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Comparison between sodium–hydrogen ion and lithium–hydrogen ion exchange in human platelets

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The membrane-coupled exchange of Li^+ and/or Na^+ for H^+ was studied in human platelets measuring intracellular pH (pH_i) with a fluorescent indicator. A Li^+-containing medium restored the internal pH of preacidified platelets to their prior pH_i control value. When Na^+ was replaced by Li^+, similar steady-state values were attained in this system, although it was transported more slowly. The K_m and V_{max} were both higher with Na^+ than with Li^+. Exchanges of Li^+ or Na^+ with H^+ were both blocked by ethylisopropylamiloride (EIPA) achieving half-maximal inhibition at submicromolar concentrations. The efflux of H^+ ions exchanged by Li^+ or Na^+ was reversible: platelets preloaded with Li^+ or Na^+ and resuspended in a choline medium exhibited an influx of H^+ sensitive to EIPA. Thrombin, an activator of Na^+/H^+ exchange, induced a rapid increase in platelet internal pH in the presence of exogenous Li^+. Thus: (1) Li^+ can be substituted for Na^+ in both the forward and the reverse exchange reaction; (2) Li^+, while having a higher affinity than Na^+ for the external site of the membrane carrier, has a lower K_m and (3) Li^+ as well as Na^+ exchange are activated by thrombin.

Introduction

An electroneutral Na^+/H^+ exchange mechanism has been demonstrated in a variety of membrane vesicles and intact cells, including platelets, lymphocytes and erythrocytes [1].

Previous kinetic characterization of the exchanger indicates that Na^+ ions bind to a single, saturable extracellular site. Both H^+ and amiloride derivatives appear to compete with extracellular Na^+ for binding to this site [2].

The exchanger has two H^+-binding sites on the cytoplasmatic side, a transport and a regulatory site. The exchanger is quiescent above a pH_i value called set point, and becomes increasingly active as pH_i is reduced as a result of H^+ binding to the intracellular regulatory site [3].

Phosphorylation of the exchanger increases the affinity of the regulatory site for H^+, shifting the set point towards alkalinity. It has been suggested that this is the working mechanism for the activation of platelet Na^+/H^+ exchange by thrombin, via G-protein-coupled receptors that activate protein kinase C [4].

Although other ions could bind to the exchanger it is not clear how they interact with the transport and regulatory sites on the intracellular and extracellular sides of the membrane [5–7].

The aim of the present study was to compare the characteristics of the exchanger in human platelets when it is driven by Na^+ or Li^+ gradients, both in direct and reverse mode of operation.

The results show differences in kinetic parameters: Li^+ is transported more slowly than Na^+ but has a higher affinity for binding sites. A high Hill coefficient for the EIPA inhibition curve was found for Na^+ but not for Li^+.

To the best of our knowledge this is the first report of the use of monensin to load a cell with Li^+, providing an alternative way to force the exchanger to operate in a reverse mode. Another new finding was that thrombin produced a similar stimulation of H^+ efflux when the exchanger was driven by either Na^+ or Li^+ gradients.

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Abbreviations: SHR, spontaneous hypertensive rats; IC_{50}, half-maximal inhibitory concentration; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EIPA, ethylisopropilamiloride; CDF, (5 and 6)-carboxyl-4,5-dimethylfluorescein; DMSO, dimethylsulfoxide.

Materials and Methods

Platelets were obtained from samples of 25 ml of fresh human blood from healthy donors collected with 15% (v/v) of a medium containing 25 g/l citric acid, 50 g/l sodium citrate and 20 g/l glucose. Platelet-rich plasma was prepared by centrifuging whole blood at $200 \times g$ for 15 min at room temperature. Likewise, platelet pellets were obtained by centrifuging at $800 \times g$ for 12 min.

Different media were used: the control Na^+ solution contained 140 mM NaCl, 4.7 mM KCl, 2.1 mM $MgSO_4$, 1.35 mM $CaCl_2$ and 10 mM Hepes at pH 7.4; the Ca^{2+}-free solutions had the same composition as the Na^+ solution, except that 1 mM acetylsalicylic acid is substituted for $CaCl_2$. Lithium and choline solutions, made by the total isosmotic replacement of NaCl by lithium or choline chloride, respectively, and titrated at the indicated pH, were otherwise identical with the control medium.

The platelet pellet was gently resuspended in Ca^{2+}-free medium supplemented with 20 μM 5-(and 6)carboxyl-4,5-dimethylfluorescein (CDF) diacetate and incubated at 37°C for 30 min. Under these conditions, this permeant ester diffuses through the cell membrane, and is thereafter cleaved in situ by native intracytoplasmic esterases. The trapped probe contains ionizable groups that hinder diffusion out of the cell or into other subcellular organelles, thus ensuring that the fluorescent marker remains cell-associated and sequestered within the cytoplasmatic compartment.

After probe loading, the platelets were pelleted again, washed once in the Ca^{2+}-free solution and resuspended in 0.5 ml of either the Na^+ solution or the choline medium (pH 6). To induce intracellular acidification an 1-h preincubation at room temperature in the low-pH media was used. This condition involves the inactivation of the forward Na^+/H^+ exchange, and H^+ accumulation from cytoplasmatic metabolic reactions or influx through cell membrane.

For fluorescent labelling determinations, 50 μl of platelet suspension were diluted to 1.0 ml with the indicated medium in a cuvette stabilized at 37°C. An Aminco Bowman spectrofluorimeter was used with excitation and emission wavelengths at 490 nm and 535 nm, respectively.

The nigericin/K^+ method of Thomas et al. [8] was employed for calibration of cytoplasmatic pH. In each preparation platelet fluorescence was measured in four samples after replacement of 130 mM NaCl with KCl plus 10 μM nigericin titrated to different pH values within the range 6.30–7.40. In these solutions the extracellular pH became equilibrated with pH_i within less than 2 min. The pH vs. fluorescence regression line was used to calculate experimental pH_i by interpolation.

The reversibility of the exchange was studied in platelets equilibrated for 30 min at 37 C in the lithium medium containing 5 μM monensin or in the sodium medium with 0.5 μM monensin plus 0.1 mM ouabain and then washed and resuspended in a solution containing the same cation with the addition of 10 mg/ml BSA in order to scavenge the ionophore. The lithium solution was kept at pH 7.4, but the Na^+ medium was adjusted to pH 7.0. This pH was chosen on the basis of preliminary experiments showing that such conditions prevented the intracellular alkalinization produced by the treatment with monensin and ouabain.

Monensin rapidly equilibrated internal and external ionic content. To estimate the intracellular activity of these cations a null point titration was done: samples of the cation-loaded platelets were added to warm media containing decreasing concentrations of Na^+ or Li^+ and increasing concentrations of choline; at 140 mM cation concentration alkalinization was attained in the presence of 50 μM monensin, with lower cation concentrations an increasing acidification was observed. The concentration of extracellular Na^+ or Li^+ at which monensin has no effect on pH_i was determined by interpolation. This value was used as an estimation of the intracellular cation concentration.

The effect of thrombin was tested in platelets resuspended in choline medium at pH 7.4 and diluted with Na^+ or Li^+ solutions to allow a 3-min preincubation at 37°C before the addition of 2 U/ml of thrombin.

The amiloride derivative ethylisopropylamiloride (EIPA) was used to investigate the role of the Na^+/H^+ exchanger in the changes of pH_i of platelets. This compound is a selective inhibitor of the Na^+/H^+ exchanger and completely blocks the pH changes at a concentration of 60 μM. To reduce the possibility of non-specific effects of the solvent 6 μM EIPA was used in experiments with thrombin.

The stock solutions of EIPA and CMF acetate were made in dimethylsulfoxide, 50% glycerol was used for thrombin and ethanol for nigericin and monensin.

Results are given as mean ± S.E. Differences were analyzed by means of the Student's *t*-test and considered significative if the *P* value was smaller than 0.05. When the effect of a treatment was evaluated changes in pH were calculated to eliminate differences between the initial pH values of preparations.

Results

In order to characterize the forward Na^+, Li^+/H^+ exchange reaction, CDF-loaded platelets were acidified by incubation in a choline solution at pH 6.0 and then were resuspended in Na^+, Li^+ or choline media at pH 7.4. The pH_i measured in the acid-treated platelets (6.60 ± 0.07) increases in the Li^+ solution, reaching a steady value in 3 min (7.29 ± 0.07). The

same final pH_i was obtained in a Na^+ solution (7.32 ± 0.04), that is similar to the pH_i of control, no preacidified platelets (7.12 ± 0.03).

The pH_i remains practically unaltered when choline was used to replace the extracellular cation, or when the exchanger was blocked by EIPA 60 μM (Fig. 1).

The addition of 0.5 mM monensin showed that recovery of pH_i stopped before the exchange was in electrochemical equilibrium. Fig. 1 also shows the ability of monensin to act as a cationic shuttle allowing the exchange of H^+ for Li^+ or Na^+, but not for choline.

The reversibility of the Li^+/H^+ exchange is shown in Fig. 2. In the experiment depicted, platelets loaded with Li^+ by preincubation in monensin were resuspended in a medium containing choline at pH 7.4, (final $[Li^+]_o = 7$ mM).

Under an outwardly-directed Li^+ gradient the pH_i was observed to decrease gradually from 6.51 ± 0.06 to 6.19 ± 0.08 after 2 min. Parallel suspensions incubated under the same conditions but in the presence of EIPA did not show significative pH_i changes (6.52 ± 0.05 to 6.45 ± 0.06, n.s.).

Gradual increases in $[Li^+]$ reduced the pH_i change (data not shown) and when $[Li^+]_o$ was maintained at 140 mM the pH_i was stable, around 6.46.

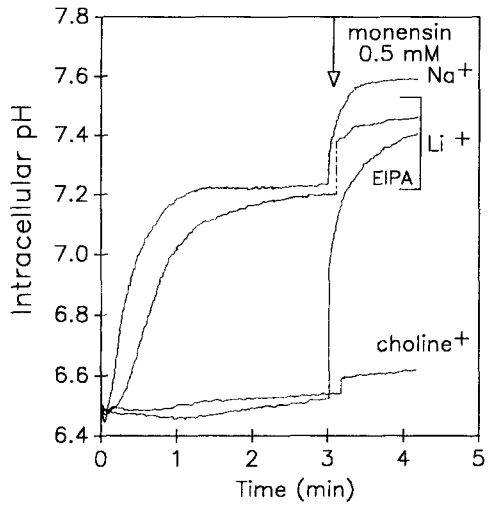

Fig. 1. Recovery from acidification by exposure to media containing Na^+ or Li^+ at pH 7.4. The curves show the time-course of recovery of pH_i of platelets after 1 h of preincubation in an acidic Na^+-free solution (choline at 140 mM, pH 6.0, room temperature), the platelets were resuspended in a medium containing either Na^+ (left ascending curves) or Li^+ (right curve) at 133 mM, pH 7.4, 37°C. The slope immediately after the inflection point was considered the initial velocity, since the Li^+ curve has a sigmoidal shape at the beginning. The steady curves show parallel experiments with choline or Li^+ in the presence of EIPA 60 μM. After 3 min the record was interrupted (arrow) to add monensin at a final concentration of 0.5 mM showing the capacity of ionophore to exchange Li^+ or Na^+ for H^+. Traces from a single experiment are representative of six similar assays.

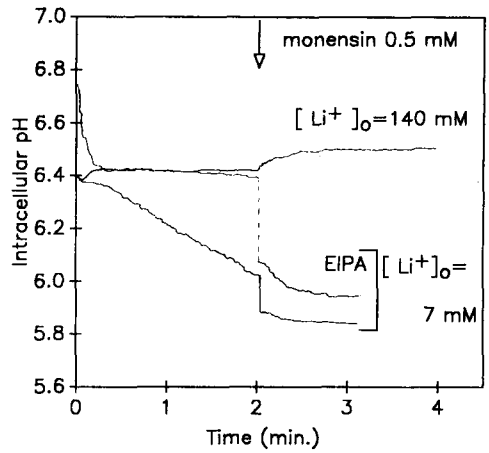

Fig. 2. Reversibility of Li^+/H^+ exchange. Platelets were preloaded with Li^+ as described in Materials and Methods and resuspended at pH_o 7.4, at the indicated extracellular lithium concentration. Note that 60 μM EIPA inhibits the acidification. Monensin addition allows to visualize the direction of the Li^+ gradient.

In a similar situation, platelets preloaded with Na^+ resuspended in choline to reduce the external Na^+ concentration to 7 mM exhibits an acidification from pH_i 7.00 ± 0.03 to 6.65 ± 0.06 in 2 min. EIPA blocks the change (pH_i moves from 6.98 ± 0.01 to 6.92 ± 0.03), and the pH_i remains stable when $[Na^+]_o$ was kept at 140 mM (from 7.15 ± 0.03 to 7.18 ± 0.04). These results indicate that the Na^+, Li^+/H^+ exchanger was playing a significant role in the observed changes in pH_i by means of its reverse mode of operation.

The addition of monensin was followed by an additional acidification at low $[Li^+]_o$ or $[Na^+]_o$ (e.g., 7 mM, the lowest final concentration used), and by an alkalinization when the external concentration of these cations was 140 mM. Changing gradually the proportions of choline and Li^+ or Na^+ the concentration of the transported cation at which the pH_i remained unaltered after monensin could be obtained. This null point was a $[Li^+]$ concentration of 97 ± 2 mM and would be used as an estimation of $[Li^+]_i$, if we assume that pH_i will remain stable in the absence of a Li gradient, in spite of the presence of the ionophore. In the case of Na^+, the null-point titration indicates that the $[Na^+]_i$ was 91 ± 9 mM.

In the forward mode of operation of the exchanger, the rate of pH_i recovery was dependent on both the nature of the extracellular cation and its concentration. Fig. 3 shows the least-square fitting of the data of the initial velocity to a Michaelis-Menten model. The K_m obtained from these curves were 46 ± 6 mM for external Na^+ and 35 ± 19 mM (S.E. of global fit) for external Li^+, considering only the points for $[Li^+] < 100$ mM. The V_{max} was 0.94 ± 0.15 units of pH/min for Na^+. The fitting indicated that in the presence of Li^+ the V_{max} will be 26.9% of the value found for Na^+, but

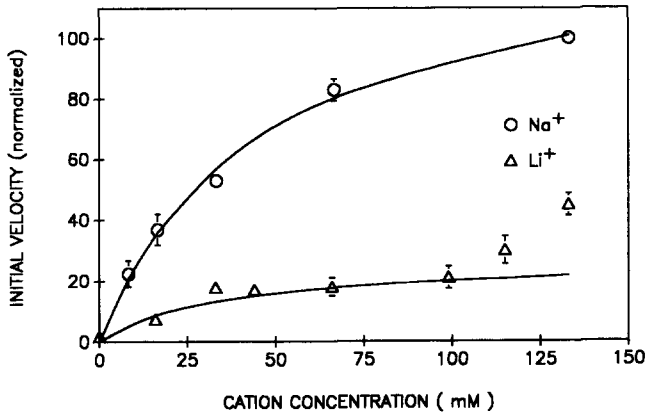

Fig. 3. Dependence of the Na^+, Li^+/H^+ exchange rate on the external concentration of monovalent cation. The initial velocity of the pH shift relative to the value at 133 mM Na^+ during the recovery of preacidified platelets in each experiment is plotted as a function of the concentration of Na^+ (○) or Li^+ (△). Means ± S.E. of six assays are given. Solid lines represent the least-square non-linear global fitting to a Michaelis-Menten model.

Li^+ curves showed a non-michaelian pattern increasing the velocity at high Li^+ concentrations.

The recovery of pH_i induced by the increase in cation concentration to 133 mM is inhibited by EIPA (Fig. 4). The half-maximal inhibitory concentration (IC_{50}) of EIPA was 213 ± 20 nM and 367 ± 99 nM in the presence of Na^+ and Li^+, respectively ($P < 0.05$). The Hill coefficient for the inhibition curves were 2.0 ± 0.3 and 1.2 ± 0.1 ($P < 0.05$), respectively.

Platelets preincubated for 3 min. with Li^+ 133 mM and pH 7.4 were stimulated by incubation with 2 U/ml of thrombin. After a brief fall in intracellular fluorescence, which may be the result of a change in cell shape [9], there was a progressive increase of pH_i. After 2 min, the rise amounted to 0.215 ± 0.014 pH units. Moreover in the presence of 6 μM EIPA, the effect of thrombin was markedly reduced with a final value of pH increase of only 0.064 ± 0.023 pH units. Similar results were obtained when Na^+ was used in place of Li^+ (Fig. 5)

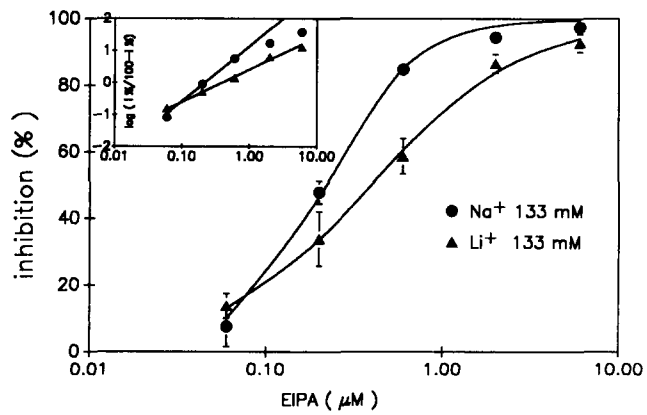

Fig. 4. Inhibition of Na^+, Li^+/H^+ exchange by EIPA. Preacidified platelets were transferred to Na^+- or Li^+-containing medium at pH 7.4 either with or without EIPA. The reduction in the initial velocity of pH recovery observed in the presence of the inhibitor, expressed as a percent of the corresponding control rate (inhibition %), is plotted as a function of EIPA concentration (μM). Mean ± S.E. (when greater than symbols) are shown. (●), Na^+; (▲), Li^+; each at 133 mM. Solid lines represent the least-square non-linear fitting to a Hill model. Inset: Hill plot of the same data.

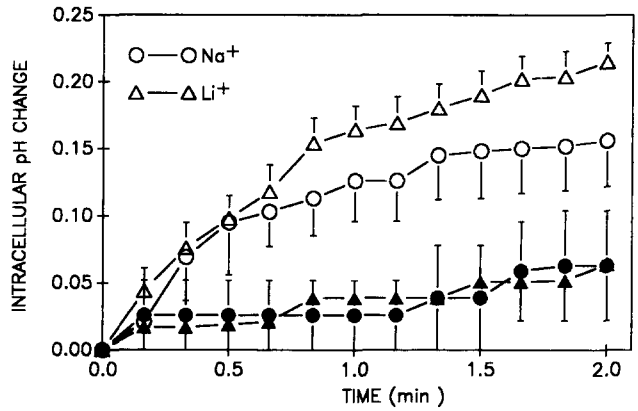

Fig. 5. Increase in platelet pH_i after treatment with thrombin. Platelets preincubated for 3 min in Na^+ (circles) or Li^+ (triangles) at 133 mM either with (solid symbols) or without (open symbols) 6 μM EIPA, were stimulated by treatment with 2 U/ml of thrombin. Data are means ± S.E. of three assays.

Discussion

The alkalinization of preacidified platelets in a Li^+-containing medium and the acidification of Li^+-loaded platelets is consistent with the functioning of a Li^+/H^+ exchange, effected by the same mechanism that normally exchanges Na^+ with H^+.

This assertion is supported by the following findings: (1) The rate of pH_i recovery in preacidified platelets increases progressively as a function of external (i.e., inwardly directed) gradients of Na^+ and Li^+; (2) The rate of acidification of cation-preloaded platelets is reduced as the external concentration of Na^+ or Li^+ increases, (i.e., reduction of outwardly directed gradient); (3) EIPA, a competitive inhibitor of Na^+/H^+ antiport also inhibits the H^+ countertransport induced by inward- and outward-directed Li^+ gradients.

Earlier studies indicated that Li^+ interacts directly with the Na^+/H^+ exchanger in both isolated membranes and intact cells [5–7]. In these studies a higher affinity for Li^+ than for Na^+ was reported, with the hierarchy of binding affinities for the external transport site being $H^+ \gg$ amiloride $\gg Li^+ > NH_4^+ > = Na^+ \gg K^+$, Rb^+, Cs^+, choline [10].

In membrane vesicles from intestinal microvilli, the K_m for Li^+ was found to be 10-times lower than the K_m for Na^+ [5], but values for these parameters do not differ so much in whole cells [10].

In our experiments, the affinity for Li^+ in whole human platelets was lower than for Na^+, considering the points were $[Li^+]_o < 100$ mM.

Nonetheless, despite the fact that this affinity for Li^+ is high, there is a marked reduction in its V_{max}. With respect to the induction of platelet swelling in propionate salts, Livne et al. [11] found that Li^+ was 32% as effective as Na^+.

This observation is consistent with the reduction in the initial rate of pH_i recovery in our own experiments (Fig. 3). This reduction in the rate of H^+ exchange on the part of Li^+ might result from either decreased translocation velocity for the antiport carrying Li^+ or a slower dissociation rate for Li^+ from its internalized binding site after passage into the cell. Once inside the cell, Li^+ or Na^+ could conceivably bind to an internal regulatory site (a locus at which the bound effector alters the activity of the Na^+, Li^+/H^+ carrier but is not necessarily transported itself), because intracellular Li + accumulation is slower at $[Li^+]_o < 100$ mM the initial velocity might reflect the behavior of cation-depleted cells. Experimentally determined 'initial velocity' could be affected by intracellular accumulation of Li^+ at $[Li^+]_o > 100$ mM. The sigmoidal appearance of the recovery curve as a function of time (Fig. 1) also suggests an activation by the intracellular accumulated Li^+.

The interaction of EIPA with the Na^+/H^+ exchanger has been previously assumed to be competitive [12]. As theoretically expected for a cation of higher affinity, the IC_{50} of the inhibitor is greater for Li^+ than for Na^+.

EIPA competes for the external cation site, lowering the initial rate of recovery. Theoretically this inhibition should have a Hill coefficient close to the unity, and this is the case when Li^+ was the transported ion. But Na^+ exchanges faster, is accumulated intracellularly and interacts with intracellular sites. This secondary effect of Na^+ is also inhibited by EIPA, leading to a high Hill coefficient for Na^+/H^+ exchange. The intracellular action of Li^+ on the initial velocities is almost absent because of its slow accummulation.

Consistent with this possibility, it was previously reported that amiloride is a mixed inhibitor of Na^+/H^+ exchange and a single competitive inhibitor of Li^+/H^+ exchange [5]. The overall results from these kinetic observations by us and by others [5,13] seem to indicate the existence of cooperative interactions of Na^+ or Li^+ in the intracellular side of the exchanger.

Carboxylic ionophores such as monensin promote an electrically neutral diffusion of the cations that allowed cellular loading. Because the movements of extracellular Na^+ is faster than the egress of intracellular K^+, in the presence of an inwardly-directed gradient a transient egress of H^+ occurs raising temporarily the intracellular pH [14]. We took advantage of these phenomena to estimate the intracellular concentration by null-point titration.

The initial pH_i in the reverse mode experiment was similar to the one used in the forward mode for Li^+. Unfortunately, in the experiments of reverse mode operation, the loading maneuvers and/or the presence of ouabain to inhibit the extrusion of Na^+ produce an intracellular alkalinization that made it difficult to perform a quantitative analysis of the results.

The risk of developing hypertension in humans may be linked to an inherited membrane abnormality that is present in different types of cells [15–17]. For this reason several laboratory tests have been developed in an attempt to detect some marker of this membrane defect in hypertensive subjects. As a result of such efforts, an increased Na^+/H^+ exchange activity has been documented in platelets [15,16], and leucocytes of hypertensive humans [17] and of spontaneously hypertensive rats (SHR) [18]. Recently, this increase was attributed to an increased protein kinase C activity in hypertensive subjects [19] without change in the number of transporters. Several reports have described an alteration of phosphoinositide metabolism by Li^+ [20–22] and it is important to know if the change of Na^+ for Li^+ interferes with the regulatory mechanism of this potential predictor of hypertension risk.

The present experiments demonstrate that thrombin, an agonist that stimulates Na^+/H^+ countertransport via protein kinase C-catalyzed phosphorylation [24–26] is able to activate Li^+/H^+ in platelets. Therefore, the change of Na^+ by Li^+ does not interfere with the regulatory mechanisms involved in the activation of the exchanger.

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References

1 Gende, O.A. and Cingolani, H.E. (1991) Arch. Int. Physiol. Biochim. 99, 95–98.
2 Aronson, P.S., Nee, J. and Suhm, M.A. (1982) Nature 299, 161–163
3 Kinsella, J.L. and Aronson, P.S. (1980) Am. J. Physiol. 238, F461–F469.
4 Livne, A., Aharonovitz, O., Fridman, H., Tsukitani, Y. and Markus, S. (1991) Biochim. Biophys. Acta 1068, 161–166.
5 Ives, H.E., Yee, V.J. and Warnock, D.G. (1983) J. Biol. Chem. 258, 9710–9716.
6 Kinsella, J.L. and Aronson, P.S. (1981) Am. J. Physiol. 241, C220–C226.
7 Aronson, P.S., Shum, M.A. and Nee, J. (1983) J. Biol. Chem. 258, 6767–6771.

8 Thomas, J.A., Buchsbaum, R.N, Zimniar, A. and Racker, E. (1979) Biochemistry 18, 2210–2218.
9 Siffert, W., Siffert, G., Scheir, P. and Akkerman, J.W. (1989) Biochem. J. 258, 521–521.
10 Paris, S. and Pouyssegur, J. (1983) J. Biol. Chem. 258, 3503–3508.
11 Livne, A., Grinstein, S. and Rothstein, A. (1987) Thromb. Haemost. 58, 971–977.
12 Wright, E.M., Wright, S.H., Hirayana, B. and Kipper, I. (1982) Proc. Natl. Acad. Sci. USA 79, 7514–7517.
13 Green, J., Yamaguchi, D.T., Kleeman, C.R. and Mualen, S. (1988) J. Gen. Physiol. 92, 239–261
14 Henderson, P.J.F., McGivan, J.D. and Chappell, J.B. (1969) Biochem. J. 111, 521–535.
15 Livne, A., Balfe, J.W., Veitch, R., Marquez-Julio, A., Grinstein, S. and Rothstein, A. (1987) Lancet i, 533–536.
16 Rosskopf, D., Morgenstern, E., Scholz, W., Oswald, V. and Siffert, W. (1991) J. Hypertens. 9, 231–238.
17 Ng, L.L., Dudley, C., Bomford, J. and Hawley, D. (1989) J. Hypertens. 7, 471–475.
18 Feig, P.V., D'occhio, M.A. and Boylan, J.W. (1987) Hypertension 9, 282–288.
19 Livne, A., Aharonovitz, O. and Paran, E. (1991) J. Hypertens. 9, 1013–1019.
20 Vickers, J.D., Kinlough-Rathbone, R.L. and Mustard, J.F. (1984) Biochem. J. 224, 399–405.
21 Parker, J.C. (1986) J. Gen. Physiol. 87, 189–200
22 Davis, B.A., Hogan, E.M. and Boron, W.F. (1992) Am. J. Physiol. 263, C246–C256
23 Zavoico, G.B. and Cragoe, E.J. (1988) J. Biol. Chem. 263, 9635–9639.
24 Siffert, W. and Akkerman, J.W. (1988) J. Biol. Chem. 263, 4223–4227.
25 Hwang, C.L., Cogan, M.G., Cragoe, M.G. and Ives, H.E. (1987) J. Biol. Chem. 262, 14134–14140.
26 Livne, A.A., Sardet, C. and Pouyssegur, J. (1991) FEBS Lett. 284, 219–222.