Biochimica et Biophysica Acta 939 (1988) 349–354
Elsevier

BBA 73943

Leucine transport-induced activation of the Na^+/H^+ exchanger in human peripheral lymphocytes

Yasuhide Mitsumoto, Kumie Sato and Tetsuro Mohri

Department of Physiological Chemistry, School of Pharmacy, Hokuriku University, Kanazawa, Ishikawa (Japan)

(Received 12 October 1987)
(Revised manuscript received 30 December 1987)

Key words: Leucine transport; pH, cytoplasmic; Sodium ion–proton exchanger; (Human lymphocyte)

Adjustment of amino-acid-induced cytoplasmic pH decrease by the Na^+/H^+ exchange system in human lymphocytes has been studied using a fluorometric technique to monitor the intracellular pH change. When the interior of lymphocytes is acidified by addition of nigericin to medium, cytoplasmic pH is immediately corrected toward its resting value. This recovery of the cytoplasmic pH depends on extracellular Na^+ and is inhibited by amiloride. A temporary (less than 2 min) decrease in the cytoplasmic pH, followed by a slow recovery phase, was observed in incubation with 1.0 mM leucine in Na^+-containing medium. This leucine-dependent decrease of cytoplasmic pH persisted longer when amiloride was added to the medium. Cytoplasmic pH recovery from the leucine-induced acidification depends on external Na^+ concentration. Amiloride-sensitive Na^+/H^+ exchanger was stimulated by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in the lymphocytes and preincubation of the cells with TPA partially prevented the leucine-induced cytoplasmic acidification. We conclude that human peripheral lymphocytes are provided with an amino acid-H^+ cotransport system, which is cooperatively coupled to the amiloride-sensitive Na^+/H^+ exchanger to correct the cytoplasmic pH anomaly.

Introduction

A good deal of effort in the investigation on the active transport of amino acids and glucose in diverse cell membranes has been focused on the role of electrostatic and chemical concentration gradients of cations. As its result, the 'Na^+ gradient hypothesis' for the transport of certain amino acids and glucose has been proposed, dictating that Na^+ gradient toward the inside of cells generated by active Na^+ extrusion performed by the Na^+/K^+-ATPase system is utilized to drive active entries of them [1,2]. On the other hand, it has been reported that lowering the extracellular pH accelerates the uptake of amino acids such as leucine and valine by mammalian cells [3,4]. Mohri and his collaborators [5,6] have found that the membrane vesicles of a human culture cell show intravesicular acidification in coincidence with the transport of leucine and valine into the vesicles. Finally, we have reported that Na^+-independent leucine transport across the plasma membrane of the human cultured cell is specifically dependent on the electrochemical H^+ gradient across the

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate: $[H^+]_i$, intracellular H^+ concentration: BCECF, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid: pH_i, cytoplasmic pH: Mes, 4-morpholineethanesulfonic acid.

Correspondence: Y. Mitsumoto, Department of Physiological Chemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11, Japan.

membrane and cooperatively coupled to the amiloride-sensitive Na^+/H^+ exchanger in the membrane [6].

An antiport system that exchanges extracellular Na^+ for intracellular H^+, $[H^+]_i$, electroneutrally has been implicated in the regulation of cytoplasmic pH in several mammalian cell types [7–11]. This exchanger is specifically inhibited by amiloride and can utilize the energy inherent to the inward chemical gradient of Na^+, although the normal $[H^+]_i$ is above the level predicted from the electrochemical equilibrium of H^+ and Na^+ across the plasma membrane. In cells such as fibroblasts and lymphocytes, the antiport system appears to be largely quiescent in the resting state but is markedly stimulated by artificially lowering cytoplasmic pH [7,8,10]. In some cell types it can also be activated even at normal cytoplasmic pH by a number of growth-promoting agents [9,12,13] and by osmotic shrinking of cells [14,15].

In this study we have investigated the effect of leucine on the cytoplasmic pH and the amiloride-sensitive Na^+/H^+ exchanger in human peripheral lymphocytes. We propose that the activity of the Na^+/H^+ exchanger is physiologically important to accumulate leucine and other L type amino acids in the cells.

Materials and Methods

Materials. Ficoll-Hypaque was purchased from Farmacia Fine Chemicals (NJ, U.S.A.). 2,7-Bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF) acetoxymethyl ester was obtained from HSC Research Development (Toronto, Canada). Amiloride was the kind gift of Merck, Sharp & Dohme (NJ, U.S.A.). 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was purchased from Sigma Chemicals (St. Louis, MO, U.S.A.). Nigericin was obtained from Shionogi Pharmaceutical Co. (Osaka, Japan). Other reagents used here were of special grade from Wako Pure CHemical Co. (Tokyo, Japan).

Solutions. Hepes-buffered saline (buffer A) contained 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 10 mM Hepes-NaOH (pH 7.4). Hepes-buffered choline medium (buffer B) was made by replacing NaCl and NaOH of buffer A by choline chloride and KOH, respectively. BCECF acetoxymethyl ester (1 mM) and amiloride (100 mM) were dissolved in dimethyl sulfoxide and kept at 4 °C in stock. Stock solutions of TPA and nigericin were 10^{-4} M and 0.8 mM in ethanol, respectively.

Cell isolation. Lymphocytes were isolated from fresh heparinized blood samples obtained from healthy human donors (A.O., K.S. and U.K.) with their consent by Ficoll-Hypaque gradient centrifugation [16]. Cell viability determined by trypan blue exclusion was greater than 90% throughout this period and was not affected by any of the procedures or reagents used. The cells were washed and resuspended in Hepes-RPMI (HCO_3^--free) at 10^7 cells/ml, and could be maintained in this medium at room temperature for up to 6 h.

Cytoplasmic pH determination. Cytoplasmic pH was determined fluorometrically essentially according to the method described earlier [17,18]. Cells in suspension (10^7 cells/ml in Hepes-RPMI) were loaded with the probe BCECF by incubation with its acetoxymethyl ester (5 μM) for 30 min at 37 °C. After washing with buffer A twice, $1–5 \cdot 10^6$ cells were used for fluorescence determination in 2 ml of the medium specified in the results using Hitachi MPF-4 fluorescence spectrophotometer with excitation at 508 nm (5 nm slit) and emission at 530 nm (10 nm slit).

Results

Cytoplasmic pH measurement with intracellulary trapped BCECF

Intracellulary trapped pH indicator with fluorescence has some distinct advantages for measuring cytoplasmic pH in vitro [19] such as unsurpassed time resolution in a continuous record and high sensitivity to small changes in cytoplasmic pH. We exploited in the present experiments a fluorescein derivative, BCECF, as a fluorescent monitor of cytoplasmic pH. Calibration curve of cytoplasmic dye fluorescence as a function of cytoplasmic pH was obtained by the H^+ equilibration method using a high concentration of K^+ in medium and the K^+/H^+ ionophore nigericin as previously described [19,20]. The fluorescence intensity of BCECF varies in a linear manner with pH change over the range from 6.4–7.4 in the lymphocytes (Fig. 1), gradually attaining the maximum in the higher pH values.

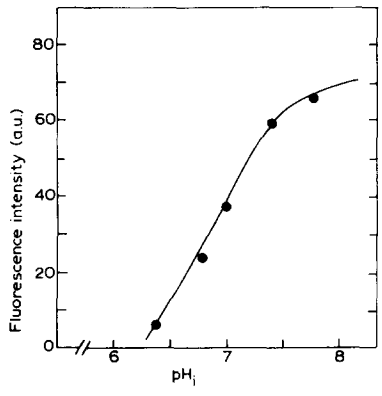

Fig. 1. Fluorescence intensity of BCECF as a function of pH_i. Lymphocytes were loaded with BCECF as described under 'Materials and Methods'. Cells were suspended at 37 °C in 10 mM buffer solutions of different pH values containing 130 mM KCl, 5 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM glucose after 3–5 min equilibration with 8 μM nigericin. Mes (pH 6.4 and 6.8), Hepes (7.0 and 7.4) and Tris (pH 7.8) were used as buffers.

This pH range favorably fits variation of the cytoplasmic pH of this cell (having its middle point at approx. 7.0).

Response of Na^+/H^+ exchanger to cytoplasmic pH

To assay the responsiveness of the Na^+/H^+ exchanger to cytoplasmic pH in human lymphocytes, the cells were treated with nigericin in buffer B (Fig. 2). Upon addition of nigericin (2 μM), cytoplasmic pH fell promptly due to exchange of cytoplasmic K^+ and external H^+ with a difference from the base of 0.670 ± 0.026 (S.E.) pH at 3 min ($n = 5$). After the ionophore was scavenged with bovine serum albumin [18], addition of Na^+ to the medium showed a rapid and notable cytoplasmic alkalinization, suggesting activation of the Na^+/H^+ exchanger (Fig. 2a). This assumption is supported by the finding that Na^+-induced cytoplasmic alkalinization was virtually canceled by addition of 1 mM amiloride (Fig. 2b). Nominally HCO_3^--free media were used throughout these experiments to minimize interference of other potential cytoplasmic pH regulatory systems such as Cl^-/HCO_3^- exchanger or Na^+/HCO_3^- cotransport and to eliminate signficant change of cellular volume [17,18].

Alteration of the cytoplasmic pH following leucine addition and effect of amiloride

Changes of the cytoplasmic pH on exposure of the cells to leucine (1 mM) in the presence of Na^+ in medium and the effect of amiloride were investigated to find out the response of the Na^+/H^+ exchange system to leucine uptake. The curve a of

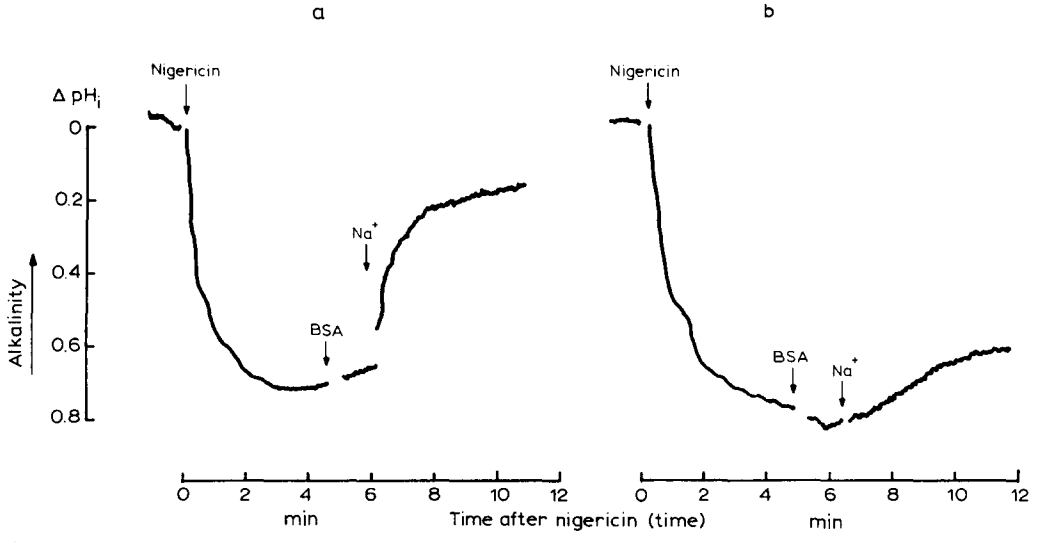

Fig. 2. Na^+-dependent cytoplasmic pH restoration in acid-loaded human lymphocytes. BCECF-loaded lymphocytes were suspended in buffer B at 37 °C. Where indicated, 2 μM nigericin was added to initiate K^+/H^+ exchange. Acid-loading was terminated by scavenging the ionophore with bovine serum albumin (5 mg/ml). Then NaCl (40 mM) was added to the medium in the presence (b) or absence (a) of 1 mM amiloride. Traces are representative of at least three experiments.

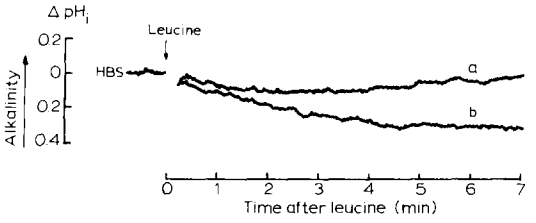

Fig. 3. Leucine-induced cytoplasmic acidification and the effect of amiloride. BCECF-loaded lymphocytes were suspended in buffer A and the fluorescence was recorded at 37 °C. Where indicated, 1 mM leucine was added to medium in the presence (b) or absence (a) of 1 mM amiloride. Traces are representative of at least five experiments.

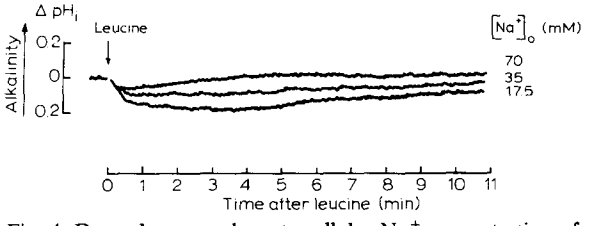

Fig. 4. Dependence on the extracellular Na^+ concentration of the cytoplasmic pH recovery from the leucine-induced acidification. Where indicated, 1 mM leucine was added to three kinds of modified buffer A different in Na^+ concentration. Osmolarity and ionic strength were kept constant using choline$^+$ as a Na^+ substitute. Other details are the same as in the legend to Fig. 3. $[Na^+]_0$, external Na^+ concentration. Traces are representative of at least four experiments.

Fig. 3 shows that leucine addition to incubation medium decreases only transiently the cytoplasmic pH (the difference is 0.101 ± 0.014 (S.E.) pH at 2 min ($n = 7$)). In a buffer A medium containing amiloride leucine-induced progressive acidification of the cell interior with time (the differences are 0.196 ± 0.003 pH at 2 min and 0.283 ± 0.043 pH at 5 min (n = 3)) (Fig. 3, curve b).

Dependence of the recovery from the leucine-induced cytoplasmic acidification on the external Na^+ concentration

Fig. 4 shows that the recovery of the cytoplasmic pH from acidity after addition of leucine is dependent on the external Na^+ concentration and 70 mM Na^+ in medium is sufficient for the complete recovery. The pH differences from the base were calculated as 0.193 ± 0.083, 0.114 ± 0.005 and 0.013 ± 0.004 at 5 min after leucine addition in 17.5, 35 and 70 mM Na^+ in medium ($n = 3$ or more). These results indicate that leucine-coupled H^+ uptake can be counteracted physiologically by H^+ extrusion by the action of the Na^+/H^+ exchanger in lymphocytes.

TPA inhibits the leucine-dependent cytoplasmic acidification

In many cell types including lymphocytes [14,17,21], the treatment with TPA has been dem-

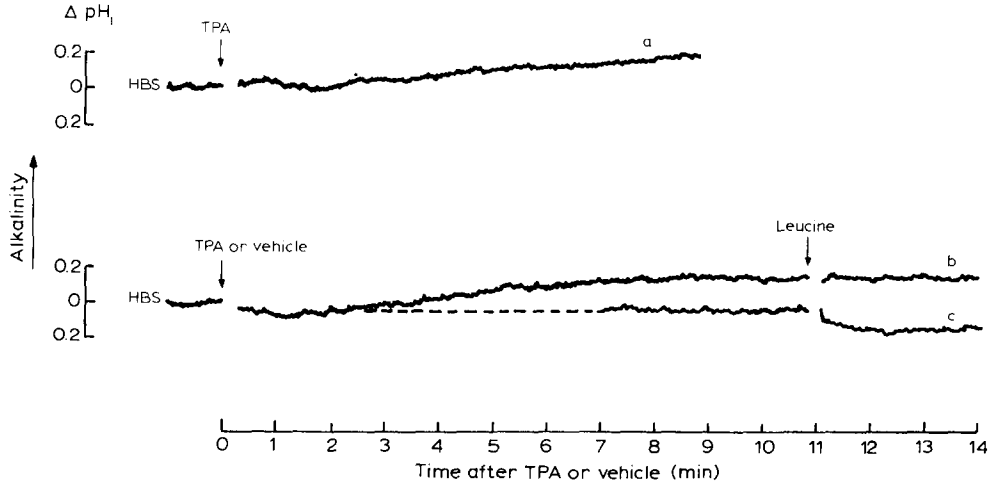

Fig. 5. TPA-induced cytoplasmic alkalinization and effect of TPA pretreatment on the leucine-induced acidification. BCECF-loaded lymphocytes were suspended in buffer A at 37 °C and the fluorescence was recorded with 10^{-7} M TPA added at arrow (a). Leucine (1 mM) was added to medium after treatment of cells with (b) or without (c) TPA for about 10 min. Traces are representative of at least four experiments.

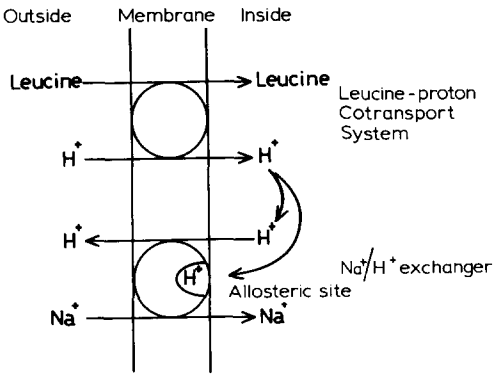

Fig. 6. A schematic presentation of functional linking of leucine-proton cotransport with the Na^+/H^+ exchanger.

onstrated to induce cytoplasmic alkalinization. Fig. 5 shows that the treatment of lymphocytes with TPA (10^{-7} M) in buffer A induces the cytoplasmic alkalinization with a short lag, giving the maximum after 7 min from TPA addition (curve a). The difference due to the treatment was 0.126 $\pm$ 0.021 (S.E.) pH at 10 min ($n = 9$) after TPA addition. The effect of TPA was abolished by addition of amiloride (data not shown). When cells were pretreated with TPA for about 10 min, leucine-induced cytoplasmic acidification was scarcely detected (less than 0.01 pH, $n = 7$) (curve b). In contrast, cytoplasmic pH of cells treated without TPA for the same period decreased on addition of leucine (curve c). The decrease of pH was 0.058 $\pm$ 0.009 (S.E.) pH at 2 min ($n = 5$) after leucine addition. We think therefore that the Na^+/H^+ exchanger activated by TPA is ready to respond to intracellular pH change due to leucine-motivated proton movement.

Discussion

Borghetti et al. [22] reported that alanine uptake through the system L increases as the extracellular pH decreases in the pig lymphocyte. We also have recognized in human lymphocytes that the uptake of leucine by them is accelerated by lowered external pH (unpublished result). Acceleration by acidic medium of the transport of amino acids utilizing the system L and intracellular acidification accompanying their uptakes have been demonstrated in other cells [4,23]. It has been revealed by us in the plasma membrane vesicles from a human culture cell that the Na^+-independent leucine transport is energized by a protonmotive force 5 and amiloride notably impaires leucine uptake by the cells in the presence of Na^+ in medium, but not in the absence, suggesting physiological importance of the Na^+/H^+ exchanger for complete functioning of the leucine transport system [6]. In the present experiments with human peripheral lymphocytes it has been demonstrated that the cytoplasmic acidification following leucine uptake can be dissipated by an intrinsic activation of Na^+/H^+ exchanger (Figs. 3 and 4), a process accelerated by TPA (Fig. 5).

The electroneutral Na^+/H^+ exchange system appears to be present in the plasma membrane of virtually all vertebrate cells, where it efficiently regulates cytoplasmic pH by virtue of its sensitivity to $[H^+]_i$ [24,25]. The functioning of cytoplasmic pH regulatory mechanism in intact cells is usually assessed by monitoring recovery of cytoplasmic pH to its basal level from a rapid acidification of the cytoplasm by the action of protonophore like nigericin. Recent evidence suggests that internal H^+ activates the Na^+/H^+ exchanger by binding to its allosteric site, which faces to the cytoplasm and is distinct from the H^+ transport site of the exchanger system [26]. Moolenaar et al [8] reported that, in quiescent human fibroblasts, the Na^+/H^+ exchanger is relatively inactive if the cytoplasmic pH is maintained around neutrality. Once cytoplasmic pH is reduced, however, the Na^+/H^+ exchanger is increasingly stimulated. Grinstein and Rothstein [25] have suggested that protonation of the allosteric site greatly enhances the rate of Na^+/H^+ antiport and adjusts upwards the 'set point' to $[H^+]_i$. It has been demonstrated by Moolenaar et al. [27] and Paris and Pouyssegur [24] that Na^+/H^+ exchange activators such as phorbol ester and some of growth factors increase the apparent affinity of the exchanger's binding site for cytoplasmic H^+. In the circumstances the lymphocytes from human blood seem to possess the Na^+/H^+ antiport system furnished with an allosteric site sensitive to cytoplasmic pH and more sensitized by the action of TPA.

The results we obtained support the proposition that the Na^+/H^+ exchanger is stimulated by

leucine uptake coupled to H^+ uptake in human lymphocytes probably through allosteric activation of the exchanger triggered by H^+ binding, as illustrated in Fig. 6.

References

1 Christensen, H.N., De Cespades, C., Handlogten, M.E. and Ronquist, G. (1973) Biochim. Biophys. Acta 300, 487–522.
2 Heinz, E., Geck, P. and Pfeiffer, B. (1980) J. Membr. Biol. 57, 91–94.
3 Shotwell, M.A., Kilberg, M.S. and Oxender, D.L. (1983) Biochim. Biophys. Acta 737, 267–284.
4 Christensen, H.N., De Cespedes, C., Handlogten, M.E. and Ronquist, G. (1974) Ann. NY Acad. Sci. 227, 355–379.
5 Mohri, T., Mitsumoto, Y. and Ohyashiki, T. (1983) Biochem. Int. 7, 159–167.
6 Mitsumoto, Y., Sato, K., Ohyashiki, T. and Mohri, T. (1986) J. Biol. Chem. 261, 4549–4554.
7 Aronson, P.S. (1985) Annu. Rev. Physiol. 47, 545–560.
8 Moolenaar, W.H., Tertoolen, L.G.J. and De Laat, S.W. (1984) J. Biol. Chem. 259, 7563–7569.
9 Moolenaar, W.H., Tertoolen, L.G.J. and De Laat, S.W. (1984) Nature (Lond.) 312, 371–374.
10 Paris, S. and Pouyssegur, J. (1983) J. Biol. Chem. 258, 3503–3508.
11 Roos, A. and Boron, W.F. (1981) Physiol. Rev. 61, 296–434.
12 L'Allemain, G., Paris, S. and Pouyssegur, J. (1984) J. Biol. Chem. 259, 5809–5815.
13 Rothenberg, P., Glaser, L., Schlesinger, P. and Cassel, D. (1983) J. Biol. Chem. 258, 12644–12653.
14 Grinstein, S., Cohen, S., Goetz, J.D. and Rothstein, A. (1985) J. Cell Biol. 101, 269–276.
15 Cassel, D., Whiteley, B., Zhuang, Y. and Glaser, L. (1985) J. Cell. Physiol. 122, 178–186.
16 Boyum, A. (1968) J. Clin. Lab. Invest. Suppl. 97, 77–98.
17 Grinstein, S., Cohen, S., Goetz, J.D., Rothstein, A. and Gelfand, E.W. (1985) Proc. Natl. Acad. Sci. USA 82, 1429–1433.
18 Grinstein, S. and Furuya, W. (1986) Am. J. Physiol. 250, 283–291.
19 Rink, T.J., Tsien, R.Y. and Pozzan, T. (1982) J. Cell Biol. 95, 189–196.
20 Thomas, J.A., Buchsbaum, R.N., Zimniak, A. and Racker, E. (1979) Biochemistry 18, 2210–2218.
21 Rosoff, P.M., Stein, L.F. and Cantley, L.C. (1984) J. Biol. Chem. 259, 7050–7060.
22 Borghetti, A.F., Tramacere, M., Ghiringhelli, P., Severini, A. and Key, J.E. (1981) Biochim. Biophys. Acta 646, 218–233.
23 Shotwell, M.A., Jayme, D.W., Kilberg, M.S. and Oxender, D.L. (1981) J. Biol. Chem. 256, 5422–5427.
24 Paris, S. and Pouyssegur, J. (1984) J. Biol. Chem. 259, 10989–10994.
25 Grinstein, S. and Rothstein, A. (1986) J. Membr. Biol. 90, 1–12.
26 Aronson, P.S., Nee, J. and Suhm, M. (1982) Nature (Lond.) 299, 161–163.
27 Moolenaar, W.H. (1986) Annu. Rev. Physiol. 48, 363–376.