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Transport of choline by Madin-Darby canine kidney cells

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Choline is an essential precursor for the synthesis of phosphatidylcholine, the most abundant phospholipid classes in renal cells, as well as for the synthesis of the osmolyte glycerophosphorylcholine. The characteristics of choline uptake in the renal epithelial cell line MDCK were investigated. In the range of physiological concentrations, choline entered MDCK cells, grown as a monolayer on solid support, via a specific sodium-independent transport system (apparent $K_m = 43$ μM, apparent $V_{max} = 284$ pmol/mg protein per 5 min). Cell ATP depletion, addition of KCl to the medium to reduce the cell membrane potential, and hemicholinium-3 (HC-3) inhibited choline uptake. Specific binding of [^{3}H]HC-3 was detected on the apical membrane of cells grown on plastic dishes, whereas it occurred only on the basolateral domain of cells grown on permeant support. When growing cells on filter, choline uptake from the basolateral side was 10-times the apical uptake. This suggests that the choline carrier present at the apical domain of cells grown on solid support is either inactivated or no longer targeted to the apical but to the basolateral membrane of MDCK cells grown on filter.

Introduction

Choline uptake represents the first step in the major pathway of biosynthesis of choline-containing phospholipids by living cells [1,2]. The kidney plays a major role in the homeostatic regulation of plasma choline levels. At physiological concentrations, the fractional excretion of choline is usually below 5% [3]. Part of the transported choline is used for the synthesis of phosphatidylcholine (PC), which is in the kidney, as in other organs, the most abundant phospholipid [1,4]. In addition, glycerophosphorylcholine (GPC), an organic osmolyte concentrated in renal medullary cells [5,6], is obtained via PC degradation [6,7]. Madin-Darby canine kidney (MDCK) cells, a renal epithelial cell line which retains differentiated properties of distal nephron segments [8,9], was shown to respond to hyperosmolarity by an increase in intracellular GPC content dependent on the presence of choline in the culture medium [5]. The characteristics of choline uptake by these cells and, more generally, by cells constituting the distal parts of the nephron, are, except for the recent data on isolated cells from rat inner medullary collecting duct (IMCD) [10], poorly documented. The present study was undertaken to characterize the uptake of choline by MDCK cells grown as confluent monolayers. The results demonstrate that, at physiological concentrations, choline is transported into MDCK cells, grown as a monolayer on plastic support, by a specific sodium-independent system, likely electrogenic, which is affected by intracellular ATP depletion. Growing cells on permeant support resulted in a basolateral uptake 10-times the apical uptake, suggesting a preferential localization of the carrier in the basolateral membrane.

Materials and Methods

Materials

[*methyl*-^{3}H]Choline chloride (81.8 Ci/mmol) was obtained from Amersham; $KH_2^{32}PO_4$ (1 Ci/mmol) was obtained from Dositek (France). [*methyl*-^{3}H]Hemicholinium-3 diacetate salt (124 Ci/mmol) was purchased from New England Nuclear. Ethanolamine, 2-dimethylethanolamine, hemicholinium-3 (HC-3) and tetraethylammonium chloride (TEA) were obtained from Sigma. 1-Pyrene butyrylcholine bromide was obtained from Molecular Probes. All other biochemicals used were of the highest purity available and obtained from regular commercial sources.

Cell culture

MDCK cells (passages 74 to 76) were grown to confluence on plastic dishes (Costar No. 3100), under a 5% CO_2/95% air atmosphere in a 50:50 mixture of

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Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 10% fetal calf serum, at 37°C [11,12]. After two passages, cells were seeded on 24-well culture dishes and grown for 5 days: the first 4 days with the complete medium in the presence of 10% fetal calf serum (the medium was changed for fresh medium every 48 h), the last day without serum. For experiments using a permeable support, cells were seeded on 0.4 μm pore Transwell (Costar n°3413), after two passages on plastic dishes, and grown as previously described [13]. Briefly, cells were grown 2 days with complete medium in the upper and lower chambers. The medium was then changed for fresh medium containing (lower chambers) or not (upper chambers) fetal calf serum. The medium was changed, 24 h prior to the experiment, for fresh serum-free medium in both the lower and the upper chambers.

Choline uptake studies

(A) Cells grown on plastic dishes. Determinations of choline uptake by confluent MDCK cells monolayer were performed with cells attached to the bottom of 24-well Petri dishes. The uptake medium contained (in mmol/l): 137 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 Hepes buffer (pH 7.2) and 4 glutamine. Sodium dependence of choline uptake was tested by replacing isoosmotically NaCl with *N*-methylglucamine. Osmolarity of each medium was maintained at 300 mosM, as determined by freezing point osmometry. Monolayers were washed four times with substrate-free transport medium at 37°C, followed by a 10 min temperature equilibration prior to the incubation at 37°C in 300 μl uptake medium containing 5 μCi/ml of [^{3}H]choline. The uptake was stopped by adding 2 ml of ice-cold substrate-free uptake medium. Monolayers were rapidly rinsed four times with this solution. The culture dishes were allowed to dry and 250 μl of 1 M NaOH was added to solubilize the cells. Incorporated radioactivity was determined by liquid scintillation counting and aliquots were collected for protein determination. All the results were corrected for diffusion, which was estimated by adding a 1000-fold excess of non-labeled choline to 50 μM of [^{3}H]choline.

(B) MDCK cells grown on filters. Choline uptake by MDCK cells grown on permeable support was measured on day 3 to 4 after confluence in a medium identical to that used for the uptake on plastic dishes-attached cells. Cells were rinsed four times with substrate free medium before incubation at 37°C, with radiolabeled choline (5 μCi/ml, 50 μM) either at the apical (100 μl) or at the basolateral (600 μl) side of filters. After 10 min of uptake, filters were washed four times with ice-cold medium and cut off and the radioactivity was determined by liquid scintillation counting. As before, the results were corrected for diffusion, which was estimated by adding, at the apical or at the basolateral side of the cells, a 1000-fold excess of non-labeled choline to 50 μM of [^{3}H]choline.

Inhibition of choline uptake by various structural analogues was tested by adding 500 μM of the selected products to the uptake medium containing 50 μM [^{3}H]choline.

Phosphate uptake studies

Phosphate uptake by cells grown on plastic dishes was determined as previously described [11]. Briefly, uptakes were measured at 37°C in a buffer solution of the following composition (mmol/l): 137 NaCl, 5.4 KCl, 2.8 $CaCl_2$, 1.2 $MgSO_4$ 15 Hepes (pH 7.4); cells were incubated in the uptake solution in presence of $K_2H^{32}PO_4$ (1 μCi/ml). The uptake was stopped by washing the cells three times with 1 ml/ well of ice-cold solution (137 mM NaCl, 15 mM Hepes (pH 7.4)). Cells were then solubilized in 250 μl 1 M NaoH and aliquots were counted by liquid scintillation.

Specific hemicholinium binding

Specific binding of [^{3}H]HC-3 was determined on cells grown either on plastic dishes or on filters. Day 5 cells grown on 24-well plastic dishes, serum deprived for 24 h, were incubated for 30 min under gentle stirring, with 0.1 to 4 nM [^{3}H]HC-3. Incubation in parallel with 10 μM unlabelled HC-3 allowed the determination of non-specific binding. Incubation was stopped by washing the monolayer with ice-cold buffer. Washing was repeated four times, each wash implying a 2 min incubation with the buffer. After the washing steps, cells were incubated for 1 h, at room temperature, in 250 μl 1 M NaOH before scintillation counting. Sodium dependence of [^{3}H]hemicholinium-3 binding was tested by replacing NaCl, isoosmotically, by *N*-methylglucamine. The inhibition of [^{3}H]hemicholinium-3 binding by choline was tested by adding 500 μM choline to the binding medium. Protein determinations were performed on cells obtained from parallel cultures, grown from the same cell suspension. For cells grown on filters, 3.8 nM [^{3}H]HC-3 was added either to the apical or to the basolateral side of the filter. Besides the non-specific binding to the cells, determined as above, basolateral labelling was corrected for [^{3}H]HC-3 adsorption to the filter by dividing by two the HC-3 adsorption on filters without cells. After the washing steps, filters were cut from the plastic holder and dipped into the scintillation liquid, 24 h before counting.

Protein determination

Protein determinations were made with the Pierce BCA protein assay reagent (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Data analysis

Results were expressed as mean ± S.E. of three to six independent experiments, in which cell sampling was done in triplicate. Comparisons were performed by a *t*-test. $P < 0.05$ was considered significant. Kinetics analysis were performed according to De Lean et al. [14] and Malo and Berteloot [15].

Results

Choline uptake by MDCK cells grown on solid support

At 50 μM, a concentration in the physiological range for the dog and numerous mammalian species [16–18], choline uptake by a confluent monolayer of MDCK cells was linear up to 30 min incubation and accounted for 398 ± 10 and 776 ± 83 pmol/mg protein for 15 and 30 min, respectively ($n = 3$). The subsequent series of experiments were performed using 5 min incubation periods. For kinetic analysis, [^{3}H]choline uptake was tested for a range of concentrations between 0.01 μM and 3 mM, on six different cell batches. As shown by Fig. 1, replacing sodium by *N*-methylglucamine in the incubation medium had no effect on choline uptake. Curve analysis of the uptake revealed the presence of a single transport system for choline. The corresponding kinetic constants were (a) in the presence of sodium: $K_{m,app} = 43.7 \pm 5$ μM, $V_{max,app} =$ 284 pmol/mg protein per 5 min; (b) when replacing sodium by *N*-methylglucamine in the medium: $K_{m,app} = 39 \pm 8.8$ μM, $V_{max,\,app} = 282$ pmol/mg protein per 5 min. These results indicated that, at physiological concentration, the choline uptake by MDCK cells was mediated by a sodium-independent transport system.

For further characterization of this system, the influence of membrane potential and of energy poisons on choline uptake were investigated. The presence of 50 mM KCl in the medium, which depolarizes the membrane of renal distal cells [10], inhibited by 22%

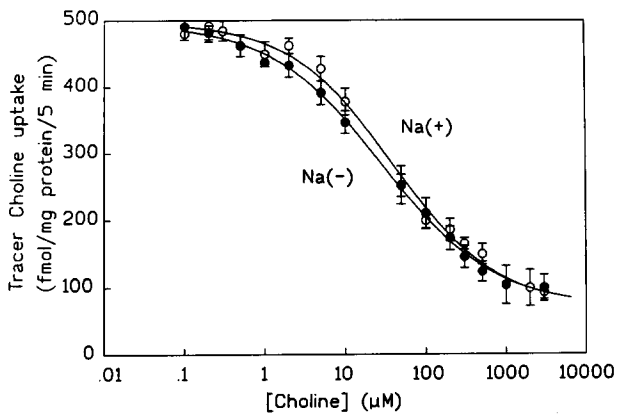

Fig. 1. Kinetics of choline uptake by MDCK cells. [^{3}H]choline uptake in absence or presence of sodium. Data are means ± S.E. of six independent experiments ($n = 6$).

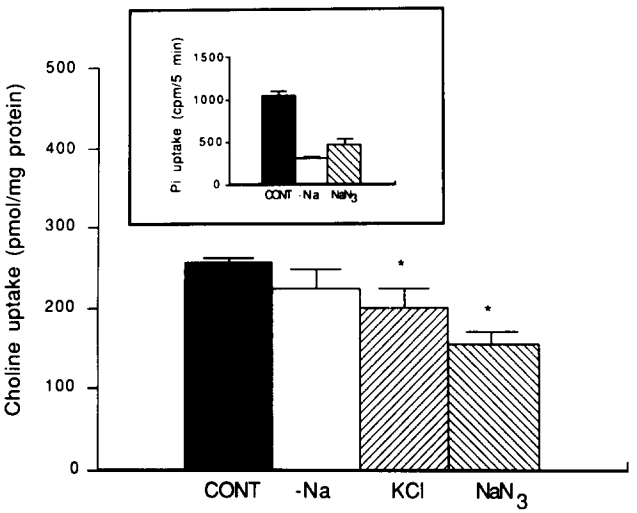

Fig. 2. Effects of external KCl and of energy poisons on choline uptake. Cells were preincubated for 30 min in the presence of 50 mM KCl or in the presence of 5 mM sodium azide plus 50 mM 2-deoxyglucose. [^{3}H]Choline uptake (50 μM) was measured after a 5 min incubation. The inset shows the ^{32}P (1 μCi/ml) uptake (5 min) by MDCK cells incubated 30 min in: phosphate-free buffer (CONT); in phosphate- and sodium-free buffer (−Na), phosphate buffer (+Na) and 5 mM sodium azide plus 50 mM 2-deoxyglucose (NaN_3). Data are means ± S.E. of three independent experiments ($n = 3$).

choline uptake (Fig. 2). This suggested that choline uptake was an electrogenic process. The incubation of MDCK cells with 5 mM sodium azide and 50 mM 2-deoxyglucose for 30 min, also inhibited significantly (35%) the choline uptake (Fig. 2). In comparison, the phosphate transport was strongly reduced either in abscence of sodium or in the presence of metabolic inhibitors (Fig. 2 inset). The decrease in the Na-coupled phosphate transport indicated the efficiency of sodium or ATP depletion.

Specificity of the choline transport system was evaluated by measuring choline uptake in the presence of a 10-times excess (500 μM) of chemically related compounds (Fig. 3): ethanolamine and 1-pyrenebutyrylcholine (a fluorescent derivative of butyrylcholine) were powerfull inhibitors of choline uptake. *N,N*-Dimethylethanolamine also inhibited choline uptake, whereas TEA, which is usually transported by the broad-specificity organic cation transporter of renal epithelial cells [19–21], and betaine had no effect. Hemicholinium-3, a compound which binds specifically to choline carriers and inhibits both sodium-dependent and sodium-independent choline transport in various cell types [10,22–25], was the most potent inhibitor of choline transport in MDCK cells (76% inhibition). This allowed the use of the [^{3}H]HC-3 for estimating the number of the binding sites. Specific binding of [^{3}H]HC-3 (Fig. 4), indicated the presence of 3.72 ± 0.95 fmol binding sites/10 mg cell protein on the apical surface of MDCK cell monolayers grown on plastic

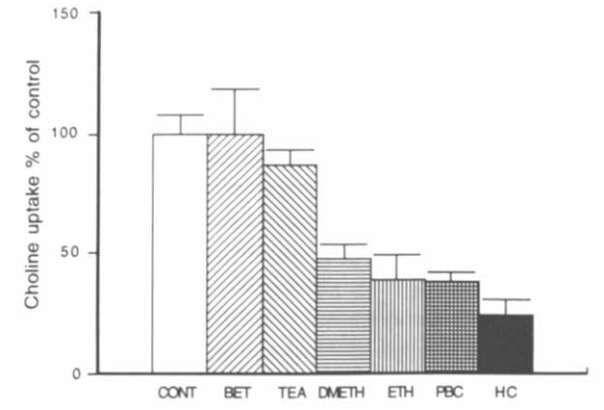

Fig. 3. Effect of structural analogues on the choline uptake by MDCK. 5 min uptakes of 50 μM [^{3}H]choline were determined in the presence of 500 μM various structural analogues. Mean $\pm$S.E. ($n = 3$).

support. Excess of choline in the assay medium completely inhibited the specific binding of [^{3}H]HC-3 to the cells (Fig. 4). In contrast, replacing sodium by *N*-methylglucamine had no effect on [^{3}H]HC-3 binding (data not shown).

Choline uptake by MDCK cells grown on a permeable support

Growing MDCK cells on plastic dishes gave no access to the basolateral side of the plasma membrane. The next series of experiments, were performed to evaluate the relative importance of apical vs. basolateral choline uptake. As shown by Fig. 5, at physiological choline concentration (50 μM), the uptake of choline by confluent monolayers grown on permeable support was about 10-times greater from the basolateral than from the apical side of the culture. The

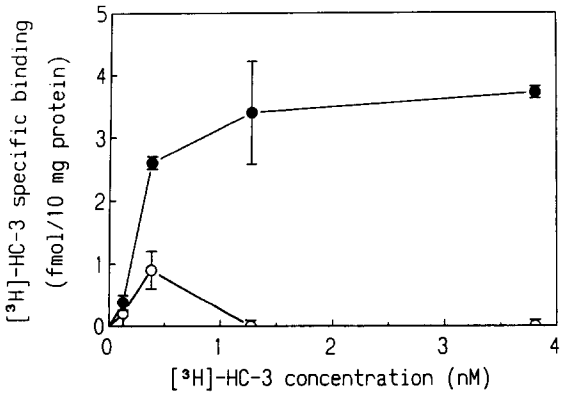

Fig. 4. Saturation curve of the apical [^{3}H]Hemicholinium-3 binding to confluent MDCK cells grown on plastic support. Cells were incubated with 200 μL [^{3}H]HC-3 at the concentration indicated either in presence or in absence of 500 μM choline. Incubations in parallel with 10 μM unlabelled HC-3 allowed the determination of non-specific binding (see Materials and Methods for details). Mean $\pm$S.E. ($n = 3$).

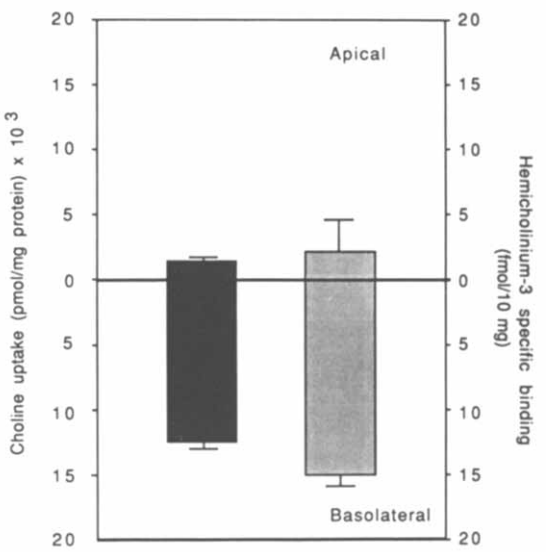

Fig. 5. Choline transport and HC-3 binding by MDCK cells grown on filters. Uptake of [^{3}H]choline (50 μM) from the apical or the basolateral side of the filters, and [^{3}H]HC-3 binding experiments were performed as described in Materials and Methods ($n = 3$).

kinetic constants determined for the transport system detected at the basolateral side of the cells were: $K_m = 39 \pm 10$ μM and $V_{max} = 2050$ pmol/mg protein per 5 min. This basolateral transport was inhibited by ethanolamine and dimethylethanolamine (data not shown). Furthermore, HC-3 inhibited choline uptake at the basolateral but not at the apical side of cells (Fig. 6). In accordance with these data, specific binding of [^{3}H]HC-3 became not statistically different from zero on the apical side, whereas it corresponded to approx. 15 fmol/10 mg of cell protein for the basolateral domain of the cells (Fig. 5). Again, TEA in excess had no effect on either basolateral or apical choline uptake (Fig. 6).

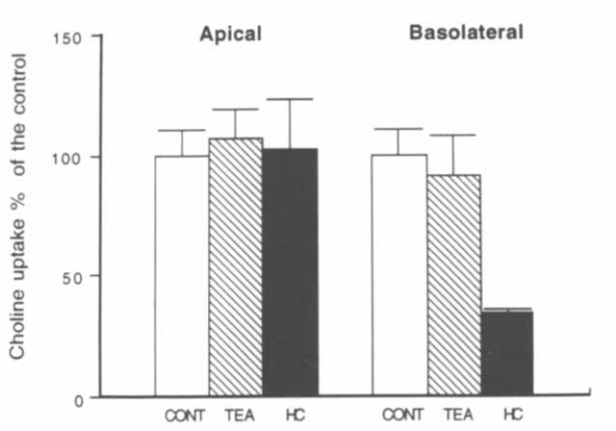

Fig. 6. Effect of TEA and HC-3 on choline uptake from the apical and the basolateral membrane of MDCK cells. 10 min uptake of 50 μM [^{3}H]choline was determined in absence or in presence of either 500 μM TEA or 500 μM HC-3 at the apical or at the basolateral side of the cells. Mean $\pm$S.E. ($n = 3$).

Discussion

The present data show that, at physiological concentrations, choline uptake in MDCK cells grown on solid support is mediated by a specific transporter, which can be inhibited by hemicholinium-3. When grown on filters, cells expressed this transport system essentially on the basolateral membrane domain. These data suggest that switching the growth condition from solid to permeant support resulted in a preferential localization of the choline transport system at the basolateral side of the cells.

As reported recently for IMCD [10] cells and rabbit brush-border membrane [26], the choline transport by MDCK cells was found to be sodium-independent, to be affected by membrane potential, and to be inhibited by molecules possessing a tertiary or a quaternary nitrogen atom, but not by betain or TEA, a recognized substrate for the broad specificity organic cations transporter of epithelial cells [20]. In addition we showed that ATP depletion by metabolic inhibitors modified the choline uptake by MDCK cells. A sodium-independent specific choline transporter, but non-electrogenic, was identified in small intestinal brush-border membrane [27]. The affinity of these three renal systems fell within a small range, i.e., from 43 μM to 80 and 97 μM, respectively for MDCK cells, IMCD and renal brush-border membrane vesicles, suggesting that they might eventually correspond to different expressions of the same transport system localized in different membrane environments.

Binding of [^{3}H]HC-3 was previously used to estimate the number of high-affinity sodium-dependent choline carrier sites in neuronal rat brain cells [28,29]. In these cells, sodium had no effect on the hemicholinium association rate but only slowed the dissociation of [^{3}H]HC-3 from the binding sites [28]. Our result supported the view that the binding of [^{3}H]HC-3 might also be a useful tool to evaluate the number of sodium-independent choline carriers in MDCK cells culture: (a) the specific binding of [^{3}H]HC-3 was saturable; (b) it was not affected by the absence of sodium; (c) it was prevented by the presence of choline in excess in the medium. Accordingly, our data suggest the presence of 3.7 fmol choline transporter sites/10 mg protein on the apical membrane of MDCK cells grown on plastic support. This value falls in the lower range of [^{3}H]HC-3 binding sites number reported for rat brain [30]. The higher number of choline transporter sites in the brain might functionally be explained by the need of choline for acetylcholine synthesis.

Data from cells grown on permeant support showed that uptake from the basolateral side was approx. 10-times the uptake from the apical side. As for cells grown on solid support, choline uptake from either side was unaffected by an excess of TEA. On the other hand, HC-3 inhibited choline uptake only from the basolateral side, which corresponded to the disappearance of a significant specific binding of [^{3}H]HC-3 on the apical membrane. In addition the basolateral choline carrier of cells grown on filter showed the same affinity as the apical transport system of cells grown on plastic support. This suggested that the choline transporter, active initially on the apical membrane of cells grown on solid support, was preferentially expressed on the basolateral membrane of cells grown on filters. In addition, for these cells, besides an increase in the absolute number of HC-3 binding sites, efficiency (transport rate/number of HC-3 binding sites) of choline mediated transport was increased approx. 2-fold compared to cells grown on plastic dishes. Experiments performed in the last few years have shown that the cellular polarization of confluent MDCK cells grown on solid support is not as complete as that obtained when cells are grown on filters [31]. For cells grown on solid support, a fraction of transport systems and receptors might well be present on the apical membrane, i.e., facing the growth medium, to allow the entry of cell nutrients. Accordingly, the presence of HC-3 binding sites on the apical domain of plastic dishes grown MDCK cells might correspond to defects in the sorting mechanisms of the high-affinity choline carrier. Alternatively, as recently reported for the sodium-potassium ATPase in the same cell line [32], loss of the transport activity and/or the accessibility of the carriers present in the apical membrane, associated with an exclusive localization of the active transport system in the basolateral membrane might have resulted from differences both in the lipid environment and in the relationships between the transporter and the cytoskeleton, which develop upon growth on filters. Finally, the enhanced efficiency of the HC-3-sensitive choline transport system when localized in basolateral membranes could be related to the higher fluidity of this membrane domain [12,33]: activity of the red blood cell choline carrier was shown to be modulated by benzyl alcohol [34] a fluidizing agent. In conclusion, the present data show that, for physiological concentrations, choline uptake by MDCK cells occurs via a sodium-independent specific transport system. Inactivation or relocalization of this transport system to the basolateral membrane domain when cells are grown on permeant supports provides a valuable tool for studies on the relationships between choline transport and choline headgroup phospholipid synthesis in renal epithelial cells.

References

1 Toback, F.G.(1984) Am. J. Physiol. 246, F249–F259.
2 Kent, C.(1990) Prog. Lip. Res. 29, 87–105.
3 Acara, M. and Rennick, B. (1973) Am. J. Physiol. 225, 1123–1128
4 Banfic, H., Pokrajac, N. and Irvine, R.F. (1985) Biochim. Biophys. Acta 833, 223–228.

5 Nakanishi, T., Balaban, R.S. and Burg, M.B.(1988) Am. J. Physiol. 255, C181–C191.
6 Garcia-Perez, A. and Burg, B.M. (1991) 71, 1081–1115.
7 Schmolke, M., Bornemann, A. and Guder, G.W. (1991) Contrib. Nephrol. 95, 255–263.
8 Cereijido, M., Robins, E.S., Dolan, W.J., Rotuno, C.A. and Sabatini, D.D. (1978) J. Cell. Biol. 77, 853–880.
9 Handler, J.S., Perkins, F.M. and Johnson, J.P. (1980) Am. J. Physiol. 238, F1–F9.
10 Bevan, C. and Kinne, R.K.H. (1990) Pflugers Arch. 417, 324–328.
11 Friedlander, G., Shahedi, M., Le Grimellec, C. and Amiel, C. (1988) J. Biol. Chem. 263, 11183–11188.
12 Le Grimellec, C., Friedlander, G. and Giocondi, M.C. (1988) Am. J. Physiol. 255, F22–F32.
13 Zlatkine, P., El Yandouzi, E.H., Op den Kamp, J.A.F. and Le Grimellec, C. (1991) Biochim. Biophys. Acta 1065, 225–230.
14 De Lean, A., Munson, P.J. and Robbard, D. (1978) Am. J. Physiol. 235, E97–E102.
15 Malo, C. and Berteloot, A. (1991) J. Membr. Biol. 122, 127–141.
16 Altman, P.L. (1961) Blood and other body fluids, biological handbooks (Dittmer, D.S., ed.), pp.92–93, FASEB, Washington D.C.
17 Bligh, J. (1952) J. Physiol. 117, 234–240.
18 Klein, J., Koppen, A., Loffelholz, K. and Schmitthenner, J. (1992) J. Neurochem. 58, 870–876.
19 Fauth, C., Rossier, B. and Roch-Ramel, F. (1988) Am. J. Physiol. 254, F351–F357.
20 Rennick B.R. (1981) Am. J. Physiol. 240, F83–F89.
21 Wright S.H. and Wunz, T.M. (1987) Am. J. Physiol. 253, F1040–F1050.
22 Fisher, A.B., Chander, A., Dodia, C., Reicherter, J. and Kleinzeller, A. (1989) Am. J. Respir. Cell. Mol. Biol. V1, 455–462.
23 Diamond, I. and Kennedy, E.P. (1969) J. Biol. Chem. 244, 3258–3263.
24 Haga, T. and Noda, H. (1973) Biochim. Biophys. Acta. 291, 564–575.
25 Breer, H. and Knipper, M. (1990) J. Neurobiol. 21, 269–275.
26 Wright, S.H., Wunz, T.M. and Wunz, T.P. (1992) J. Membr. Biol. 126, 51–65.
27 Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. and Semenza, G. (1978) Biochim. Biophys. Acta 506, 136–154.
28 Sandberg, K., Coyle, J.T. (1985) Brain Res. 348, 321–330.
29 Manaker, S., Wieczorek, C.M. and Rainbow, T.C. (1986) J. Neurochem. 46, 483–488.
30 Vickroy, T.W., Roeske, W.R., Gehler, D.R., Wamsley, J.K. and Yamamura, H.I. (1985) Brain Res. 329, 368–373.
31 Ojakian, G.K., Romain, R.E. and Herz, R.E. (1987) Am. J. Physiol. 253, C433–C443.
32 Wilson-Hammerton, R., Krzeminski, K.A., Mays, R.W., Ryan, T.A., Wollner, D.A. and Nelson, W.J. (1991) Science 254, 847–850.
33 Le Grimellec, C., Giocondi, M.C., Carriere, B., Carriere, S. and Cardinal, J. (1982) Am. J. Physiol. 242, F246–F253.
34 Martin, K. (1977) in Membrane Transport in Red Cells (Ellory, J.C., ed.), pp. 101–113, Academic Press, New York.