が Cl^- チャネルであるとともに外向き整流性 Cl^- チャネル(ORCC)，容積調節性 Cl^- チャネル(VRCC)や容積調節性 ATP 放出を制御する。さらに，1995 年には糖尿病治療薬であるスルホニル尿素剤の作用点がインスリン分泌調節のスイッチとして働く ATP 感受性 K^+ チャネルのサブユニットである *SUR1*(*ABCC8*)であることが明らかになった[3]。SUR 1 は，それ自身がトランスポーターやチャネルではなく，細胞内 ADP 濃度を感知する受容体として，K^+ チャネルの開閉を制御していると考えられる。

トランスポーター，チャネル，受容体の機能の違いは図 2 のように例えるとわかりやすい。トランスポーターは，例えば手押しポンプのようなものであり，水を汲み上げるには棒を押したり引いたりし続ける必要がある。つまり，ATP 加水分解による構造変化

表 1　主なヒト ABC 蛋白質遺伝子の分類と関連する疾病

通称遺伝子名	新遺伝子名	アミノ酸数	機能・特徴/過剰発現・異常による表現型
ABCA サブファミリー			
ABC1	*ABCA1*	2261	コレステロール・リン脂質輸送/HDL 欠損症
ABC2	*ABCA2*	2436	脳オリゴデンドロサイト特異的発現
ABC3	*ABCA3*	1704	肺サーファクタント分泌/新生児サーファクタント欠損症
ABCR	*ABCA4*	2273	レチノイン酸輸送/黄斑部変性症
ABCA7	*ABCA7*	2146	コレステロール・リン脂質輸送
ABCB サブファミリー			
MDR1, PGY1	*ABCB1*	1280	生体異物排出ポンプ/がん細胞の多剤耐性
TAP1	*ABCB2*	808	抗原ペプチドの ER 内への輸送
TAP2	*ABCB3*	653	抗原ペプチドの ER 内への輸送
MDR2/3	*ABCB4*	1279	ホスファチジルコリン輸送/肝内胆汁うっ滞症
ABC7	*ABCB7*	752	鉄イオウ複合体の輸送(ミトコンドリア)
SPGP, BSEP	*ABCB11*	1321	胆汁酸輸送/肝内胆汁うっ滞症
ABCC サブファミリー			
MRP1	*ABCC1*	1531	グルタチオン/グルクロン酸抱合体排出/がん細胞の多剤耐性
MRP2/cMOAT	*ABCC2*	1545	グルタチオン/グルクロン酸抱合体排出/体質性黄疸
MRP3	*ABCC3*	1527	グルタチオン/グルクロン酸抱合体排出
MRP4	*ABCC4*	1325	抗ウイルス核酸誘導体輸送
MRP5	*ABCC5*	1437	核酸類縁体輸送
MRP6	*ABCC6*	1503	?/弾性繊維性仮性黄色腫
CFTR	*ABCC7*	1480	クロライドチャネル/嚢胞性線維症
SUR1	*ABCC8*	1581	ATP 感受性 K^+チャネルサブユニット/低血糖症
SUR2	*ABCC9*	1549	ATP 感受性 K^+チャネルサブユニット
ABCD サブファミリー			
ALDP	*ABCD1*	745	極長鎖脂肪酸輸送/副腎白質ジストロフィー
ALDR	*ABCD2*	740	ペルオキシソーム膜に局在
PMP70	*ABCD3*	659	極長鎖脂肪酸輸送
ABCG サブファミリー			
ABCG1	*ABCG1*	638	コレステロール・リン脂質輸送
ABCP, BCRP	*ABCG2*	655	抗がん剤耐性，エストロゲン輸送/phototoxin 感受性
ABCG5	*ABCG5*	651	植物ステロール排出/高シトステロール血症
ABCG8	*ABCG8*	673	植物ステロール排出/高シトステロール血症

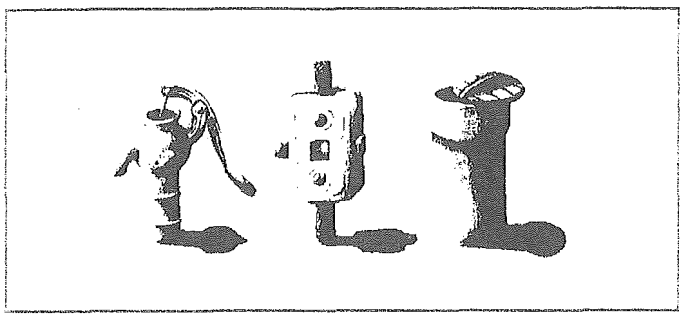

図2　ABC蛋白質の3つの機能（ポンプ，受容体，チャネル）

と物質輸送が厳密に共役している。チャネルは，構造変化でふた(ゲート)を開閉するが，イオンを輸送するのに構造変化を繰り返す必要はなく，土管のようである。ATPは輸送の駆動力ではなく，チャネル開閉の調節のために使われていると思われる。受容体はスイッチのようであり，SUR1の場合は細胞内ATPとADPがリガンドとして結合することによって構造が変化し[5]，チャネルサブユニットであるKir 6.2の開閉を制御する。それぞれのABC蛋白質が類似の二次構造とよく保存されたATP結合領域をもつにもかかわらず，なぜトランスポーター，チャネル，受容体という異なった役割を果たしているのかは興味深い。

さらに最近，多くのABC蛋白質がさまざまな脂質の輸送に関与しており[7]，それらの異常が動脈硬化症[8]，黄斑部変性症，新生児肺サーファクタント欠損症[9]やアルツハイマー病などと関連することがわかってきた。ABC蛋白質の研究はこれからさらに重要性を増すと思われる。

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4. 脂質輸送とABCタンパク質

植田和光，高橋　圭，小林　綾，松尾道憲

ABCタンパク質（ABCトランスポーター）の1つであるABCA1は高密度リポタンパク質（HDL）の形成に必須であり，その異常はタンジール病を引き起こす．その発見によって，リン脂質やコレステロールなどの脂質の膜を介した輸送は濃度勾配に従った自由拡散ではなく，トランスポーターが関与していることが明らかになった．ABCA1以外に少なくともABCA3，ABCA7，ABCG1，ABCG4，ABCG5，ABCG8，ABCB4などのABCタンパク質が脂質輸送に関与していることがわかっており，さらにその数が増えつつある．脂質動態の分子メカニズムの解明はこれから佳境に入ろうとしている．

はじめに

リン脂質やコレステロールは，細胞膜の構成成分であると同時にステロイドホルモン，脂溶性ビタミン，胆汁酸などの前駆体として細胞の増殖と生存に必須である．動物はそれら脂質を体内で合成するだけでなく，毎日の食餌によって外界から摂取しており，小腸から肝臓，肝臓から末梢組織，末梢組織から肝臓へなど，さまざまな組織間を脂質は移動する．そのためには，脂質はさまざまな組織で細胞膜を越えて輸送されなければならない．脂質の細胞膜を越えた輸送は，取り込みに関しては低密度リポタンパク質（LDL）受容体やSR-BIなどの受容体を介する経路が主であるのに対して，排出は濃度勾配に従って自由拡散によって行われると考えられてきた．しかし，ABCタンパク質※1の遺伝子の1つである*ABCA1*が血中HDLがなくなる遺伝病であるタンジール病※2の原因遺伝子として同定されたことによって，脂質輸送へのトランスポーターの関与が認知されることになった．本稿では，脂質，そのなかでもコレステロールの膜を介した輸送に関与するABCタンパク質に焦点をあてて概説する．

[キーワード&略語]
ABCタンパク質，コレステロール，トランスポーター
HDL：high density lipoprotein（高密度リポタンパク質）
LDL：low density lipoprotein（低密度リポタンパク質）
ABC：ATP binding cassette
WHAM：Wisconsin hypoalpha mutant

1 ABCA1

1）ABCA1とHDL形成

末梢組織ではコレステロールは細胞内で合成されるとともに肝臓からLDLの形で供給される．ステロイド合成を行う組織以外ではコレステロールは異化されない．そのため高密度リポタンパク質（HDL）として肝臓へ逆転送される経路が，末梢組織からコレステロールが放出される唯一の経路である（図1）．

タンジール病や家族性低HDL症の患者の*ABCA1*遺伝子に変異が同定され，HDLが形成されない原因が，*ABCA1*の異常であることが1999年に明らかにされた[1)~3)]．*ABCA1*遺伝子は1994年に単離されたが，機能としてはホスファチジルセリンを脂質二重層の外層へフリップしアポトーシス細胞の貪食に関与すると

Lipid transport and ABC proteins
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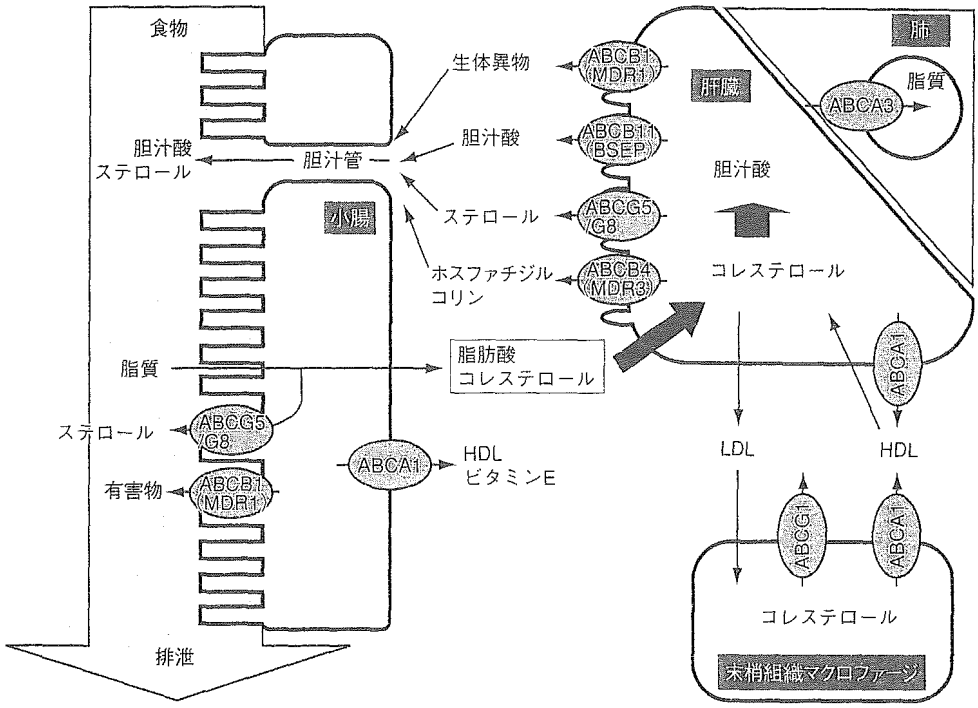

図1　脂質ホメオスタシスに関与する主なABCタンパク質
さまざまなABCタンパク質が組織特異的に発現し，膜を介して脂質を輸送することによって体内の脂質ホメオスタシスが保たれている

という説[4]，アニオンを輸送する活性をもつ[5]という説が提唱されていた．タンジール病の原因遺伝子としての発見によって，ABCタンパク質であるABCA1がHDL新生に重要な役割を果たしていること，ABCA1がリン脂質だけでなくコレステロールも輸送している可能性が示唆された．

ABCA1を安定発現させた培養細胞の培養液に脂質の結合していないアポリポタンパク質A-Ⅰ（ApoA-Ⅰ）を加えると，ApoA-Ⅰにコレステロールとリン脂質が結合したHDLが培地中に出現する[6,7]．ApoA-Ⅰは，約240アミノ酸からなる水溶性のタンパク質で，その大部分がαヘリックス構造をとっている．そのαヘリックス構造の片面に疎水性のアミノ酸が並んでおり，リン脂質とコレステロール複合体をApoA-Ⅰが疎水面を内側にして取り囲むことによって血中に溶かすと考えられている．ABCA1を発現していない細胞にApoA-Ⅰを添加しても全くHDLは形成されないことから，新生HDL（pre β-HDLと呼ばれる）の形成にはABCA1が必須であることが明らかになった．

2）ABCA1によるHDL形成メカニズム

これまでにタンジール病や家族性低HDL症の患者の*ABCA1*遺伝子に多くの変異が同定されているが，その多くが第1細胞外ドメインやATP結合領域にマップされている[8]（図2）．ABCA1の第1細胞外ドメインのアミノ酸配列はニワトリからヒトまで非常によく保存されており，ApoA-Ⅰと直接相互作用していることが考えられる．また，ATP結合領域の変異に

※1　ABCタンパク質

ABCトランスポーターとも呼ばれる．200アミノ酸にわたって配列のよく保存されたATP結合領域を1分子内に2つ有し（図3），12～17の膜貫通αヘリックスをもつ膜タンパク質の総称であり，いずれもATPによって駆動または制御される．細菌から植物，哺乳動物まで広い生物種に分布している．ヒトでは49のABCタンパク質遺伝子が同定されており，それぞれの遺伝子の異常が疾病と関連している．

※2　タンジール病

*ABCA1*遺伝子の変異によって引き起こされる常染色体劣性の遺伝病．臨床症状としては，オレンジ色の扁桃腫大，肝脾腫，角膜混濁，末梢神経障害がみられ，虚血性心疾患の頻度が高い．*ABCA1*にヘテロに変異をもつ人は，年齢とともに冠動脈壁の厚さの増加が正常人と比べて有意に大きいことが疫学調査からわかっている．

よってHDLが形成されなくなること，ABCA1がATP加水分解活性をもつこと[9]，HDLが形成されなくなるATP結合領域の変異によってATP加水分解活性を失うこと（投稿中）も明らかになっている．それらの事実から予想されるABCA1の作用メカニズムは，以下のようなものであろう．つまり，①ApoA-ⅠがABCA1の細胞外ドメインと相互作用する，②ABCA1がATP加水分解することによって構造変化する，③膜中のコレステロールとリン脂質がABCA1によって押し出され，ApoA-Ⅰと複合体を形成する，である．

しかし，タンジール病でみつかった第1細胞外ドメインのアミノ酸置換のうち，R587WあるいはQ597R変異をもつABCA1は細胞膜には発現せず，大部分が小胞体に留まっていた[9]．それゆえ，これらの変異はApoA-Ⅰとの相互作用の異常ではなく，局在の異常を引き起こすと考えられる．一方，W590S変異体は野生型と同様に主に細胞膜上に局在した．さらに，細胞外ドメインに対する抗体との反応性から正しいトポロジーをもつことがわかった[9]．しかし，W590S変異体は野生型と同様にApoA-Ⅰとクロスリンクされるため，ApoA-Ⅰとの相互作用には影響しないことが予想されている．

残念ながら実際にABCA1の第1細胞外ドメインがApoA-Ⅰとの相互作用に直接関与していることを示す証拠は得られていない．また，ABCA1がATPのエネルギーを用いてリン脂質とコレステロールをどのように輸送しているのかについても議論が分かれており，ABCA1を介したpre β-Ⅰ HDL形成のメカニズムはいまだ明らかではない．

3）ABCA1の局在と細胞内リサイクリング

C末端にGFPを融合させたABCA1-GFPをHEK293細胞に安定発現させると，主に細胞膜に発現し，一部細胞内コンパートメントに局在する[9)10)]．シクロヘキシミドで新規タンパク質合成を阻害した後に，膜タンパク質のエンドソームから細胞膜への移動を阻害するモネンシンで処理すると，時間を追って細胞内コンパートメントに局在するABCA1の量が増加した[9]．この結果は，ABCA1は細胞膜とエンドソームの間をすばやくリサイクリングしていることを示唆している．

タンジール病患者から単離された細胞では，そのような後期エンドソーム循環が起こらない[11]．その結果，後期エンドソームにはコレステロールが蓄積する．さらにそれらは界面活性剤では引き抜かれないため，おそらくスフィンゴミエリンとともにミクロドメインを形成していると思われる．興味深いことにそのコレステロールが蓄積した後期エンドソームにはニーマンピック病の責任遺伝子産物であるNPC1が大量に局在していた．そのような状態の細胞にABCA1発現アデノウイルスを感染させると，後期エンドソーム循環が正常になり，ApoA-Ⅰ依存コレステロール排出も検出された．正常細胞に発現させたABCA1-GFPの存在するエンドソームにはApoA-Ⅰが取り込まれており，NPC1を含むエンドソームは減少していた．以上の結果から，ABCA1は，NPC1を含んだ後期エンドソーム中の脂質をエンドサイトーシスされたApoA-Ⅰと相互作用できるように変換し，最終的には新生HDLとして細胞外へ排出しているというモデルが考えられる[11]．しかし，コレステロールの細胞内動態とABCA1の関連はいまだ不明な点が多い．

4）ABCA1とコレステロールの小腸吸収

WHAM（Wisconsin hypoalpha mutant）と名づけられたニワトリは血中にHDLが存在しない．おそらくそのためにカロテノイドが血中に保持されないため白い皮膚と白いくちばしをもつ．遺伝子解析の結果，そのニワトリのABCA1の第1細胞外ドメイン中の89番目のアミノ酸がグルタミン酸からリジンに置換（図2）していることが明らかになった[12]．このWHAMニワトリでは，食餌中のコレステロールが小腸からほ

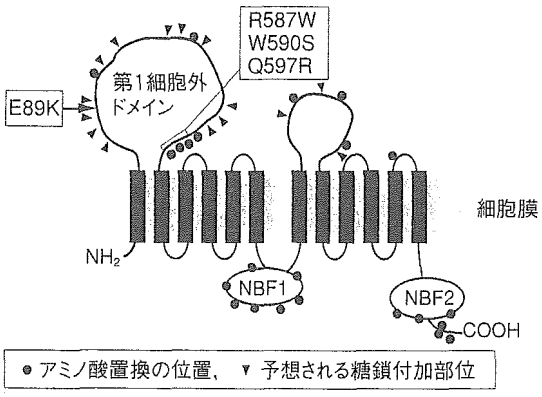

図2　ABCA1の予想される二次構造とタンジール病および家族性低HDL血症を引き起こすアミノ酸置換
E89K変異は血中にHDLが存在しないWHAMニワトリでみつかったアミノ酸置換．R587W，W590S，Q597Rについては本文参照

とんど吸収されない．哺乳動物では，食物中の脂質成分はそのほとんどがカイロミクロンとして小腸から吸収され，まずリンパ液に入り，血液を介して肝臓へ運ばれると考えられている．それに対し，鳥類ではコレステロールは小腸から直接血中にHDLとして吸収され，即座に肝臓へと運ばれるのである．ヒトの腸由来の培養細胞であるCaco-2においても，ABCA1は細胞の側面と基底側膜に発現している[13]．以上の結果は，進化の過程でABCA1は小腸からコレステロールを吸収する役割を担ってきており，ヒトにおいてもコレステロールはカイロミクロンを介した経路だけでなく，一部はHDLとして血液中に直接吸収されている可能性がある．また，ABCA1欠損マウスでは血中のビタミンEが大幅に減少することから[14]，ABCA1はビタミンEなどの脂溶性ビタミンの消化管上皮における吸収にかかわっている可能性がある．

2 ABCA7

*ABCA7*は*ABCA1*と相同性が高く，*ABCA1*と同じくコレステロール蓄積によって発現誘導される遺伝子として単離された[15]．われわれはほぼ同時期に，ABCA1の第1細胞外ドメインのアミノ酸番号270～449の間のアミノ酸配列が，自己免疫疾患であるシェグレン症候群の自己抗原SS-Nと高い相同性をもつことを見出し，それをコードしている遺伝子の全長cDNAを単離した．そしてそれは*ABCA7*と同一の遺伝子であった[6]．*ABCA7*は*ABCA1*と比べて発現の組織特異性が高く，胸腺，リンパ節，骨髄，末梢白血球，脾臓，脳などで8～9 kbのpoly（A）RNAとして発現しており，免疫系の組織および脳で何らかの機能を果たしていることが示唆された．また，*ABCA7*は組織特異的にスプライシングされている[16]．ヒトABCA7を培養細胞に安定発現させると，ABCA1の場合と同様にApoA-Ⅰに依存してリン脂質とコレステロールを細胞外へ排出する[16][17]．米国のグループは，マウスのABCA7を培養細胞に発現させるとリン脂質は排出するが，コレステロールは排出しないと報告した[18]．ABCA7の機能には種特異性があるのかもしれない．ABCA7によるHDL形成の生理的意味はいまだ不明である．

3 ABCG1

ABCA1はApoA-Ⅰ依存的にHDLを形成するが，形成されるのはコレステロール含量の少ないpre β-HDLである．そこにさらにコレステロールが含有されることによって成熟型HDLが形成される．ABCG1はその過程に関わっていることが示唆された[19]．われわれは，ABCG1がABCA1とは異なって，ApoA-Ⅰ

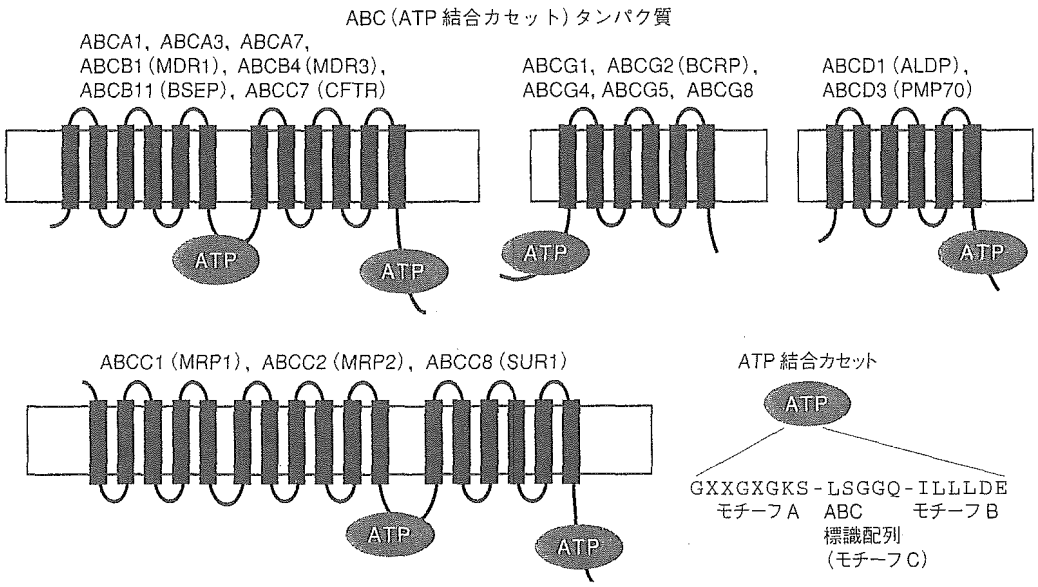

図3 代表的なヒトABCタンパク質の予想される二次構造
モチーフA，モチーフB，モチーフCはATP結合領域中で特に保存されたアミノ酸配列

がなくてもリン脂質とコレステロールを培地中に放出する活性ともつことを見出している（投稿中）．ABCG1は次に述べるABCG5，ABCG8と同様にハーフサイズのABCタンパク質であり（図3），ABCG5/ABCG8がヘテロ二量体として機能するのに対して，ホモ二量体として機能する（投稿中）．また，ABCG1と高い相同性をもつABCG4も同様の活性をもつ可能性が示唆されている[19]．

ABCG5/ABCG8

われわれは，コレステロールと同様にシトステロールを代表とする植物性ステロールなどの非動物性ステロールを毎日摂取している．その量は200～400 mg/日であり，コレステロールとほぼ同量である．しかし，摂取したコレステロールの50～60％が小腸から吸収されるのとは対照的に，植物性ステロールは5％以下（20 mg以下）しか吸収されない．その仕組みの中心はABCG5，ABCG8であり[20][21]，ABCG5/ABCG8ヘテロ二量体が植物性ステロールの小腸からの吸収を防ぐとともに，胆汁中に排出している（図1）．*ABCG5*と*ABCG8*遺伝子の変異は植物性ステロールの異常な蓄積を引き起こし，常染色体劣性の高シトステロール血症という脂質代謝異常症を引き起こす[20][21]．

おわりに

本稿では，コレステロール輸送に関与しているABCタンパク質に絞って紹介した．しかし，これら以外にも脂質の輸送に関与するABCタンパク質には，図1に示したように，胆汁中にホスファチジルコリンを分泌するABCB4（MDR3），肝臓中でコレステロールが酵素的に変換され生成した胆汁酸を分泌するABCB11（BSEP）[22]，ペルオキシソーム内へ長鎖および極長鎖脂肪酸を輸送するABCD2（ALDP）やABCD3（PMP70）[23][24]，肺サーファクタント脂質の分泌に関与しているABCA3[25]などが知られている．ヒトは49のABCタンパク質遺伝子をもっており，生理的基質の不明なABCタンパク質がまだ多く残されている．また，ABCタンパク質とほかの脂質輸送に関わるタンパク質の協調性などに関しては，まだほとんど解明されていない．脂質の動態の分子メカニズムの解明はこれから佳境に入ろうとしている．

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植田和光：1978年京都大学農学部農芸化学科卒業，1985年から2年間米国NIH国立癌研究所のPastan研究室に留学し，真核生物の最初のABCタンパク質であるヒト*MDR1*遺伝子に出会った．現在，京都大学大学院農学研究科応用生命科学専攻教授．ヒトのABCタンパク質のそれぞれの生理的役割を解明するとともに，三次元構造に基づいて基質認識機構，ATP加水分解と輸送の共役機構を解明したい．脂質恒常性に関与するABCタンパク質の活性調節によって健康を維持する方法を見出したい．自分がもっと呆ける前に，脳内のABCタンパク質の重要性を解明しアルツハイマー病を防ぎたい．

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Letters to the Editor

Pilsicainide in breast milk from a mother: comparison with disopyramide and propafenone

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Information about the transfer of antiarrhythmic drugs into milk is available. However, results for the same drug sometimes vary. This variation may be due to factors such as interpatient variation of drug transfer or the small number of patients in the studies. Equations are useful for predicting drug transfer into milk [1]. Pilsicainide is a new class I antiarrhythmic drug used in Japan. We collected serial specimens of plasma and milk after a single dose of three different antiarrhythmic drugs, including pilsicainide, in a healthy mother. We also compared the results with an equation for predicting the milk/plasma ratio.

One healthy nursing mother (one of the authors, M.W.; body weight 56 kg) took a single oral dose of either pilsicainide (50 mg), disopyramide (100 mg) or propafenone (150 mg) after a 12-h fast. The wash-out period between each dose was 4 weeks and the order of the section was randomly chosen. She was prohibited from breast feeding on trial days. Blood and milk samples were collected at 1, 2, 3, 4, 6, and 12 h after each dosing. Breast milk samples were collected by manual expression and milk pH was measured anaerobically. For sampling milk, breasts were emptied of milk at each time point and a stirred aliquot was taken for analysis. Protein binding of each drug was measured after microultrafiltration. Concentrations of pilsicainide, propafenone, 5-(OH)propafenone and disopyramide were measured using high-performance liquid chromatography [2, 3] or enzyme immunoassay [4]. The detection limit was 0.06 $\mu g\ ml^{-1}$, 10 $ng\ ml^{-1}$, 10 $ng\ ml^{-1}$, and 0.07 $\mu g\ ml^{-1}$, for pilsicainide, propafenone, 5-(OH)propafenone and disopyramide, respectively. The milk/plasma ratio of each drug (M/P_{obs}) was calculated by the ratio of $AUC_{0-12\,h}$ for milk and plasma, obtained by the trapezoidal method. Pharmacokinetic parameters of pilsicainide after a single oral administration were estimated using the least square method applied to a one-compartment open model with first-order absorption and elimination. The maximum and minimum serum concentrations of pilsicainide at steady state were calculated by the estimated pharmacokinetic parameters using computer software. The body weight-adjusted relative infant dose of the drug was calculated from the assumption that a baby takes 150 $ml\ kg^{-1}\ day^{-1}$ of milk. Finally, we estimated the M/P ratio (M/P_{pred}) according to the previously reported equations of Begg [1], and compared the results with actual M/P_{obs}. The Ethics Committee of the Medical School approved the protocol.

The pilsicainide concentration in milk was higher than that in plasma at each observation point, whereas disopyramide and propafenone concentrations in milk were lower than those in plasma (Table 1). Estimated C_{max}/C_{min} at steady state, M/P_{obs}, protein binding, milk pH, octanol/water partition coefficient at pH 7.2 and pKa used for the prediction are also shown.

The value of M/P for disopyramide varies widely (from 0.4 to 0.9) among reports [5–7]. Because the protein binding ratio of disopyramide is nonlinear with respect to concentration [8], this difference might be due to the different concentrations of the drugs used in these reports. The M/P_{obs} of pilsicainide is highest among the three drugs and concentrations were detected at 12 h in both plasma and milk after a single dosing. This is the first report on the M/P ratio of pilsicainide. $AUC_{0-12\,h}$ of pilsicainide in milk was 4.7 $\mu g\ h^{-1}\ ml^{-1}$. If we assume that a baby takes 150 $ml\ kg^{-1}\ day^{-1}$ of milk, the estimated amount of ingested drug is 0.05875 mg, which means that the body weight-adjusted relative infant dose is 7% (Table 1). Because 10% is the notionally accepted cut-off for an average drug, this value for pilsicainide is just within the accepted range. The M/P_{obs} for propafenone was lowest among the three in this study. There is one report for propafenone [9] and the value is

Table 1
Pharmacokinetic profiles of three antiarrhythmic drugs

	Concentration 1 h		2 h		3 h		4 h		6 h		12 h		Estimated C_{max}/C_{min} at steady state		$AUC_{0-12\,h}/AUC_{0-\infty}$		pH of milk	Protein binding ratio (%)	pKa*	Octanol/ water partition coefficient*	M/P obs†	M/P pred‡	Relative infant dose (%)
	M	P	M	P	M	P	M	P	M	P	M	P	M	P	M	P							
Pilsicainide ($\mu g\ ml^{-1}$)	0.84	0.42	0.67	0.41	0.55	0.31	0.48	0.27	0.36	0.21	0.16	0.10	1.19/0.40	0.65/0.24	4.70/5.25 ($\mu g\ h^{-1}\ ml^{-1}$)	2.70/2.98	7.16	12.6	10.2	1.76	1.75	1.91	7
F-pilsicainide ($\mu g\ ml^{-1}$)		0.37		0.35		0.28		0.24		0.18		0.08											
Disopyramide ($\mu g\ ml^{-1}$)	0.47	1.35	0.65	1.41	0.64	1.46	0.64	1.41	0.44	1.03	0.11	0.45	0.89/0.37	2.47/1.21	4.80/5.10 ($\mu g\ h^{-1}\ ml^{-1}$)	11.80/12.21	6.90	81.4	8.36	0.66	0.41	0.79	3
F-disopyramide ($\mu g\ ml^{-1}$)		0.26		0.28		0.26		0.25		0.18		0.07											
PROP ($ng\ ml^{-1}$)	21.9	51.6	37.4	124.0	27.0	82.0	15.5	54.7	ND	26.3	ND	ND	39.5/0.58	118.2/4.3	109.6/109.8 ($ng\ h^{-1}\ ml^{-1}$)	444.8/422.46	6.97	82.7*	9.56	68.3	0.25	0.81	0.1
5-OH-PROP ($ng\ ml^{-1}$)	2.9	49.6	102.0	89.5	73.8	66.7	45.4	41.5	19.8	26.7	ND	12.9											

*M, Milk; P, plasma; F, free; PROP, propafenone. *Information from manufacturers. †Calculated from $AUC_{0-12\,h}$ milk/$AUC_{0-12\,h}$ plasma. ‡Calculated from Ref. 2. ln M/P = −0.09 + 2.54 ln (Mu/Pu) + 0.79 ln (fu,p) + 0.46 ln K.*

comparable to that reported in this study. The equation of Begg gave a close M/P_{pred} for pilsicainide. However, since there was only one subject in this study, further studies should be conducted with a larger number of subjects to confirm these findings. We did not collect samples more than 12 h after dosing, which is a limitation of this study. $AUC_{0-12\,h}$ was between 89 and 100% of AUC_{0-8} for the three drugs. We did not measure N-monodealkylated disopyramide, which has higher cholinergic activity than the parent compound, in this study. Accumulation of this metabolite into breast milk is reported [5], which might also have occurred in our study. Although the relative infant dose for disopyramide is low, careful monitoring is needed in the clinical setting.

In conclusion, we report here for the first time the drug transfer of pilsicainide after a single dosing in a healthy mother. We also evaluated transfer of disopyramide and propafenone in an identical mother. Begg's equation is effective for predicating the M/P-value, especially for pilsicainide. These observations may be useful for the treatment of arrhythmia in patients who are breast feeding.

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Cyclosporin A produces distal renal tubular acidosis by blocking peptidyl prolyl *cis-trans* isomerase activity of cyclophilin

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Watanabe, Seiji, Shuichi Tsuruoka, Soundarapandian Vijayakumar, Gunter Fischer, Yixin Zhang, Akio Fujimura, Qais Al-Awqati, and George J. Schwartz. Cyclosporin A produces distal renal tubular acidosis by blocking peptidyl prolyl *cis-trans* isomerase activity of cyclophilin. *Am J Physiol Renal Physiol* 288: F40–F47, 2005. First published September 7, 2004; doi:10.1152/ajprenal.00218.2004.—Cyclosporin A (CsA), a widely used immunosuppressant, causes distal renal tubular acidosis (dRTA). It exerts its immunosuppressive effect by a calcineurin-inhibitory complex with its cytosolic receptor, cyclophilin A. However, CsA also inhibits the peptidyl prolyl *cis-trans* isomerase (PPIase) activity of cyclophilin A. We studied HCO_3^- transport and changes in β-intercalated cell pH on luminal Cl^- removal in isolated, perfused rabbit cortical collecting tubules (CCDs) before and after exposure to media pH 6.8 for 3 h. Acid incubation causes adaptive changes in β-intercalated cells by extracellular deposition of hensin (*J Clin Invest* 109: 89, 2002). Here, CsA prevented this adaptation. The unidirectional HCO_3^- secretory flux, estimated as the difference between net flux and that after Cl^- removal from the lumen, was -6.7 ± 0.2 pmol·min^{-1}·mm^{-1} and decreased to -1.3 ± 0.2 after acid incubation. CsA in the bath prevented the adaptive decreases in HCO_3^- secretion and apical Cl^-:HCO_3^- exchange. To determine the mechanism, we incubated CCDs with FK-506, which inhibits calcineurin activity independently of the host cell cyclophilin. FK-506 did not prevent the acid-induced adaptive decrease in unidirectional HCO_3^- secretion. However, [AD-Ser]^8 CsA, a CsA derivative, which does not inhibit calcineurin but inhibits PPIase activity of cyclophilin A, completely blocked the effect of acid incubation on apical Cl^-:HCO_3^- exchange. Acid incubation resulted in prominent "clumpy" staining of extracellular hensin and diminished apical surface of β-intercalated cells [smaller peanut agglutinin (PNA) caps]. CsA and [AD-Ser]^8 CsA prevented most hensin staining and the reduction of apical surface; PNA caps were more prominent. We suggest that hensin polymerization around adapting β-intercalated cells requires the PPIase activity of cyclophilins. Thus CsA is able to prevent this adaptation by inhibition of a peptidyl prolyl *cis-trans* isomerase activity. Such inhibition may cause dRTA during acid loading.

bicarbonate secretion; cell pH; intercalated cell; hensin; cortical collecting duct

CYCLOSPORIN A (CSA) is a potent immunosuppressive cyclic undecapeptide whose use has changed the outcomes of transplantation and more recently autoimmune disease. Because this agent is administered for long periods and in the case of transplantation often permanently, it is not surprising that side effects began to appear. Among these complications, hypertension, renal fibrosis, and even the potential development of malignancies have clouded the salutary outcomes.

Cyclosporin can also cause distal renal tubular acidosis (dRTA) (28) independent of its "usual" nephrotoxic effects (1, 13, 17). Studies in patients undergoing treatment for liver transplantation as well as administration of CsA to experimental animals demonstrate the presence of dRTA without the presence of parenchymatous renal disease. Recently, we showed that net HCO_3^- absorption by isolated cortical collecting ducts (CCDs) from rats treated with CsA is reduced to 30% of the rate in CCDs from normal (32). The mechanism for this effect of CsA is also unknown. Cyclosporin exerts its immunosuppressive effect by binding to cyclophilin A, which then interacts with the Ser/Thr protein phosphatase calcineurin. It is calcineurin that mediates the blockade of transcriptional events needed for cellular immunosuppression. Besides their function as presenter proteins for CsA, cyclophilins also have an enzymatic activity; they catalyze the isomerization of peptidyl prolyl bonds, thereby accelerating slow steps in protein folding and oligomerization (9). However, it is thought that this activity is not relevant for the immunosuppressive action of CsA. In this paper, we show that CsA likely causes dRTA not by inhibiting calcineurin but rather by blocking the peptidyl prolyl *cis-trans* isomerase (PPIase) activity.

The CCD is composed of two cell types, the principal cell (responsible for salt and water transport) and the intercalated cell, which mediates acid-base transport. Intercalated cells exist in a spectrum of types with two extremes; a β-form that secretes HCO_3^- and an α-form that secretes acid. Acid-base disturbances can cause a reversal of polarity of HCO_3^- flux in the CCD of rabbits (21, 24, 25, 27, 30), rats (4), and mice (22, 23). Metabolic acidosis in rats and rabbits results in a reduction in the number of β-cells and an increase in the number of α-cells (5, 25). In rabbit CCDs incubated in vitro in acidic media, we found that individually identified β-intercalated cells were remodeled to functionally resemble α-intercalated cells (24). A similar remodeling of β-intercalated cells, in which the polarity of H^+ pumps and Cl^-/HCO_3^- exchangers is reversed, occurs in rabbit intercalated cells in cell culture and requires the deposition of polymerized hensin in the extracel-

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lular matrix (ECM). Seeding β-intercalated cells on already polymerized hensin converts them to an α-phenotype (34, 36).

We recently discovered in rabbit CCDs exposed to acidic media in vitro that hensin was deposited underneath the intercalated cells that were undergoing the conversion. Furthermore, a blocking antibody to hensin prevented the adaptation of β-intercalated cells in isolated, perfused CCDs (27). Hence, the adaptive conversion of β-intercalated cells to α-intercalated cells during acid incubation depends on ECM-associated hensin. In the present study, we demonstrate that CsA also blocks the acid-induced adaptation of β-intercalated cells. Furthermore, CsA prevented the deposition of hensin in the ECM of isolated, perfused tubules. Finally, we show that blockade of peptidyl prolyl *cis-trans* isomerization but not the calcineurin pathway is responsible for this effect. These studies are the first to directly implicate the PPIase activity of cyclophilins in the maturation of a functional protein in cells.

METHODS

Animals. Female New Zealand White rabbits weighing 1.5 to 3 kg were maintained on laboratory chow and water (30). Each rabbit was anesthetized using an intracardiac injection of pentobarbital sodium (100 mg/kg) after premedication with intramuscular xylazine (5 mg/kg) and ketamine (44 mg/kg) (27).

Microperfusion of CCDs. CCDs were microdissected and microperfused as performed in this laboratory (27, 30). Equilibration, transport and cell pH studies were performed using Burg's solution in the perfusate and bath, containing (in mM) 120 NaCl, 25 $NaHCO_3$, 2.5 K_2HPO_4, 2 $CaCl_2$, 1.2 $MgSO_4$, 5.5 D-glucose, 1 trisodium citrate, 4 sodium lactate, and 6 L-alanine, 290 ± 2 mosmol/kgH_2O, and gassed with 94% O_2-6% CO_2, yielding a pH 7.4 at 37°C (24, 27, 30). In most experiments, the bath was continually exchanged at 11 ml/h to maintain constant composition. In the case of a limited amount of synthesized inhibitor, bath osmolality was maintained within 1% by adding 60 μl water to the bath every 10 min for 1 h and replacing the bathing solution (and drug) each hour (27).

Incubations for 3 h in acid (pH 6.8 in both luminal and bathing solutions) and control (pH 7.4) media at 37°C were previously described (24, 30). The incubation at pH 6.8 yields a physiology comparable to 3-day acidosis in vivo, whereas that at pH 7.4 sustains net HCO_3^- secretion (24, 25).

Bicarbonate transport. Triplicate collections of 12–15 nl of tubular fluid were made under water-saturated mineral oil and analyzed for HCO_3^- (27, 30, 33). When J_{HCO3} was greater than 0, there was net HCO_3^- absorption; when J_{HCO3} was less than 0, there was net HCO_3^- secretion. To dissect out the coexisting fluxes of H^+ secretion by α-intercalated cells and HCO_3^- secretion by β-intercalated cells, we reversibly removed Cl^- from the luminal fluid (27, 30). CCDs were equilibrated for 5 min before collection of fluid during each experimental period. When Cl^- is removed from the lumen, HCO_3^- secretion ceases because of the inhibition of apical Cl^-/HCO_3^- exchange (30, 31); the uncovered flux is unopposed H^+ secretion. The difference between net flux and H^+ secretory flux is the HCO_3^- secretory flux (27). Measurements were repeated after the 3-h incubation (at pH 6.8 or 7.4) and compared with preincubation values. Transepithelial voltage was measured between calomel cells in 3 M KCl using the perfusion pipette as a luminal electrode (24, 27). In many of the incubations, CsA (Sigma, 5 or 10 μM) or tacrolimus (FK-506, 0.01–0.1 μM, Fujisawa) was added to the bath 10 min before the start of the acid incubation. Ten millimolar stock solutions of CsA and tacrolimus were prepared in methanol and diluted into the bathing fluid.

Cell pH studies. Cell pH was measured by excitation ratio fluorometry (490-nm/445-nm excitation; 520-nm emission) using 5–10 μM BCECF (Molecular Probes) (24, 27, 33). Fluorescence was detected in multiple intercalated cells and corrected for background (Photon Technology). Precision was enhanced by programming the averaging of two measurements during each snapshot. By examining cells in focus close to the perfusion pipette and in the wall of the tubule, we minimized movement and contaminating fluorescent signals. Readings were obtained in Burg's solution, after the reversible removal of luminal Cl^- and thereafter following the reversible removal of basolateral Cl^-. Readings after Cl^- removal were stable by 1 min and were obtained at 2 and 3 min of each period. These two readings were averaged for each period. The sequence of readings was repeated in the same identified intercalated cells after 3-h incubation.

In several of the incubations, CsA (5–10 μM), with a K_i value of 0.5 nM to inhibit the PPIase activity of cyclophilin A, and an IC_{50} of 100 nM of the CsA/cyclophilin A complex to inhibit calcineurin (6), the specific cyclophilin A inhibitor {O-[$NH_2(CH_2)_5NHC(O)CH_2$-]D-Ser}^{8}-CsA, ([AD-Ser]^8 CsA, or its inactive congener, CsH-Ac) was added to the bath. Ten micromolar [AD-Ser]^8 CsA inhibited <5% of calcineurin in the presence of saturating concentrations of cyclophilin A, and the inhibition of the PPIase activity exhibited an IC_{50} of 3.2 nM (Zhang Y and Fischer G, unpublished observations). CsH-Ac is a derivative of the completely inert cyclosporine compound CsH (8). The O-acetylation in position 1 of CsH allowed us to remove traces of CsA in the CsH samples but did not change the inertness of the compound. Ten micromolar CsH-Ac showed <5% inhibition of cyclophilin PPIase activity and of calcineurin (11). Stock solutions of the latter agents (1 mM) were prepared in 50% ethanol/50% water and diluted into media on the morning of study. The maximum amount of ethanol added to the bath would have been 0.5%, a concentration previously found to have no effect on tubule function (25). For the studies in isolated, perfused tubules, we used concentrations of inhibitors that were much higher than the IC_{50} shown above. These concentrations prevented the phenotype switching of cultured intercalated cells in vitro (data not shown). One likely reason for the need for much higher concentrations is that these compounds are hydrophobic, and all of our media contained albumin from fetal bovine serum at substantial concentrations. Hence, the free concentration of these agents could not be determined.

Calibrations using the nigericin and high-potassium buffer technique (24, 33) were performed at the end of each experiment.

Confocal fluorescence microscopy. Microperfused CCDs were incubated for 3 h at pH 6.8 in the presence or absence of CsA or [AD-Ser]^8 CsA, as described above, and then labeled extracellularly with anti-hensin antibody at 1:100 dilution in PBS/1% BSA by exposure at 4°C for 4–5 h, followed by fixation in Prefer (Anatech). After permeabilization by 0.1% Triton X-100, β-ICs in these CCDs were counterlabeled with rhodamine-peanut agglutinin (20 μg/ml), followed by secondary FITC-goat anti-guinea pig IgG (1:75) for 1–2 h at room temperature in the dark. Each tubule was transferred to a slide in 90% glycerol in PBS with 0.1% phenylenediamine (to prevent quenching), placed in coverslips, and examined using an Axiovert 100 laser-scanning confocal microscope (model LSM 410; Carl Zeiss) (15, 26, 27, 36). Images were collected using a ×40 objective (real magnification ×100), and 1-μm optical sections were obtained and analyzed by the Zeiss LSM-PC software. The final images were processed with Adobe Photoshop software.

Analysis and statistics. Data are presented as means ± SE. Paired and unpaired comparisons were performed using standard statistical software (Excel, Microsoft, Bellevue, WA). Significance was asserted when P values were <0.05.

RESULTS

Adaptation to in vitro acid incubation. When CCDs from normal rabbits are perfused and bathed in vitro in solutions simulating an ultrafiltrate of rabbit plasma at pH 7.4, they generally secrete HCO_3^- (25, 27, 30). After a 3-h incubation at

pH 6.8, these same tubules reverse their HCO_3^- transport rates to net absorption (24, 27, 31). We confirmed these results in seven newly studied CCDs, finding that the baseline rate of HCO_3^- transport reversed from a secretory flux of -3.5 ± 0.1 pmol·min^{-1}·mm^{-1} to a net absorptive flux of 2.6 ± 0.1 pmol·min^{-1}·mm^{-1} (Fig. 1, *panel 1*). When Cl^- was removed from the lumen before acid incubation, HCO_3^- secretion was inhibited, revealing an absorptive H^+ flux of 3.3 ± 0.1 pmol·min^{-1}·mm^{-1}. The difference between this flux and baseline represents the HCO_3^- secretory flux of -6.7 ± 0.2 pmol·min^{-1}·mm^{-1}. After acid incubation, the H^+ flux was 3.9 ± 0.2 pmol·min^{-1}·mm^{-1}, and the computed HCO_3^- secretory flux was significantly reduced to -1.3 ± 0.2 pmol·min^{-1}·mm^{-1}, similar to what has been recently published from our laboratory (27). In sum, acid incubation in vitro again caused the CCDs to reverse polarity of HCO_3^- flux from secretion to absorption, with the major adaptation being a reduction in HCO_3^- secretory flux from -6.7 to -1.3 pmol·min^{-1}·mm^{-1}, and the minor adaptation being a significant 0.6 pmol/min increase in H^+ secretion. Also, there was a significant decrease in luminal electronegativity from -2.9 to -2.4 mV, compatible with increased electrogenic H^+ secretion.

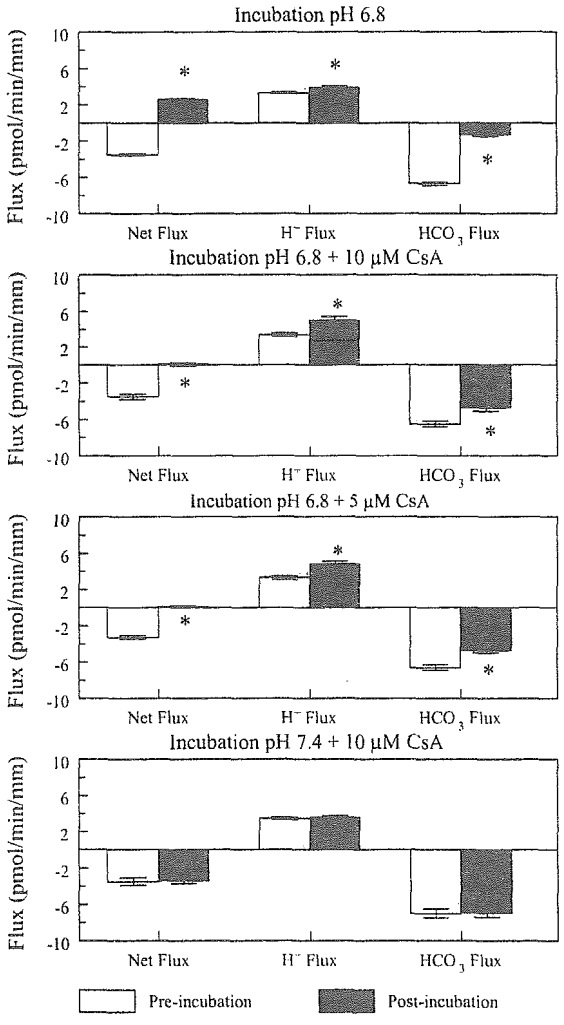

Fig. 1. Effect of Cyclosporin A (CsA) on HCO_3^- transport. Open bars are preincubation and filled bars are postincubation. Net HCO_3^- flux is *left* pair, unidirectional H^+ secretory flux is *middle* pair, and unidirectional HCO_3^- secretory flux is *right* pair. *Panel 1*: effect of incubation at pH 6.8 [$n = 7$ cortical collecting ducts (CCDs)]. *Panel 2*: incubation at pH 6.8 with 10 μM CsA ($n = 5$). *Panel 3*: effect of 5 μM CsA during incubation at pH 6.8 ($n = 6$). *Panel 4*: effect of 10 μM CsA during incubation at pH 7.4 ($n = 4$). *Statistical differences by paired *t*-test, $P < 0.05$.

CsA at 10 μM in the bath prevented the adaptive decrease in HCO_3^- secretion (Fig. 1, *panel 2*). In five CCDs, the baseline net flux was -3.5 ± 0.3 pmol·min^{-1}·mm^{-1}, and after acid incubation plus 10 μM CsA, it was 0.08 ± 0.2 pmol·min^{-1}·mm^{-1}. The H^+ secretory flux significantly increased from 3.4 ± 0.2 to 5.0 ± 0.4 pmol·min^{-1}·mm^{-1}, as has been observed previously after acid incubation (27). However, the adaptive decrease in HCO_3^- secretory flux was attenuated by 75% (-6.4 ± 0.3 to -4.7 ± 0.3 pmol·min^{-1}·mm^{-1} or a decrease of only 1.7 pmol·min^{-1}·mm^{-1}), compared with acid incubation alone. Transepithelial voltage significantly decreased from -2.9 ± 0.1 to -2.7 ± 0.2 pmol·min^{-1}·mm^{-1}, reflecting the increase in H^+ secretion.

Similar findings were obtained in six CCDs using 5 μM cyclosporin (Fig. 1, *panel 3*). The baseline net flux of -3.3 ± 0.2 changed to 0.1 ± 0.1 pmol·min^{-1}·mm^{-1}, and this was accompanied by a significant increase in H^+ secretory flux from 3.3 ± 0.2 to 4.8 ± 0.3 pmol·min^{-1}·mm^{-1}. The HCO_3^- secretory flux decreased only 29% from -6.6 ± 0.3 to -4.7 ± 0.3 pmol·min^{-1}·mm^{-1}, a change of 1.9 pmol·min^{-1}·mm^{-1}. Transepithelial voltage became significantly less negative (-2.8 ± 0.1 to -2.5 ± 0.1 mV), as H^+ secretion was increased in the adaptation.

Control incubation of four CCDs for 3 h at pH 7.4 in the presence of 10 μM cyclosporin did not change the net, H^+ secretory, or HCO_3^- secretory fluxes (Fig. 1, *panel 4*) or the transepithelial voltage (data not shown).

Adaptation of apical and basolateral Cl^-:base exchange. We examined the same intercalated cells before and after acid incubation to determine their adaptation to low pH. The vast majority of cells loaded from the lumen with BCECF-AM showed a reversible alkalinization in response to the removal of luminal Cl^- (Fig. 2), compatible with their being β-intercalated cells (27). Incubation at pH 6.8 × 3 h inhibited apical Cl^-/HCO_3^- exchange, manifested by a complete loss of this alkalinization (Fig. 2, *top* tracing, and Fig. 3, *panel 3*). The ΔpH before incubation averaged 0.42 ± 0.03 pH units, and this was reduced to 0.03 ± 0.02 units in 20 cells from four CCDs. In contrast, incubation at pH 7.4 × 3 h did not change apical anion activity (ΔpH 0.50 ± 0.03 to 0.46 ± 0.03; Fig. 3, *panel 1*) in 10 cells from 3 CCDs.

CsA (10 μM) in the bath prevented the loss of apical Cl^-/HCO_3^- activity (Fig. 2, *bottom* tracing, and Fig. 3, *panel 4*). The ΔpH before acid incubation averaged 0.50 ± 0.02, and after incubation was 0.44 ± 0.02 pH units in 21 cells from 4 CCDs. Similarly for a 3-h incubation at pH 7.4, CsA did not change the ΔpH in response to removal of luminal Cl^- (0.38 ± 0.02 to 0.42 ± 0.02 pH units) in 30 cells from 3 CCDs (Fig. 3, *panel 2*).

In response to the removal of bath Cl^-, β-intercalated cells show a decrease in cell pH (27) as shown in Fig. 2. This sensitivity was lost after pH 6.8 incubation (before: -0.45 ± 0.04, after: -0.04 ± 0.03 pH units; Fig. 4, *panel 3*). In addition, some cells now showed an alkalinization in response

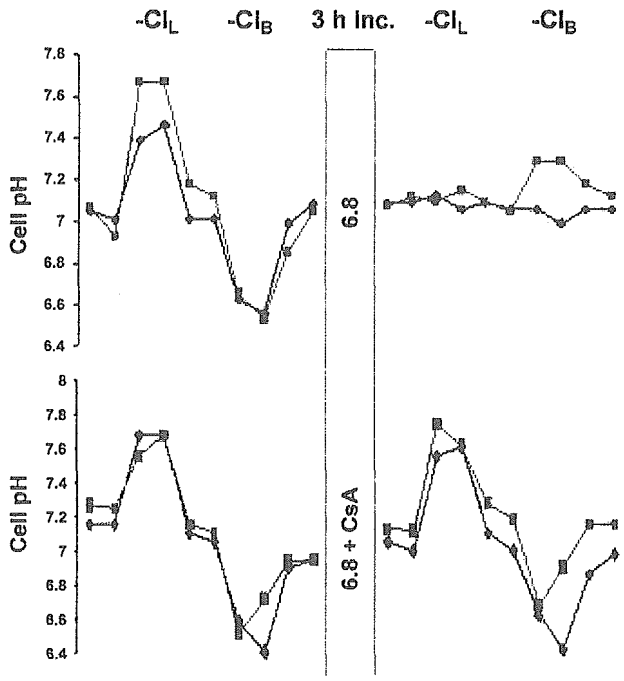

Fig. 2. Tracings of pH of individual cells studied under baseline conditions (Burg's solution in bath and lumen), Cl^--free lumen ($-Cl_L$), baseline, Cl^--free bath ($-Cl_B$), before and after the 3-h incubation (3-h Inc.). *Top*: 2 cells studied before and after 3-h incubation at pH 6.8. Note that 1 cell now alkalinizes on removal of bath Cl^- ($-CL_B$) after the 3-h incubation. *Bottom*: tracings of 2 cells studied before and after 3-h incubation at pH 6.8 in the presence of 10 μM CsA (6.8 + CsA).

to removal of bath Cl^- (see Fig. 2, *top* tracing). In contrast, incubation at pH 7.4 did not affect this sensitivity (before: -0.45 ± 0.03, after: -0.49 ± 0.03 pH units; Fig. 4, *panel 1*).

CsA in the bath prevented the loss of response to basolateral removal of Cl^- (Fig. 4, *panel 4*). The ΔpH before acid incubation was -0.43 ± 0.03, and after incubation was -0.43 ± 0.03 pH units. In a control study, 3-h incubation at pH 7.4, CsA slightly but significantly increased the ΔpH in response to the removal of basolateral Cl^- (before: -0.41 ± 0.03, after: -0.49 ± 0.3; Fig. 3, *panel 2*).

Mechanism of action of CsA. Cyclophilin A, the cytosolic receptor of CsA in immunosuppression, has two biochemical activities. In the cytosol, it forms the presenter protein for CsA whereby the resulting cyclophilin A/CsA complex specifically inhibits calcineurin. On the other hand, it catalyzes peptidyl prolyl *cis-trans* isomerizations, which often represent rate-limiting steps in the folding of proteins. Because the active site of cyclophilin A is involved in these processes, CsA blocks both the PPIase and protein phosphatase catalytic functions. We used another presenter protein complex known to block calcineurin activity, tacrolimus (FK-506/FKBP12 heteroassociate), to test whether the effect of CsA had been on the calcineurin pathway. We examined the effect of 0.01–0.1 μM (10–100 ng/ml) tacrolimus added to the bathing solution during the 3-h acid incubation (Fig. 5). The adaptive pattern resembled that of pH 6.8 alone. Baseline net HCO_3^- flux converted from -3.6 ± 0.2 pmol·min^{-1}·mm^{-1} secretion to 2.9 ± 0.2 pmol·min^{-1}·mm^{-1} absorption, whereas H^+ secretory flux significantly increased from 3.9 ± 0.2 to 4.9 ± 0.2 pmol·min^{-1}·mm^{-1}. Accompanying the increase in H^+ secretion was a significant decrease in luminal electronegativity (-2.9 ± 0.1 to -2.3 ± 0.1 mV). The HCO_3^- secretory flux adapted normally, decreasing from -7.5 ± 0.3 to -1.9 ± 0.2 pmol·min^{-1}·mm^{-1}, a decrease of 5.6 pmol·min^{-1}·mm^{-1}. These results demonstrate that CsA did not block adaptation through the calcineurin pathway. It is well known that tacrolimus does not influence the PPIase activity of cyclophilins (7, 12).

Recently, CsA derivatives have been developed that block the PPIase activity of cyclophilin A without affecting the calcineurin pathway. One of these substances, [AD-Ser]^8 CsA, was used here to test its effect on the adaptation of β-intercalated cells to acid treatment. A structural analog of [AD-Ser]^8 CsA, CsH-Ac, a derivative of the inert CsH (8), does not block the PPIase activity of cyclophilins and was used as a control. In 12 cells from 3 CCDs, CsH-Ac did not prevent the adaptation to low pH: before incubation, the increase in cell pH on luminal Cl^- removal was 0.46 ± 0.01 pH units, and after pH 6.8 incubation the increase in pH was completely blunted: 0.03 ± 0.03 pH units (Fig. 6, *bottom* tracing, and Fig. 7, *bottom*). Similarly, the effect of the inactive congener, CsH-Ac, was comparable to that of pH 6.8 alone on removal of bath chloride. Before incubation with CsH-Ac, cell pH decreased by

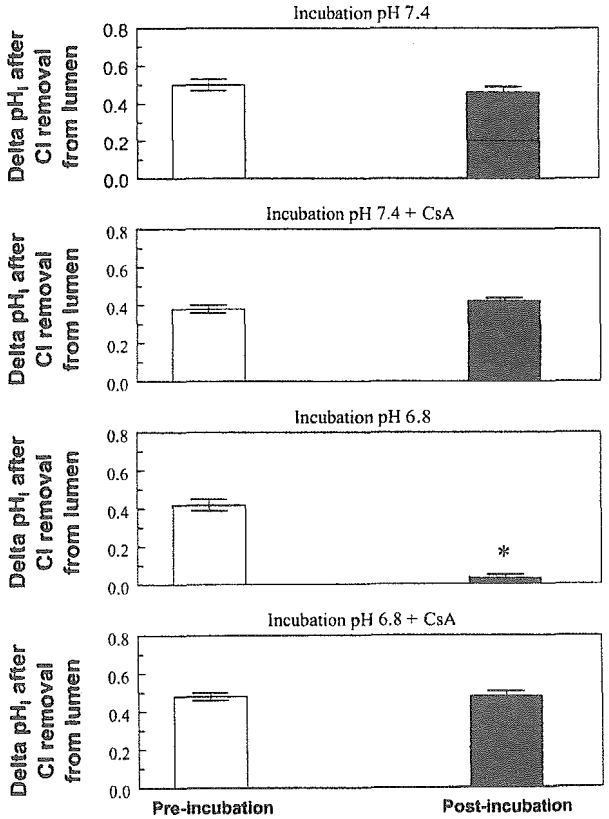

Fig. 3. Summary data showing the effect on delta intracellular pH (ΔpH_i) of removing Cl^- from the lumen (ΔpH was calculated from the difference in cell pH before and after removing Cl^- from the lumen). Open bars are preincubation and filled bars are postincubation values. *Panel 1*: incubation at pH 7.4 (n = 10 cells in 3 CCDs). *Panel 2*: incubation at pH 7.4 plus 10 μM CsA (n = 30 cells in 3 CCDs). *Panel 3*: incubation at pH 6.8 (n = 20 cells in 4 CCDs). *Panel 4*: incubation at pH 6.8 plus 10 μM CsA (n = 21 cells in 4 CCDs). *Significantly different from preincubation value by paired t-test, $P < 0.05$.

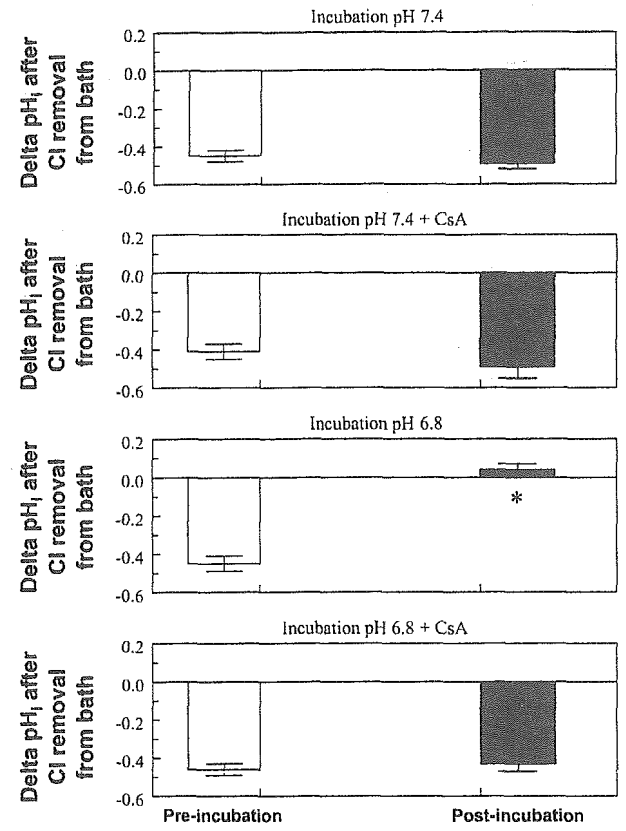

Fig. 4. Summary data showing the effect on ΔpH_i of removing Cl^- from the bath (ΔpH was calculated from the difference in cell pH before and after removing Cl^- from the bath). Open bars are preincubation and filled bars are postincubation values. *Top panel 1*: incubation at pH 7.4. *Panel 2*: incubation at pH 7.4 plus 10 μM CsA. *Panel 3*: incubation at pH 6.8. *Bottom panel 4*: incubation at pH 6.8 plus 10 μM CsA. The numbers of cells and CCDs are the same as in the legend to Fig. 3. *Significantly different from preincubation value by paired *t*-test, $P < 0.05$.

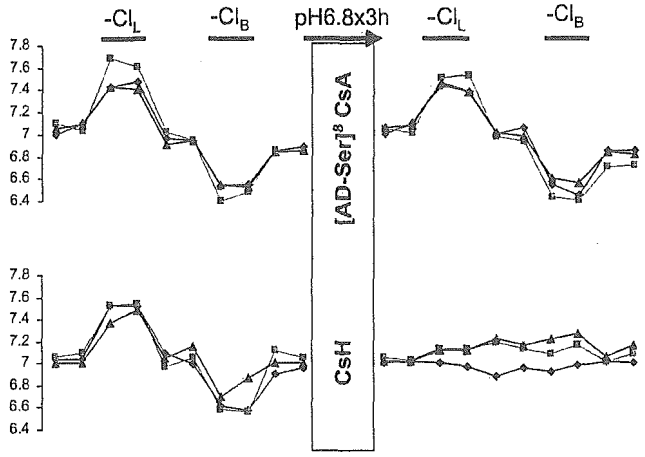

Fig. 6. Tracings of pH of individual cells studied under baseline conditions (Burg's solution in bath and lumen), $-Cl_L$, baseline, $-Cl_B$, before and after the 3-h Inc. *Top*: 3 cells studied before and after 3-h Inc. at pH 6.8 in the presence of 10 μM [AD-Ser]^8 CsA. *Bottom*: tracings of 3 cells studied before and after 3-h Inc. at pH 6.8 in the presence of 10 μM CsH (inactive agent).

-0.46 ± 0.02 pH units in response to removal of bath Cl^-, but after incubation the decrease was completely attenuated (0.01 ± 0.03 pH units, $n = 12$ cells from 3 CCDs) (Figs. 6 and 8, *bottom*).

In contrast the potent monofunctional PPIase inhibitor [AD-Ser]^8 CsA prevented the adaptation. In 36 cells from 4 CCDs, the removal of luminal Cl^- raised cell pH by 0.45 ± 0.05 pH units before incubation. After a 3-h incubation at pH 6.8 in the presence of 10 μM [AD-Ser]^8 CsA, adaptation of β-intercalated cells was prevented: cell pH increased by a similar value of 0.42 ± 0.04 pH units (Fig. 7, *top*). The tracing of representative cells shows virtually no effect of the pH 6.8 incubation in the presence of [AD-Ser]^8 CsA (Fig. 6, *top* tracing). Incubation at pH 6.8 plus 10 μM [AD-Ser]^8 CsA in the bath also

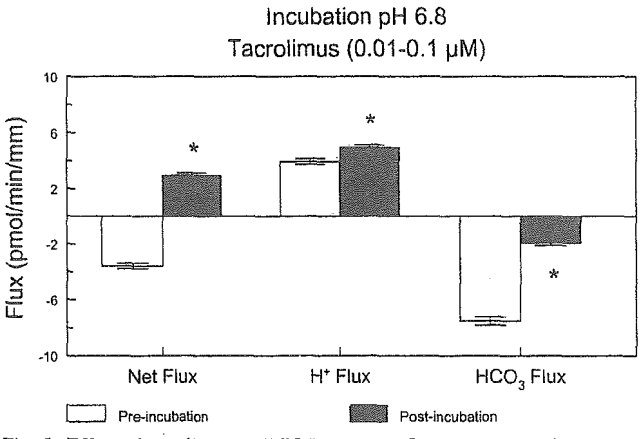

Fig. 5. Effect of tacrolimus on HCO_3^- transport. Open bars are preincubation and filled bars are postincubation. Net HCO_3^- flux is *left* pair, unidirectional H^+ secretory flux is *middle* pair, and unidirectional HCO_3^- secretory flux is *right* pair ($n = 6$ CCDs). *Significantly different from preincubation value by paired *t*-test, $P < 0.05$.

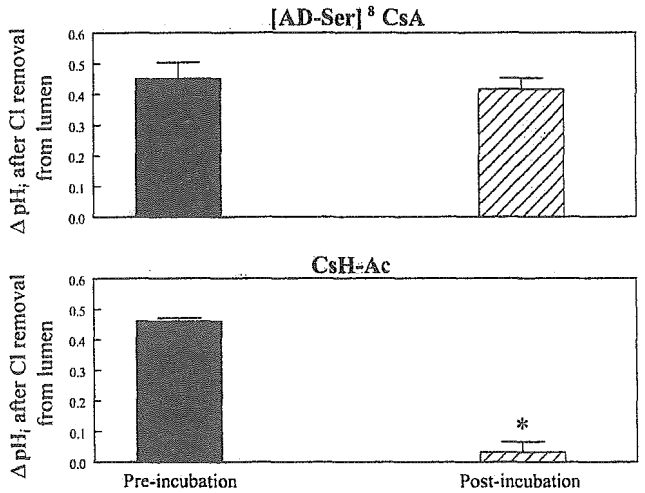

Fig. 7. Summary data showing the effect on ΔpH_i of removing Cl^- from the lumen (ΔpH was calculated from the difference in cell pH before and after removing Cl^- from the lumen). Filled bars are preincubation and slashed bars are postincubation values. *Top*: incubation at pH 6.8 in the presence of the PPIase inhibitor [AD-Ser]^8 CsA ($n = 36$ cells in 4 CCDs). *Bottom*: incubation at pH 6.8 plus 10 μM CsH (inactive agent, $n = 12$ cells in 3 CCDs). *Significantly different from preincubation value by paired *t*-test, $P < 0.05$.

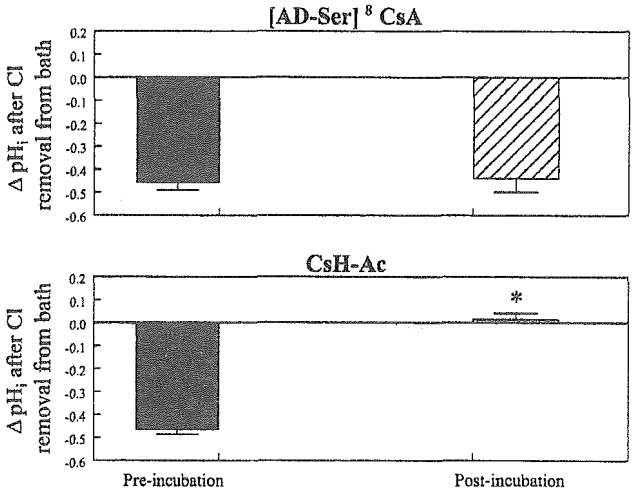

Fig. 8. Summary data showing the effect on ΔpH_i of removing Cl^- from the bath (ΔpH was calculated from the difference in cell pH before and after removing Cl^- from the bath). Filled bars are preincubation and slashed bars are postincubation values. *Top*: incubation at pH 6.8 in the presence of the PPIase inhibitor, [AD-Ser]^8 CsA. *Bottom*: incubation at pH 6.8 in the presence of 10 μM CsH (inactive agent). The numbers of cells and CCDs are the same as in the legend to Fig. 7. *Significantly different from preincubation value by paired *t*-test, $P < 0.05$.

prevented adaptive changes in response to removal of bath Cl^-. Before incubation, the removal of bath Cl^- reduced cell pH by -0.46 ± 0.03 units, and after incubation at pH 6.8 plus 10 μM [AD-Ser]^8 CsA there was a comparable acidification of -0.44 ± 0.06 pH units ($n = 36$ cells in 4 CCDs) (Figs. 6 and 8, *top*).

Deposition of extracellular hensin. CCDs were incubated 3 h at pH 6.8 in the presence or absence of CsA and stained for extracellular hensin (green) and peanut agglutinin (PNA, red). Control CCDs were dissected in pH 7.4 media, not incubated, and immediately stained for extracellular hensin and then rhodamine PNA (Fig. 9, *top left*). There was minimal staining for extracellular hensin and prominent red PNA caps in a confocal 1-μm image. Figure 9, *top right*, shows a CCD after incubation at pH 6.8. Hensin staining was increased and appeared "clumpy" or clustered. The PNA caps were less prominent. Figure 9, *bottom left*, shows a CCD after incubation at pH 6.8 in the presence of 10 μM CsA. Hensin staining appeared decreased compared with Fig. 9, *top right*, and the red PNA caps were more prominent; the appearance resembled more closely that at pH 7.4 in the *top left*.

Incubation of CCDs at pH 6.8 in the presence of the PPIase inhibitor [AD-Ser]^8 CsA also prevented the acid-induced increase in extracellular hensin staining (less green staining in Fig. 9, *bottom right*). Note also that the acid-induced reduction in PNA labeling (red) was also prevented.

DISCUSSION

Identification of the genes responsible for human dRTA has recently been complemented by a number of gene deletions that cause the same syndrome in mice. Of the human genes, several subunits of the H^+-ATPase, the anion exchanger AE1 and carbonic anhydrase II, all cause defects in the well-described H^+ secretory pathway by the α-intercalated cell. Studies in human dRTA have identified further heterogeneity where the loci were not linked to the ATPase, AE1 or carbonic anhydrase (19). Identification of these genes promise to identify new mechanisms that mediate acid-base transport in the CCD. Our finding that the mechanism by which cyclosporin causes dRTA provides an additional new mechanism for the causation of dRTA, namely, a defect in the adaptation of β-intercalated cells to acidosis. Other studies in collecting duct function have recently identified pendrin as an apical anion exchanger expressed in the β-intercalated cell. Mutations in this gene would not be expected to cause acidosis, rather they ought to cause distal renal tubular alkalosis, although this has not been seen in either humans or mice except under conditions of mineralocorticoid loading (35).

We recently discovered that the mechanism of conversion of a β- to α-intercalated cell is critically dependent on the deposition of an extracellular matrix protein, hensin (reviewed in Ref. 2). Hensin is expressed in intracellular vesicles of all cells of the collecting duct but only underneath the α-intercalated cells of the CCDs and in medullary collecting tubules is it present in the ECM (27). Moreover, during the adaptation of β-intercalated cells to lowering the bath pH, hensin becomes deposited in the ECM under these β-cells. Deposition of hensin in the ECM requires extensive polymerization (14, 15). We discovered that addition of labeled monomeric hensin to α-phenotype intercalated cells in the in vitro cell culture system resulted in the formation of higher order multimers. In other words, there exists an ECM activity capable of polymerizing hensin (14). We propose that this activity is among the cyclophilins, enzymes known to assist protein folding and oligomerization. At present, this speculation is entirely based on the fact that acid treatment in the presence of specific inhibitors of the PPIase function of cyclophilins did not lead to deposition of hensin in the ECM and consequently no adaptation of β-intercalated cells. A structurally related CsA derivative, CsH-Ac, which lacks active site blockade of cyclophilin A, was completely inactive in preventing the adaptation of β-intercalated cells. All we know at present is that CsA and PPIase inhibitors are blocking the process "upstream" of the deposition of hensin. But the following arguments suggest that the speculation is plausible.

Mature hensin contains eight SRCR domains separated from each by a proline-rich string of 23 amino acids. Productive formation of polymerized hensin from monomers with the underlying slow prolyl *cis-trans* isomerizaion reactions of the proline-rich segments of hensin might benefit from PPIase catalysis by avoiding aggregation-prone folding intermediates. Importantly, prolyl *trans*-to-*cis* isomerization was shown to be the prerequisite for dimerization of the antibody domain C(H3). In refolding experiments, the species with the wrong *trans* isomerization state accumulated as a monomeric folding intermediate (29).

For the extracellular polymerizing activity to be a PPIase of the cyclophilin type, the protein would obviously have to be secreted. There are three cyclophilins known to be directed to the extracellular space. The original member, cyclophilin A, was found to be a cytosolic enzyme. Two homologs, cyclophilins B and C, were discovered, and each of these proteins has a signal peptide allowing it to be secreted by the classical protein transport pathway. Both of these proteins can be secreted, although it is thought that cyclophilin B is most often located in the endoplasmic reticulum. Cyclophilin C was dis-

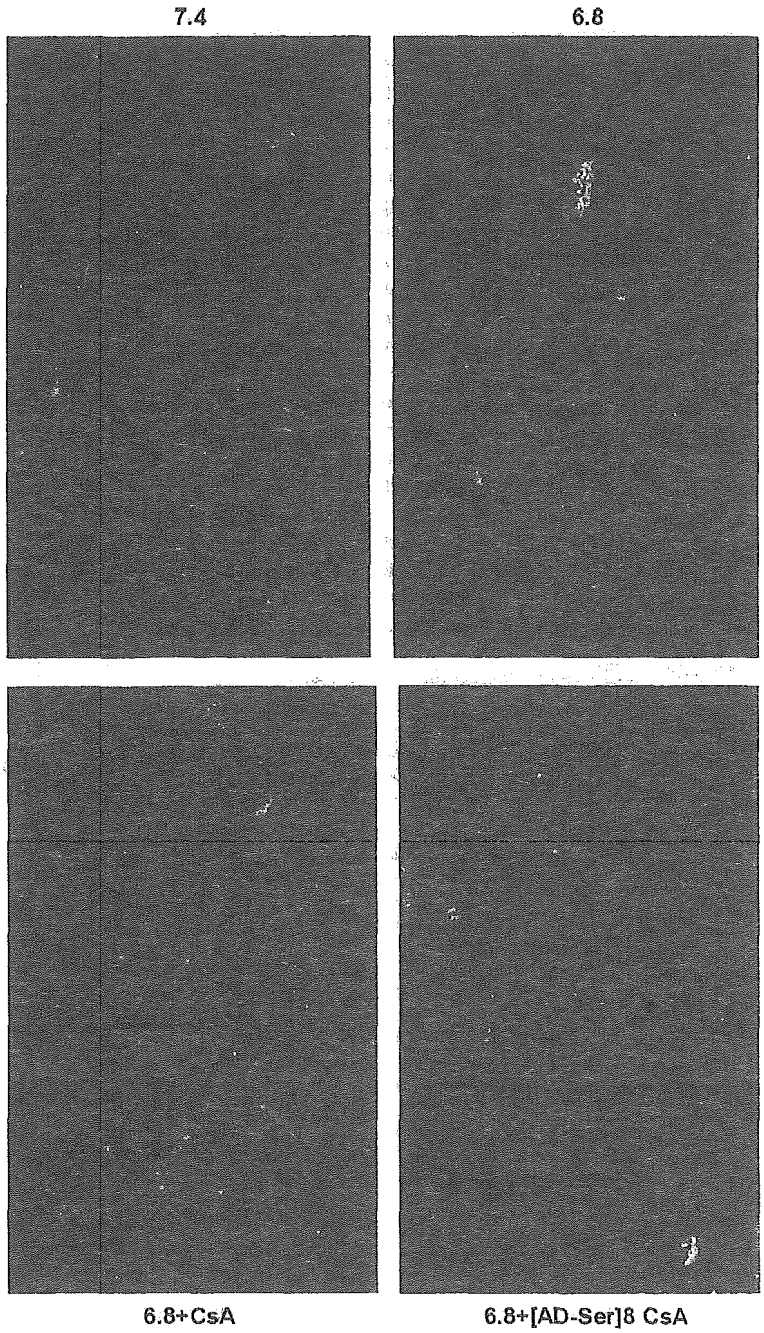

Fig. 9. Confocal microscopic photomicrographs of CCDs labeled for extracellular hensin (green) and peanut agglutinin (red). *Top left*: CCD exposed to pH 7.4. *Top right*: CCD incubated at pH 6.8 × 3 h. *Bottom left*: CCD incubated at pH 6.8 plus 10 μM CsA. *Bottom right*: CCD incubated at pH 6.8 plus 10 μM [AD-Ser]^8 CsA.

covered as a protein that binds to a 77-kDa protein whose sequence reveals that it contains an SRCR domain (10). Some studies have shown that cyclophilin B is secreted and acts to mediate adhesion of lymphocytes to the ECM (3). There has been a recent report that even cyclophilin A can be secreted (18). These studies have shown that oxidative stress causes release of cyclophilin A from vascular smooth muscle. We point out that there are a number of similarities between oxidative stress and acidosis.

CsA exerts its immunosuppressive effect by binding to cyclophilin A. a cytoplasmic protein with IIPase enzymatic activity. CsA also binds to and inhibits the enzymatic activity of the several other cyclophilin homologues present in mammalian genomes. The cyclophilin:cyclosporin complex interacts with the calcium-regulated Ser/Thr protein phosphatase calcineurin, blocking its dephosphorylating activity. Calcineurin is critical for the immune response where it dephosphorylates the cytoplasmic form of the transcription factor nuclear factor of activated T cells, allowing it to translocate to the nucleus and activate the transcription of immune response genes including IL-2 (8, 20).

FK-506 (tacrolimus) is another immunosuppressive agent whose targets are the protein family of FK binding proteins (FKBP's) with the most prominent cytosolic member FKBP12.

FK-506 is completely inert toward cyclophilins (7, 12). However, the FK-506/FKBP complex also interacts with calcineurin, blocking its enzymatic activity and interrupting the same pathway as CsA/Cyclophilin A. It is well known that cyclophilins are widely distributed in cells and that calcineurin is critical for a number of physiological actions in a variety of cells and tissues. There is at present no doubt that the mechanism of immunosuppressive effect of CsA and FK-506 is mediated through the calcineurin pathway. However, despite a wealth of biochemical and molecular studies on this system it has been difficult to define the mechanism of the side effects of CsA. For instance, CsA is associated with hyperkalemia, but it is not possible to conclude whether the effect is simply due to the acidosis (with its effect on re-distribution of cell K^+ or inhibition of K^+ secretion) or is a direct effect on a specific K^+ transporting cell. There is increasing evidence that nephrotoxicity associated with immunosuppressive therapy by CsA can be mediated by inhibition of the PPIase activity of cyclophilin A (16). However, could blockade of this enzymatic activity be responsible for at least some of the other complications? Our present studies are the first to directly suggest a role for this enzymatic activity in causing one of the known complications of CsA treatment. Future work will have to determine whether the other complications are also related to this effect.

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Regulation of cholesterol 7α-hydroxylase mRNA expression in C57BL/6 mice fed an atherogenic diet

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Abstract

The nuclear receptors liver X receptor (LXR) α and farnesoid X receptor (FXR) are positive and negative regulators of cholesterol 7α-hydroxylase (CYP7A1) transcription, respectively. To clarify their roles in the regulation of CYP7A1 in mice, we investigated mRNA expression of their target genes in the livers of C57BL/6 mice fed the following five diets for 2 weeks: a standard diet, cholic acid, cholesterol, cholesterol + high fat, or an atherogenic diet (cholic acid + cholesterol + high fat). The mRNA level of ATP-binding cassette transporter (ABC) A1 gene, one of LXRα target genes, significantly increased on the diets containing cholic acid and/or cholesterol + high fat, but not on the diet containing cholesterol alone. On the other hand, the mRNA levels of the FXR target genes ABCB11, ABCC2, and short heterodimer partner increased only on the diet containing cholic acid with or without cholesterol + high fat. Surprisingly, cholesterol alone or cholesterol + high fat did not affect CYP7A1 mRNA level, whereas cholic acid with or without cholesterol + high fat greatly reduced the level. Thus, in the atherogenic diet-fed mice, cholic acid component is needed for the FXR activation, and FXR dominantly regulates CYP7A1 transcription.

Keywords: CYP7A1; Liver X receptor; Farnesoid X receptor; Cholic acid; ATP-binding cassette transporter A1; Short heterodimer partner

1. Introduction

Cholesterol 7α-hydroxylase (CYP7A1) is a liver-specific enzyme which catalyzes the first and rate-limiting step in the classical bile acid synthetic pathway [1]. The enzyme converts cholesterol into 7α-hydroxycholesterol, and subsequent enzymatic steps lead to the conversion of 7α-hydroxycholesterol mainly into cholic acid. Because bile acid synthesis is the major pathway responsible for the maintenance of whole body cholesterol homeostasis, the regulation of CYP7A1 activity seems to be important. Actually, dietary cholesterol markedly stimulates CYP7A1 transcription in rats [2–5]. On the other hand, feeding cholesterol to hamsters, guinea pigs, rabbits, and monkeys does not lead to the stimulation (or even leads to the suppression) of CYP7A1 activity [6–9]. Thus, the effect of dietary cholesterol on CYP7A1 activity differs between the species.

Recently, it has been shown that the expression of CYP7A1 gene is controlled by the transcription factors belonging to the nuclear receptor superfamily [1]. Liver X receptor (LXR) α, whose endogenous ligands are the oxysterols, positively regulates CYP7A1 transcription, while farnesoid X receptor (FXR), whose potent ligands are the bile acids, negatively does. Interestingly, LXRα-mediated up-regulation of CYP7A1 dominates FXR-mediated stimulus in cholesterol-fed rats [10], while FXR is the dominant regulator in cholesterol-fed rabbits [11]. Therefore, such difference in

Abbreviations: ABC, ATP-binding cassette transporter; CYP7A1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LRH, liver receptor homolog; LXR, liver X receptor; PXR, pregnane X receptor; SHP, short heterodimer partner

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