e S2 de rins de ratos, em glomérulos e em células intercaladas de coelhos [112]. NHE-2, NHE-3 e NHE-4 são formas específicas de células epiteliais. NHE-3 está presente em membrana luminal (bordo em escova) de epitélio intestinal e de túbulos proximais renais de coelhos, como bem demonstrado por imunohistoquímica [21]. NHE-5 não está presente em epitélios, sendo particularmente abundante em tecido neural; NHE-6 é encontrado em mitocôndrias.

Tendo em vista as modificações funcionais tubulares em tecido renal remanescente, que resultam em aumento da secreção de H^+ em túbulos proximais previamente relatadas, decidimos avaliar a expressão da isoforma 3 do trocador Na^+/H^+ (NHE-3) em tecido renal remanescente (sob estímulo hipertrófico), após remoção de 50% de massa renal.

Materiais e métodos:

Ratos machos, Wistar, com peso entre 100-130g (aproximadamente dois meses de idade), são anestesiados com éter e submetidos a remoção do rim esquerdo

(nefrectomia unilateral). Estes animais são comparados com animais pseudo-operados (sham), submetidos a incisão lombar e manuseio do pedículo renal.

A remoção do tecido renal remanescente para extração de RNA total é feita 4h e 24h após a nefrectomia unilateral ou a pseudo-operação.

No estudo foram usados 40 ratos: 10 pseudo-operados e 10 uninefrectomizados sendo extraídos após 4 horas, e 10 pseudo-operados e 10 uninefrectomizados e extraídos depois de 24 horas.

Os "northern-blots" com 10 μg de RNA total, hibridizados com sonda para NHE-3 marcada com ^{32}P-dCTP, mostram banda proeminente de aproximadamente 5,6 kb, correspondente a NHE-3/mRNA. Hibridizamos também os "northern-blots" com a sonda de GAPDH e 18 S, usados como controle interno da quantidade de RNA utilizada.

As hibridizações são feitas a 42^{o}C, em solução 5 X SSC, contendo formamida 50% e dextran-sulfato 5%. As lavagens são feitas em elevada estringência (65^{o}C - 0,1 ou 0,05 X SSC/0,1% SDS), tanto nas hibridizações com GAPDH como com NHE-3.

As sondas para NHE-3 e GAPDH foram sintetizadas por PCR em nosso laboratório, a partir de cDNA de rim de rato, utilizando oligonucleotídeos sintetizados com base na sequência de nucleotídeos publicada para este transportador [91] e para GAPDH, em ratos. A especificidade de ambas foi confirmada por sequenciamento do DNA clonado. A sonda para 18 S foi sintetizada a partir de um segmento cDNA de 18S, obtida por PCR, através da utilização de primers que continham também sequências dos promotores para as RNAs polimerases T3, T7 e SP6 (Ambion).

Em um primeiro momento, GAPDH foi utilizado como controle interno, para demonstrar a igualdade da quantidade de RNA de ratos controles e uninefractomizados. No entanto, percebemos que a uninefrectomia afetava também os níveis de mRNA-

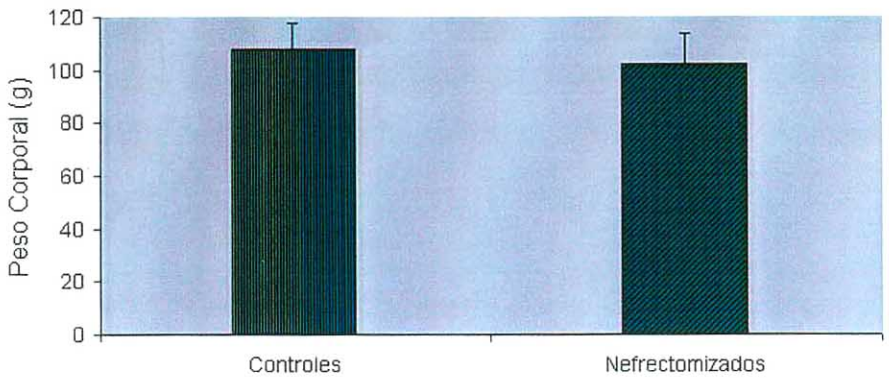
GAPDH. Decidimos então re-hibridizar nossas membrana como uma sonda específica para o RNA ribossomal 18S, já que, sendo esse, de longe, muito mais abundante que qualquer mRNA, é ideal como marcador da quantidade de RNA fixada à membrana.

Para análise através de "western blot" foram utilizados mais 15 animais (peso em 90 e 100g): 5 animais controles; 5 animais submetidos a uni-nenfrectomia e remoção do rim remanescente após 4 horas e 5 animais submetidos a uni-nefrectomia e remoção do rim remanescente após 24 horas. O rim D desses animais eram removidos e imediatamente colocados em solução gelada contendo inibidores de proteases e homogeneizados para extração de membranas celulares totais ("crude fraction") e "brush border". Essas frações celulares foram submetidas a eletroforese em SDS-PAGE, transferidas para membrana de nylon (Immobillon) e colocadas em contado com anticorpos monoclonais específicos para NHE3 e para vilina (esse último utilizado como controle interno, para verificação da quantidade de proteína presente na membrana).

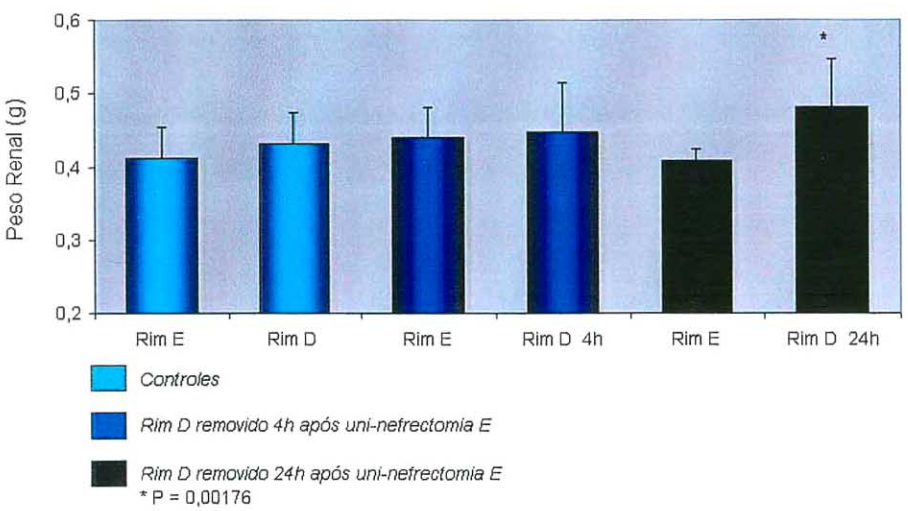
Os dados são apresentados como média ± erro padrão das razões de densitometrias medidas para as bandas correspondentes a NHE-3/18S e GAPDH/18S. Os valores de animais uninefrectomizados foram comparados com os de animais pseudo-operados correspondentes, utilizando teste t.

Resultados:

Os animais submetidos a uninefrectomia e os pseudo-operados apresentavam o mesmo peso corporal (figura 1)



O peso do rim D, rim residual nos animais uninefrectomizados, mostrou-se significativamente aumentado após 24 horas (figura 2).



As análises de Northern blot de RNA total de rins residuais extraídos 4 h horas após uninefrectomia E, mostram aumento de 106,4% nos níveis de mRNA de NHE-3 em córtex renal. Após 24 h, no entanto, observa-se redução nos níveis de mRNA de NHE-3 de cerca de 40% tanto em córtex como em medula.

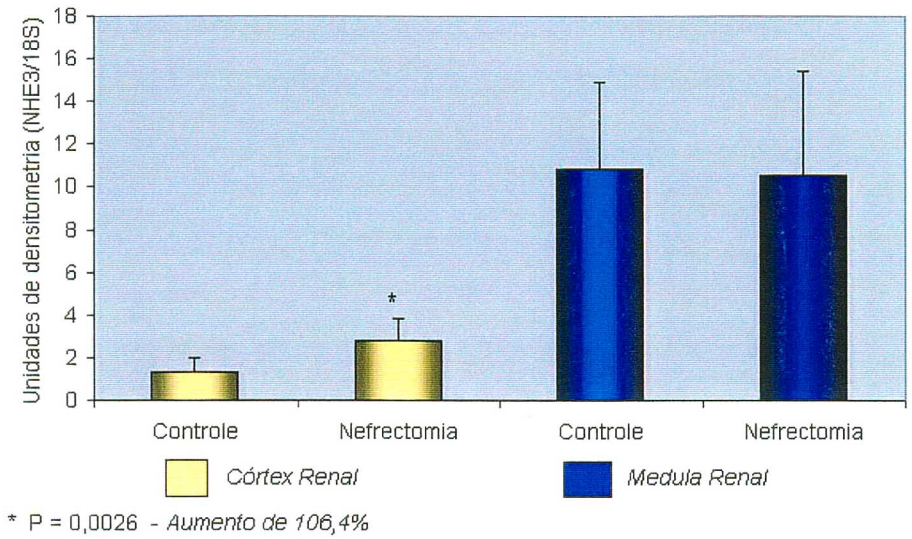


Figura 3

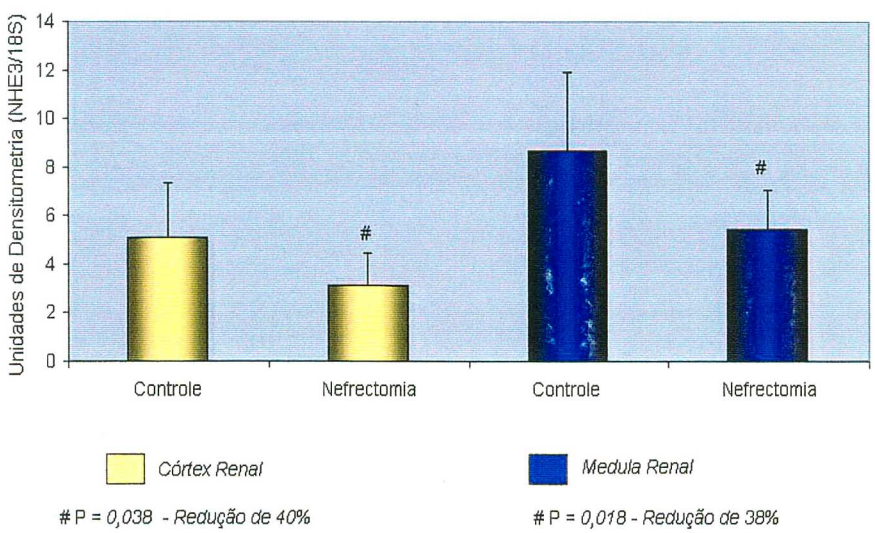


Figura 4

As análises de Northern blot em que utilizamos sonda para GAPDH, mostram aumento de 70% nos níveis de mRNA de GAPDH em córtex renal (figura 5). Após 24 h, no entanto, observa-se redução também nos níveis de mRNA de GAPDH de cerca de 20%, apenas em córtex (figura 6).

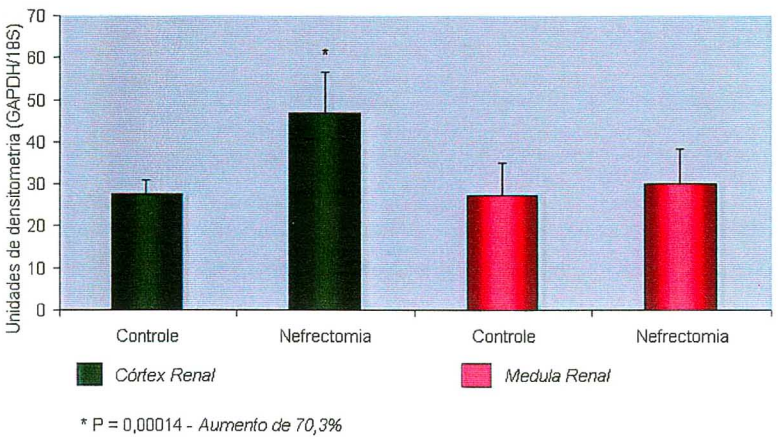


Figura 5

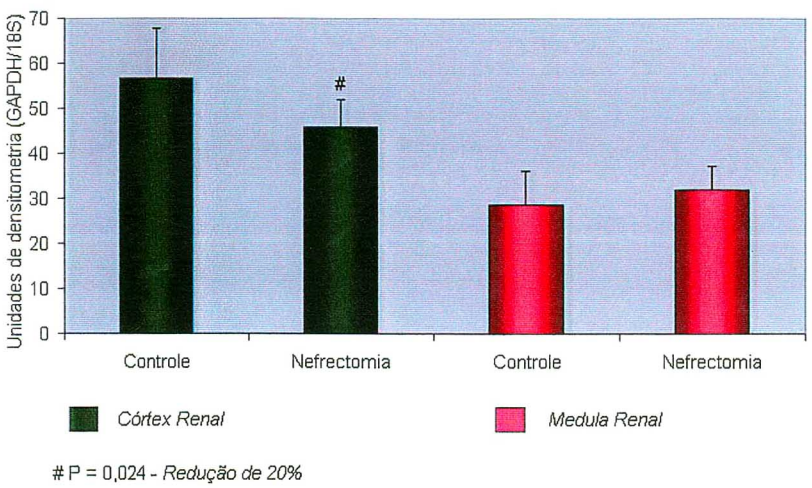


Figura 6

Os dados referentes a *Western blot* só agora estão sendo finalizados. Nossos dados preliminares mostram aumento significativo da quantidade da proteína NHE-3 em preparações de vesículas de bordo em escova, tanto após 4 horas quanto após 24 horas da nefrectomia unilateral.

A atividade de NHE-3 em vesículas de bordo em escova está sendo avaliada através da medida da captação de $^{22}Na^{+}$.

Além das análises feitas até agora nesses animais, pretendemos verificar o quanto o aumento de massa renal se deve também a hiperplasia, realizando novos experimentos em que avaliaremos a incorporação de deoxiuridina marcada no núcleo celular das células tubulares renais desses animais. Além disso, pretendemos avaliar os níveis de mRNA e de proteína NHE-1, visto ser esta a isoforma de NHE mais relacionada com as condições caracterizadas como hiperplasia/hipertrofia, como comentamos na revisão apresentada no início. Recentemente, em estudos com modelo de infarto do miocárdio em ratos, Kusumoto e col. relataram que a inibição de NHE-1 com cariporide reduz significativamente a hipertrofia do miocárdio [66].

Discussão preliminar:

Quando iniciamos estudos em rim hipertrófico pouco se sabia sobre os mecanismos celulares envolvidos nesta hipertrofia dita compensatória. Especulava-se se esta forma de hipertrofia estaria ou não relacionada a eventos do ciclo celular. Atualmente está claro que neste tipo de hipertrofia renal, assim como na observada em rins de animais diabéticos, há aumento significante de proteínas inibidoras de quinases dependentes de ciclinas (ciclin dependent kinases - CDK), tais como p21 $^{WAF1/CIP1}$ e

$p26^{KIP1}$. Estas proteínas inibidoras de CDKs se ligam a vários complexos ciclina-CDK, inibindo suas atividades e impedindo a progressão do ciclo celular [76;131].

Fine e col. formularam uma hipótese na qual o aumento da atividade do permutador Na^+/H^+ em membrana luminal seria essencial na ativação do processo hipertrófico no rim remanescente [45;46]. Entretanto, Mackovic-Basic e col. demonstraram a dissociação entre a ativação do permutador Na^+/H^+ e a hipertrofia renal em ratos submetidos a denervação. A denervação do rim remanescente impedia o aumento da atividade do permutador Na^+/H^+ mas não impedia a hipertrofia [73].

Independentemente do fato do permutador Na^+/H^+ estar envolvido como desencadeador do processo hipertrófico, o que não nos parece ser , o que nossos resultados mostram é um aumento da atividade de síntese de mRNA nas primeiras 4 horas, que não parece ser específica, mas um resultado do aumento global da atividade de síntese, como resultado de estímulos para a entrada na fase G1 do ciclo celular. A atividade aumentada do permutador Na^+/H^+ nesta fase poderia até ser um dos agentes facilitadores para a entrada na fase G1, mas não o único. O ciclo celular é interrompido na fase G1 pelo aumento dos inibidores de CDKs.

O aumento, já descrito anteriormente, na atividade do permutador Na^+/H^+ de membrana apical, como nossos dados demonstram, se deve ao aumento da quantidade da proteína NHE-3 presente em bordo em escova. Este aumento da quantidade de NHE-3 pode ser resultado de aumento de mRNA nas primeiras 4 horas, mas não após 24 horas.

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