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CONTROL OF PASSIVE PERMEABILITY OF CHINESE HAMSTER OVARY CELLS BY EXTERNAL AND INTRACELLULAR ATP

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External ATP causes passive permeability change in several transformed cells, but not in untransformed cells. We studied the effect of external ATP on the passive permeability of CHO-K1 cells, a transformed clone of Chinese hamster ovary cells. Treatment of the cells with external ATP alone did not produce a permeability change, and this was observed only when a mitochondrial inhibitor, such as rotenone or oligomycin, was present together with ATP. These inhibitors reduced the concentration of intracellular ATP and a permeability change by external ATP was observed when intracellular ATP was decreased more than 70%. This requirement for permeability change of CHO-K1 cells was quite unique, since passive permeability change of other transformed cells so far tested was induced by ATP alone. Treatment of CHO-K1 cells with cyclic AMP analogues increased their sensitivity to external ATP about 2-fold. The roles of external and intracellular ATP in controlling passive permeability are discussed.

Introduction

The mechanism controlling passive permeability of animal cells is not well understood. Recently, it was demonstrated that addition of external ATP to monolayer cultures of several transformed cells, such as 3T6, SV3T3 and HeLa cells, caused a striking increase in passive permeability, allowing passage through the membrane of normally impermeable molecules, such as nucleotides and phosphate esters [1–6]. This effect of external ATP depended on the concentration of intracellular ATP and sensitivity to external ATP increased when the intracellular ATP concentration was reduced [7]. This permeability change was specific for ATP and was reversible, and the treated cells could grow at a normal rate after the membrane was sealed. None of these effects was observed in untransformed 3T3 cells [1–7].

To generalize the effect of external ATP on the passive permeability in transformed cells, we studied the effect of ATP on CHO-K1 cells, a transformed clone of Chinese hamster ovary cells which is useful for a genetic approach to isolate mutants defective in membrane functions [8,9]. In this paper, we report that external ATP caused passive permeability change in CHO-K1 cells only when the concentration of intracellular ATP was lowered by treatment with mitochondrial inhibitors. The effect of cyclic AMP analogues, which are known to modulate various membrane functions of CHO cells [10–12], on this permeability change is also reported.

Materials and Methods

Chemicals

All chemicals used were of reagent grade and were obtained from Sigma, St. Louis, MO, U.S.A. 2-Deoxy-[^{3}H]glucose (15 Ci/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A.

Abbreviations: CHO-K1, Chinese hamster ovary cells, clone K1; 3T6, spontaneously transformed 3T3 cells; SV3T3, 3T3 cells transformed by SV40; pNPP, *p*-nitrophenylphosphate; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone.

Cell culture

Chinese hamster ovary cells, clone K1 (CHO-K1), were obtained from Flow Laboratories Inc., Rockville, MD, U.S.A. The cells were maintained in Ham's F12 supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C in a water-saturated atmosphere of 5% CO_2 in air as described [8]. Cells cultured to a subconfluent state in 35-mm dishes in this medium for 2–3 days were used throughout the present experiments. Stock cultures were replaced every 6–8 weeks by samples from stocks frozen in liquid N_2.

Measurement of passive permeability change

Passive permeability change in CHO-K1 cells induced by external ATP was measured as described previously [1–3] by monitoring either entry and hydrolysis of pNPP or efflux of acid-soluble materials labeled with deoxy[^{3}H]glucose. Briefly, monolayer cultures of CHO-K1 cells were washed twice with 0.15 M NaCl and incubated in buffer A, adjusted to pH 8.2 or 7.2 at 23°C, containing the additions indicated in the text. Buffer A consisted of 0.1 M Tris-HCl/0.05 M NaCl/5 mg/ml dextran 500. After incubation for 10 min at 37°C, the incubation medium was replaced by 1 ml buffer A (pH 8.2) with 5 mM pNNP, and incubation was continued for 10 min at 37°C. The supernatant was then removed and mixed with 0.1 ml 1 M NaOH to measure the *p*-nitrophenol formed at 410 nm. For studying efflux, the cells were labeled for 2 h with deoxy[^{3}H]glucose (0.5 μCi/ml, 1 μM) in glucose-free F12 medium containing 10% dialyzed fetal bovine serum. The labeled cells were washed with 0.15 M NaCl, and efflux of radioactive materials into 1 ml of buffer A containing the indicated additions was measured in a scintillation counter.

Measurement of intracellular ATP

Intracellular ATP was extracted from the cells with 1 ml ice-cold 0.4 M $HClO_4$ [7], and the ATP concentration was determined enzymatically with luciferase, using a Packard Tri-Carb liquid scintillation spectrometer as described [13].

Protein was determined by the method of Lowry et al. [14] with crystalline bovine serum albumin as a standard.

Results

Passive permeability change upon addition of ATP to CHO-K1 cells

Treatment of CHO-K1 cells with ATP in buffer A (pH 8.2) at concentrations of up to 2 mM did not

TABLE I

INDUCTION OF PERMEABILITY CHANGE IN CHO-K1 CELLS BY ADDITION OF ATP WITH MITOCHONDRIAL INHIBITORS

Monolayer cultures of cells were incubated in 1 ml buffer A (pH 8.2) containing the indicated additions at 37°C for 10 min. The medium was replaced by 1 ml buffer A with 5 mM pNPP and incubation was continued at 37°C for 10 min. The supernatant was then removed for measurement of *p*-nitrophenol produced as described in Materials and Methods. For studying efflux, cells labeled with deoxy[^{3}H]glucose were incubated in buffer A (pH 8.2) containing the indicated additions at 37°C for 10 min. The supernatant was removed to measure efflux of labeled materials in a scintillation counter. The total counts extracted with 5% cold trichloroacetic acid were $81.0 \cdot 10^3$ cpm/dish. Rotenone or oligomycin was added in 3 μl ethanolic solution. The numbers of cells and the protein concentrations used in these experiments were as follows: (a) $1.87 \cdot 10^6$ cells/dish, 0.226 mg protein/dish; (b) $1.56 \cdot 10^6$ cells/dish, 0.205 mg protein/dish.

Additions to buffer A	Hydrolysis of pNPP [a] (nmol/mg protein)	Efflux [b] (cpm/dish) ($\times 10^{-3}$)
None	18.8	11.4
0.5 mM ATP	23.2	12.5
0.5 mM ATP + rotenone (3 μM)	144.2	61.4
0.5 mM ATP + oligomycin (3 μg)	147.3	57.8
0.5 mM ATP + ethanol (3 μl)	18.8	12.5
Rotenone	17.5	13.5
Oligomycin	16.3	15.4

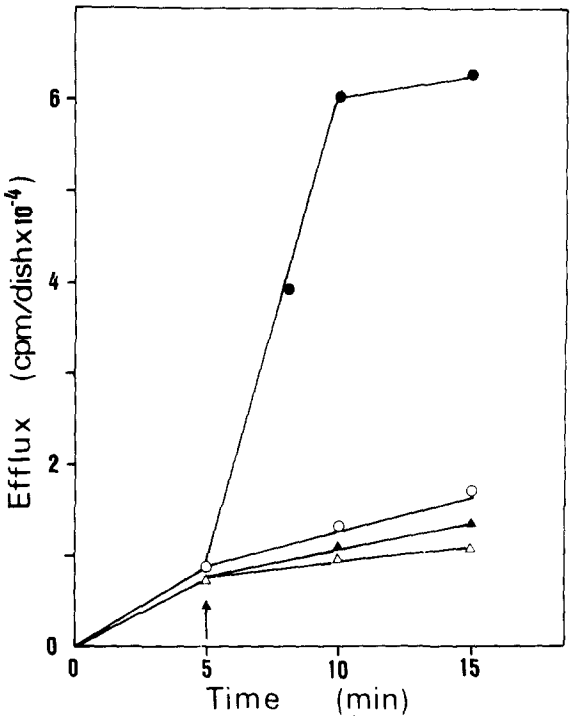

Fig. 1. Effect of ATP and rotenone on the efflux of deoxy-[^{3}H]glucose-labeled materials. CHO-K1 cells labeled with deoxy[^{3}H]glucose as described in Materials and Methods were washed and incubated at 37°C in 1 ml buffer A (pH 8.2) containing additions as indicated by the following symbols: •, 0.5 mM ATP + 3 μM rotenone; ○, 3 μM rotenone; ▲, 0.5 mM ATP; △, none. ATP was added 5 min after start of the incubation as indicated by an arrow. At the indicated time the supernatant was removed for counting. The total radioactivity within the cells which could be extracted with 5% trichloroacetic acid was 6.3 · 10^4 cpm/dish.

increase passive permeability. This concentration was more than 10-fold that used for the permeability change of transformed 3T3 cells [2]. However, when CHO-K1 cells were treated with 0.5 mM ATP with either rotenone or oligomycin, a great increase in passive permeability and subsequent hydrolysis of pNPP by alkaline phosphatase of the cells were observed (Table I). Rotenone or oligomycin alone did not produce a permeability change. The rate of pNNP hydrolysis by CHO-K1 cells under the present experimental condition was linear during incubation for 15 min at 37°C and was markedly stimulated about 7-fold when the cells were treated with ATP in the presence of a mitochondrial inhibitor. The fact that

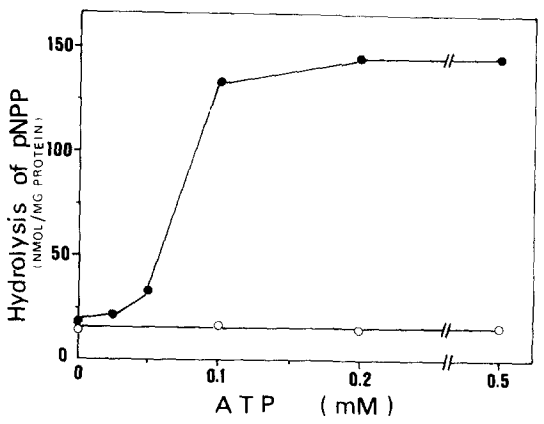

Fig. 2. Effect of added ATP on passive permeability change in CHO-K1 cells. Monolayer cultures of CHO-K1 cells were treated with buffer A (pH 8.2) containing various concentrations of ATP in the presence or absence of 3 μM rotenone at 37°C for 10 min. After incubation, the medium was removed and the cells were incubated with 5 mM pNPP in the same buffer A for 10 min at 37°C. The medium was removed to measure the *p*-nitrophenol formed as described in Materials and Methods. ○, without rotenone; •, with rotenone. The basic level of pNPP hydrolysis by the cells without rotenone could be ascribed to the activity of alkaline phosphatase located on the cell surface [1].

TABLE II

CHARACTERISTICS OF THE ATP-DEPENDENT PERMEABILITY CHANGE IN CHO-K1 CELLS

CHO-K1 cells were incubated in buffer A at either pH 8.2 or pH 7.2 in the presence of the indicated additions for 10 min at 37°C. In the experiment at 0°C, the cells were incubated with rotenone in buffer A (pH 8.2) at 37°C for 5 min and were further incubated at 0°C for 10 min in the presence of ATP. Hydrolysis of pNPP by the treated cells was measured by incubation of the cells for 10 min at 37°C in buffer A (pH 8.2) as described in Table I. Rotenone was added at 3 μM.

Additions to buffer A	Hydrolysis of pNPP (nmol/mg protein)
None (pH 8.2, 37°C)	25.3
Rotenone + 0.5 mM ATP	167.1
Rotenone + 0.5 mM GTP	24.7
Rotenone + 0.5 mM UTP	21.3
Rotenone + 0.5 mM CTP	18.5
Rotenone + 0.5 mM ATP (pH 8.2, 0°C)	25.3
Rotenone + 0.5 mM ATP (pH 7.2, 37°C)	38.7

no alkaline phosphatase activity was detected in the incubation medium showed that the enzyme remained inside the cells (data not shown). This permeability change in CHO-K1 cells by treatment with ATP plus a mitochondrial inhibitor was confirmed by measuring the efflux of deoxy[^{3}H]glucose-labeled materials from the cells. The radioactive material was mainly deoxy[^{3}H]glucose 6-phosphate, which normally cannot penetrate the plasma membrane. As shown in Table I, efflux of the radioactive materials was stimulated by incubating the cells with ATP in the presence of rotenone or oligomycin. Fig. 1 shows that the efflux of deoxy[^{3}H]glucose-labeled materials from rotenone-treated cells increased within 5 min after addition of 0.5 mM ATP. In control cultures, untreated or treated with either ATP or rotenone, only about 15–20% of the total acid-soluble radioactive materials was released. This is mainly due to the diffusion of free deoxy[^{3}H]glucose as described [7]. More than 0.1 mM ATP was required for induction of permeability change in the presence of rotenone (Fig. 2). The permeability change in CHO-K1 cells induced by added ATP was dependent on the nucleotide, pH and temperature and did not occur with other nucleotide triphosphates, at neutral pH or at 0°C (Table II). These characteristics of CHO-K1 cells were similar to those of transformed 3T3 cells [2].

The most significant difference between CHO-K1 cells and transformed 3T3 cells was that the former required a mitochondrial inhibitor besides ATP for permeability change. Previously, the suppressive effects of a fluoride-inhibitable phosphoprotein phosphatase and divalent cations on the permeability change in transformed 3T3 cells were demonstrated [1,3]. In CHO-K1 cells, however, no permeability change was observed on addition of ATP even in the presence of 10 mM NaF or 1 mM EDTA (data not shown).

Relationship between induction of permeability change by addition of ATP and change in concentration of intracellular ATP

The relation of the concentration of rotenone, amount of intracellular ATP and degree of passive permeability was examined by treating CHO-K1 cells with various concentrations of rotenone. As shown in Fig. 3, the concentration of intracellular ATP decreased to about 15% of that of untreated cells on addition of 1 μM rotenone. A permeability change on addition of 0.5 mM ATP was observed when more than 70% of the intracellular ATP was depleted with rotenone. Similar results were observed with oligomycin (Table III). In contrast, the uncoupler CCCP caused about 64% depletion of intracellular ATP, which was insufficient for induction of the permeability change. The presence of excess glucose completely abolished the effect of rotenone or oligomycin on the permeability change by increasing intracellular ATP to about half of the original concentration. As expected, deoxyglucose, which is nonmetabolizable,

TABLE III

RELATIONSHIP BETWEEN REDUCTION OF INTRACELLULAR ATP CONCENTRATION AND PASSIVE PERMEABILITY CHANGE BY ADDED ATP

The concentration of intracellular ATP was determined in CHO-K1 cells, which were treated in buffer A as indicated for 10 min at 37°C. At the same time, passive permeability change was also determined as described in Table I in cells which were incubated in buffer A with 0.5 mM ATP and the indicated additions.

Treatment	Intracellular ATP (nmol/10^6 cells) (%)	Hydrolysis of pNPP (nmol/mg protein)
buffer A	3.25 (100)	26.5
+rotenone (3 μM)	0.59 (18)	131.2
+oligomycin (3 μg)	0.63 (20)	134.0
+CCCP (10 μM)	1.20 (36)	42.0
+rotenone + 20 mM glucose	1.60 (49)	25.7
+rotenone + 20 mM deoxyglucose	0.40 (12)	116.8
+oligomycin + 20 mM glucose	1.68 (53)	30.7

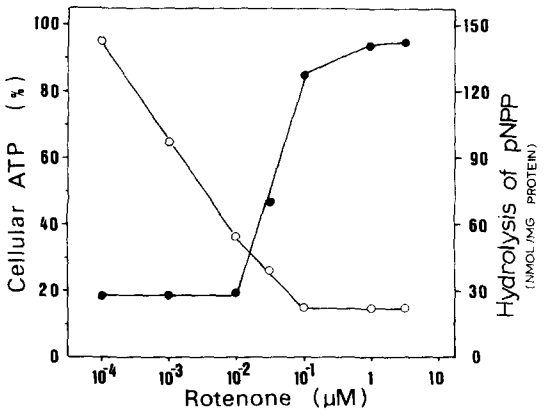

Fig. 3. Effect of rotenone on intracellular ATP and passive permeability change by external ATP. CHO-K1 cells were treated with various concentrations of rotenone in buffer A (pH 8.2) for 10 min at 37°C and the amounts of intracellular ATP of treated cells were analyzed as described in Materials and Methods. At the same time, permeability change of cells incubated with 0.5 mM ATP and various concentrations of rotenone for 10 min at 37°C was determined as described in Fig. 2. ○, amount of intracellular ATP(percentage of control). The 100% value of cells incubated with buffer A alone was 3.20 nmol/10^6 cells; ●, hydrolysis of pNPP by cells treated with 0.5 mM ATP and rotenone.

had no such effect. All these results strongly suggest that in CHO-K1 cells depletion of intracellular ATP to below a critical level is essential for induction of permeability change by external ATP.

TABLE IV

PASSIVE PERMEABILITY CHANGE INDUCED BY ADDED ATP IN CHO-K1 CELLS TREATED WITH DIBUTYRYL CYCLIC AMP

CHO-K1 cells were cultured for 2 days in the presence of 1 mM dibutyryl cyclic AMP. Passive permeability change in these cells was determined as described in the legend of Table I by measuring hydrolysis of pNPP in cells treated as indicated. Similar results were obtained in cells treated with 1 mM 8-bromo cyclic AMP.

Additions to buffer A	Hydrolysis of pNPP (nmol/mg protein)
None	25.1
0.5 mM ATP	26.0
0.5 mM ATP + rotenone (3 μM)	213.4
0.05 mM ATP + rotenone (3 μM)	210.5
Rotenone	25.5

Effect of cyclic AMP analogues on passive permeability change

It is reported that cyclic AMP analogues modulate various membrane-related functions, including cell morphology and nutrient transport of CHO cells [10–12]. Dibutyryl cyclic AMP or 8-bromo cyclic AMP changed the morphology of CHO-K1 cells from a epithelial-like form to a elongated fibroblast-like form as reported. Even in cells treated with 1 mM dibutyryl cyclic AMP for 2 days, passive permeability change was observed under the conditions described for untreated cells (Table IV). However, the sensitivity of treated cells increased and 0.05 mM ATP was enough to produce passive permeability change in the presence of rotenone.

Discussion

The present results showed that added ATP increased passive permeability of normally impermeable molecules in CHO-K1 cells in the presence of a mitochondrial inhibitor. More than 0.1 mM ATP was required for this permeability change. However, in the absence of the inhibitor, external ATP did not cause the permeability change even at concentrations of up to 2 mM. This effect of ATP on passive permeability change in CHO-K1 cells was quite unique, since the permeability change is induced by as low as 0.1 mM ATP alone in other transformed cells, such as 3T6 and SV3T3 [1–6]. In these transformed cells, the sensitivity to external ATP increased several-fold when intracellular ATP was depleted [7]. However, permeability of untransformed 3T3 cells was unaffected by ATP under the present conditions (Ref. 7 and unpublished data).

The molecular mechanism of the permeability change induced by external ATP is unknown. The permeability change in CHO-K1 cells was specific for ATP and was dependent on temperature and pH in the incubation medium (Table II), as reported for 3T6 cells [2]. These characteristics suggested that the specific reaction(s) with ATP in the plasma membrane control the permeability change. Previous studies on the mechanism in 3T6 cells demonstrated that phosphorylation by external ATP of membrane proteins could be involved in the permeability change [15,16]. More recently, a possible membrane protein with molecular weight of 45 000, the phosphoryla-

tion of which might control the permeability change, was observed (Kitagawa, T., unpublished data). The most plausible model for the permeability change is that the phosphorylated protein(s) is involved in formation of aqueous channels through the membrane, allowing the passage of nucleotides and phosphate esters. However, other mechanisms remain possible.

Mitochondrial inhibitors acted to lower the concentration of intracellular ATP of CHO-K1 cells. The permeability change was induced by external ATP when more than 70% of the intracellular ATP was depleted (Fig. 3 and Table III). These results may indicate that the mechanism by which intracellular ATP controls passive permeability differs in CHO-K1 cells and transformed 3T3 cells. There are several reports on the effects of intracellular ATP on various membrane functions, such as control of cell shape [17], concanavalin-A agglutination [18], cap-formation [19] and drug-permeability [20]. Of these, it is of interest to note that drug-permeability in CHO-K1 cells increased on lowering the intracellular ATP concentration, which was associated with dephosphorylation of a membrane protein [21]. In addition, it was recently demonstrated that intracellular ATP had a role in maintaining a permeability barrier, which might be regulated by phosphorylation of membrane proteins [22]. These facts may indicate that the difference in sensitivities to external ATP of CHO-K1 cells and transformed 3T3 cells is due to different states of phosphorylation of their membrane, affecting protein phosphorylation by external ATP. This sort of regulation of membrane permeability by phosphorylation of membrane proteins at both the inner and outer surface was previously suggested [7] and is summarized in a recent review [23].

Cyclic AMP analogues modulate various membrane-relating functions of CHO cells, including morphology and nutrient transport [10–12], though the effect is contradictory in other systems [23]. These changes by cyclic AMP are possibly mediated by cyclic AMP-dependent protein kinases. Dibutyryl cyclic AMP-treated CHO-K1 cells showed passive permeability change on addition of ATP and rotenone with about 2-fold increase in sensitivity to external ATP. This effect of cyclic AMP analogues could be ascribed to increased sensitivity of the cells to mitochondrial inhibitors (Kitagawa, T. and Akamatsu, Y., unpublished results).

The present studies on the effect of ATP on CHO-K1 cells indicate several interesting facts. First, sensitivity of transformed cells to external ATP is detected when permeability is tested under the present conditions. Second, the roles of external and intracellular ATP are clearer. Further studies with the ATP effect will provide useful information not only on regulation of passive permeability, and the role of protein phosphorylation, but also on surface changes associated with transformed cells.

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References

1 Rozengurt, E. and Heppel, L.A. (1975) Biochem. Biophys. Res. Commun. 67, 1581–1588
2 Rozengurt, E., Heppel, L.A. and Friedberg, I. (1977) J. Biol. Chem. 252, 4584–4590
3 Makan, N.R. (1978) Exp. Cell Res. 114, 417–427
4 Makan, N.R. and Heppel, L.A. (1978) J. Cell. Physiol. 96, 87–94
5 Kitagawa, T. (1980) J. Cell. Physiol. 102, 37–43
6 Kitagawa, T. (1980) Biochem. Biophys. Res. Commun. 94, 167–173
7 Rozengurt, E. and Heppel, L.A. (1979) J. Biol. Chem. 254, 708–714
8 Kao, F.T. and Puck, T.T. (1968) Proc. Natl. Acad. Sci. USA 60, 1275–1281
9 Thompson, L.H. and Baker, R.M. (1973) in Methods in Cell Biology (Prescott, D.M., ed.), vol. 6, pp. 209–281, Academic Press, New York
10 Hsie, A.W. and Puck, T.T. (1971) Proc. Natl. Acad. Sci. USA 68, 358–361
11 Hsie, A.W., Jones, C. and Puck, T.T. (1971) Proc. Natl. Acad. Sci. USA 68, 1648–1652
12 Lecam, A., Gottesman, M.M. and Pastan, I. (1980) J. Biol. Chem. 255, 8103–8108
13 Stanley, P.E. and Williams, S.G. (1969) Anal. Biochem. 29, 381–392
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
15 Dicker, P., Heppel, L.A. and Rozengurt, E. (1980) Proc. Natl. Acad. Sci. USA 77, 2103–2107
16 Makan, N.R. (1981) J. Cell. Physiol. 106, 49–61
17 Sheetz, M.P. and Singer, S.J. (1977) J. Cell. Biol. 73, 647–659

18 Vlodavasky, I., Inbar, M. and Sacks, L. (1973) Proc. Natl. Acad. Sci. USA 70, 1780–1784
19 Pozzan, T., Corps, A.N., Montecucco, C., Hesketh, T.R. and Metcalfe, J.C. (1980) Biochim. Biophys. Acta 602, 558–566
20 See, Y.P., Carlsen, S.A., Till, J.E. and Ling, V. (1974) Biochim. Biophys. Acta 373, 242–252
21 Carlsen, S.A., Till, J.E. and Ling, V. (1977) Biochim. Biophys. Acta 467, 238–250
22 Makan, N.R. (1979) J. Cell. Physiol. 101, 481–492
23 Plagemann, P.G.W. and Wohlheuter, R.W. (1980) in Current Topics in Membrane and Transport (Bronner, F. and Kleinzeller, A., eds.), vol. 14, pp. 226–330, Academic Press, New York