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HYPEROSMOTIC ACTIVATION OF THE Na^+–H^+ EXCHANGER IN A RAT BONE CELL LINE: TEMPERATURE DEPENDENCE AND ACTIVATION PATHWAYS

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SUMMARY

1. The hyperosmotic activation of the Na^+–H^+ exchanger was studied in an osteoblast-like rat cell line (RCJ 1.20). The activation was monitored by recording the intracellular pH (pH_i) changes employing double excitation of the pH-sensitive fluorescent dye 2′7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM).

2. Exposure of the cells to a hyperosmotic HCO_3^--free medium at 37 °C produced an initial cytosolic acidification of 0·05 pH units followed by a lag period and an alkalinization overshoot of about 0·2 pH units, without a concomitant change of the free cytosolic calcium $[Ca^{2+}]_i$ by the use of Fura-2 calcium-sensitive probes. This response was completely inhibited by amiloride (0·33 mM) or by Na^+ depletion from the external medium and insensitive to the extracellular Cl^- replacement, indicating the involvement of a Na^+–H^+ exchanger in the hyperosmotic response.

3. Hyperosmotic stimuli (200 mosM sucrose) applied in the temperature range of 17–37 °C demonstrated a shortening of the lag period preceding alkalinization and an increased rate of proton extrusion upon temperature elevation. The biochemical reaction underlying the lag period and the proton extrusion resulted in apparent activation energies of 19 and 29 kcal mol^{-1}, respectively, as calculated from the appropriate Arrhenius plots.

4. Stimulation of the exchanger under isosmotic conditions by 25 nM 4β-phorbol 12-myristate 13-acetate (PMA) and 0·1 mM vanadate resulted in an amiloride-sensitive pH_i increase of about 0·08 pH units. The hyperosmotic stress was additive to the stimulatory effects of these agents, suggesting an independent hyperosmotic activation pathway.

5. The hyperosmotic activation of the Na^+–H^+ exchanger was independent of cAMP, cGMP, cytosolic Ca^{2+} and protein kinase C. Thus, none of the classical transduction mechanisms seem to be involved directly in the hyperosmotic activation of the antiporter.

6. The pH_i response induced by the hyperosmotic stress was abolished by two

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calmodulin inhibitors, W-7 and chlorpromazine (50% inhibition, K_i at 28 and 20 μM, respectively), 20 μM cytochalasin B, but not by 10 μM colchicine. The results suggest the involvement of actin and calmodulin-like structural elements of the cytoskeleton in the transduction process leading to the activation of the Na^+–H^+ exchanger.

INTRODUCTION

The Na^+–H^+ exchanger is involved in the homeostatic regulation of cell volume and intracellular pH (Cala, Anderson & Cragoe, 1988). This exchanger is one of the transporters activated in the primary response of cells to growth factors (Grinstein, Rotin & Mason, 1989). A hyperosmotic challenge leads to an initial cell shrinkage resulting in a coupled regulation of cellular volume and a pH increase due to activation of the exchanger (Hoffman & Simonsen, 1989). The intracellular pH response to a hyperosmotic load consists either of an initial acidification followed by an alkalinization or of an alkalinization alone, and is preceded in both cases by a brief lag period (Cassel, Whiteley, Zhuang & Glaser, 1985; Grinstein, Rothstein & Cohen, 1985). The mechanism underlying the activation of the Na^+–H^+ exchanger by the hyperosmotic stimulus, is yet unknown, though a phosphorylation step was suggested to occur along the activation pathway (Grinstein, Cohen, Goetz & Rothstein, 1985).

Modulations of the intracellular pH are of prime importance to bone cell function. The Na^+–H^+ exchanger is considered one of the main regulators of the intracellular pH of osteoblasts in addition to a chloride–bicarbonate exchanger (Green, Yamaguchi, Kleeman & Muallem, 1988, 1990; Redhead, 1988). It was already suggested that in these cells the cytosolic pH is involved in directing the osteoblastic activity towards bone remodelling (Green *et al.* 1990). These processes of bone remodelling are driven by mechanical stresses imposed on bone under physiological conditions (Wolff, 1892; Clinton & Lanyion, 1985). Mechanical loads on bone may lead to direct effects on the cell membrane consisting of either stretching or folding (Marcus, 1987). The folding process can be simulated by applying a hyperosmotic stress, which also modifies the intracellular pH. In order to study the activation of the antiporter by the hyperosmotic stress, a well-established RCJ 1.20 osteoblastic cell line was employed (Aubin, Heersche, Merrilees & Sodek, 1982). These cloned cells were previously shown to respond to bone-related hormones (Ferrier, Illeman & Zakshek, 1985) and hyperosmotic stress (Dascalu, Nevo & Korenstein, 1991).

The current study characterizes the Na^+–H^+ exchanger of osteoblasts and examines the influence of temperature on the hyperosmotic activation. The transduction of the hyperosmotic stimulus via the various secondary messengers is investigated. We demonstrate the involvement of actin and calmodulin inhibitors in the mediation of the response to the osmotic load.

METHODS

Cell culture

A cell line of rat calvaria, RCJ 1.20 (Aubin *et al.* 1982), was cultured in Modified Eagle Medium (MEM) containing 7·5% fetal calf serum (FCS), 4·5 mg ml^{-1} glutamine, 50 units ml^{-1} penicillin and 200 μg ml^{-1} of streptomycin. The cells used in the experiments were seeded at an initial density of

1×10^5 cells cm^{-2} and kept at 37 °C under an atmosphere of 5% CO_2 in air. Three to four days after plating, cells were collected from confluent cultures by 5–10 min trypsinization (0·05% trypsin and 0·02% EDTA). The proteolytic digestion was stopped by 2% FCS and the cells were briefly (1–2 s) sedimented at 7000 *g* and resuspended in MEM containing 0·2% FCS. Prior to fluorimetry, cells were incubated at 37 °C for 2 h. Cell viability was determined by Trypan Blue and experiments were performed when Trypan Blue was excluded from ⩾ 95% of the cells.

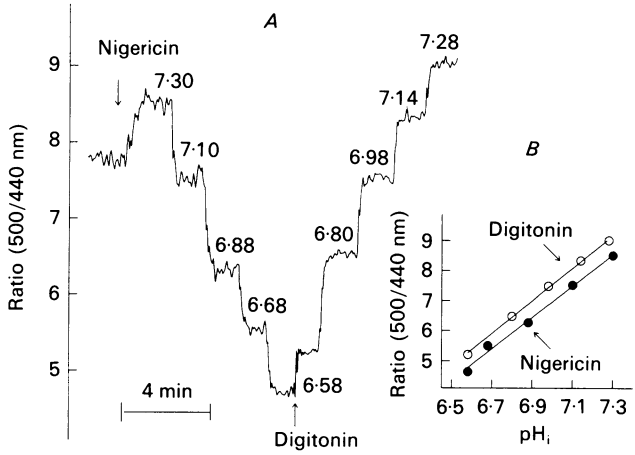

Fig. 1. Calibration of pH_i in BCECF-loaded cells. *A*, cells suspended in an isosmotic 130 mM KCl solution (see Methods) were treated with nigericin and digitonin, as indicated. The pH was varied by titration with HCl or KOH, respectively. *B*, pH dependence of the fluorescence ratio in calibrations employing nigericin or digitonin. Data are means ± S.E.M. ($n = 6$). When absent, the error was smaller than the symbol.

Fluorimetric procedures and calibration

Cells in suspension were loaded for 30 min at 37 °C with 2–3 μM BCECF for Fura-2 AM. Subsequently, cells were washed twice (7000 *g*) and suspended at $5–6 \times 10^7$ cells ml^{-1} in a 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES)-buffered solution. Each fluorimetric measurement employed $1–2 \times 10^6$ cells ml^{-1} under continuous stirring at the desired temperature (17–37 °C). Fluorescence ratios of BCECF-AM (excitation, 440 and 500 nm; emission, 530 nm, slits 4 nm) and Fura-2 AM (excitation, 340 and 380 nm; emission, 510 nm, slits 4 nm) were monitored with an SLM 8000 spectrofluorimeter. The on-line data were recorded and analysed on an XT computer. Fluorescence background under the conditions of BCECF-AM experiments was < 5%. Fluorescence background measured under the same conditions of Fura-2 AM measurements was subtracted from the measured fluorescence values. BCECF-loaded cells were used for 30 min maximum and the leakage during this interval was less than 10%.

Intracellular pH calibration was performed in all experiments by lysing the cells with 50 μM digitonin at various extracellular pH (pH_o). It is known that the excitation spectrum of intracellular BCECF is red shifted as compared to the extracellular dye (Paradiso, Negulescu & Machen, 1986; Calonge & Ilundáin, 1990). Accordingly, the pH_i of the same sample of cells was recalibrated by the nigericin method (Thomas, Buchsbaum, Zimniak & Racker, 1979). Nigericin (10 μM) was added to cells kept in an isosmotic 130 mM KCl solution and the resulting $pH_{i=o}$ was lowered by HCl addition. Subsequently, the cells were lysed with digitonin (50 μM) and the pH was varied with KOH ($n = 6$, Fig. 1*A*). These experiments were summarized in a plot of pH dependence *versus* fluorescence ratio in calibrations employing both nigericin and digitonin (Fig. 1*B*). Thus, the digitonin data were corrected by a factor of 0·08 pH units due to a digitonin-induced red shift of the BCECF excitation spectrum. The basal pH_i of RCJ bone cells suspended in a HEPES-buffered medium ($pH_o = 7·3$) was 6·94 ± 0·01 ($n = 28$, $T = 37$ °C).

Fura-2 AM signals were calibrated by loading the cells with ionomycin (5 μM) in a 2 mM Ca^{2+} solution to obtain maximum fluorescence followed by addition of 10 mM of EGTA (pH adjusted to > 8·5) in order to obtain the minimal fluorescence. The intracellular calcium was calculated as previously described (Grynkiewicz, Poenie & Tsien, 1985) employing a Ca^{2+}–Fura-2 dissociation

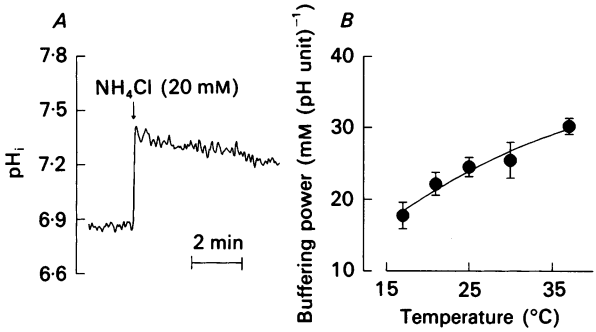

Fig. 2. Buffering power (β_i) determination in Na^+-free medium where Na^+ was equimolarly replaced by choline. *A*, the initial alkalinization was obtained following addition of 20 mM NH_4Cl to cells kept at 37 °C (in this sample experiment $\beta_i \approx 32$ mM (pH unit)$^{-1}$. *B*, the change of β_i with temperature within the range of 17–37 °C. Values represent the means ± S.E.M. of four experiments.

constant of 224 nM. The basal intracellular calcium concentration was 84·6 ± 3·1 nM ($n = 4$). The intracellular sodium, measured by the null-point titration method (Babcock, 1983) was 11·3 ± 2·5 mM ($n = 4$).

The intracellular buffering power (β_i) was measured by the addition of 20 mM (NH_4Cl) to a nominally CO_2–HCO_3^--free solution in the absence of Na_o^+ (Roos & Boron, 1981). The β_i was calculated from the initial alkalinization produced by the added NH_4^+. The pH_i peak of alkalinization was back-extrapolated to the time of exposure to NH_3 (a procedure likely to yield an overestimation of the buffering power) as follows:

$\beta_i = [NH_4^+]_i/\Delta pH_i$. The intracellular concentration of ammonium, $[NH_4^+]_i$ is calculated from: $[NH_4^+]_i = [NH_3]_i \times 10^{(pK_a - pH_i)}$. It was assumed that $[NH_3]_o = [NH_3]_i$, where $[NH_3]_o$ was calculated from the Henderson–Hasselbalch equation. The pK_a of NH_4^+ (9·27 at 37 °C) was corrected for the temperature changes. β_i of the cells was 30·20 ± 1·13 mM (pH unit)$^{-1}$ ($n = 6$) at 37 °C (Fig. 2*A*) and varied significantly with temperature (a comparison of β_i at 37 and 17 °C yields a $P < 0.001$; Fig. 2*B*). Accordingly, the calculation of apparent activation energies employs fluxes, as determined by: $J_{H^+} = (dpH/dt) \times \beta_i$.

Solutions and materials

All solutions were HCO_3^- free. The Na^+ solution contained (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES and 0·2 % FCS at pH 7·3. Choline, K^+ and gluconate solutions were prepared by isosmotic replacement of Na^+ by choline or K^+ and chloride by gluconic acid. The hyperosmotic effect was produced by sucrose, mannitol or sorbitol at the specified osmolalities. The osmolarity of the isosmotic media was 300 ± 5 mOSM (as measured by a Wescor 5100 vapour pressure osmometer). The pH meter measurements were calibrated at the different temperatures using the known variation with temperature of a standard buffer (9887 Titrisol, Merck). The pH of a HEPES-buffered solution at each desired temperature was corrected using an electrode that has been calibrated in a standard buffer at the appropriate temperature.

The Na^+–H^+ exchange activity was assessed by the NH_4^+ prepulse technique. The cells were exposed to extracellular NH_4^+ (20 mM) resulting in an instantaneous alkalinization followed by a slow recovery of the pH_i. A minimal aliquot of NH_4^+-loaded cells (40 μl) was resuspended 10–15 min later in an isosmotic HEPES medium devoid of NH_4^+ (2000 μl) leading to an immediate

acidification due to NH_3 efflux out of the cells with a concomitant entrapment of the protons in the cytosol and an ensuing pH_i recovery. The amiloride-sensitive initial rate of alkalinization in the recovery reflects the antiporter activity. Pre-incubation periods for the different agents varied from 5 min for ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide, hydrochloride (W-7), chlorpromazine and calcium ionophores to 15 min for the cyclic nucleotides, forskolin, 1-(5-isoquinoline sulphonyl)-2-methyl-piperazine, dihydrochloride (H-7) and indomethacin. Cells were pre-incubated with cytochalasin B for 45 min. The cellular volume was measured by a Coulter Counter type D industrial with an orifice of 100 μm. Chlorpromazine and W-7 resulted in an instantaneous change in the baseline fluorescence ratio (alkalinization of 0·14 pH units) without a following pH recovery. Accordingly, this artifact was subtracted from the pH calculations.

BCECF-AM was purchased from Molecular Probes, Inc., Eugene, OR, USA. Fura-2 AM, nigericin, amiloride, 4,4′-diisothiocyanostilbene-2-2′-disulphonic acid (DIDS), ouabain, monensin, furosemide, 8-bromo-cAMP, mono-butyryl cGMP, forskolin, A23187, ionomycin, EGTA, vanadate, 4β-phorbol 12-myristate 13-acetate (PMA), 4α-phorbol, 12,13-didecanoate (4α-phorbol), 1-(5-isoquinoline sulphonyl)-2-methyl-piperazine, dihydrochloride (H-7), bovine serum albumin (BSA), indomethacin, colchicine, cytochalasin B, chlorpromazine, and W-7 were obtained from Sigma.

The results are presented either as representative tracing of fluorimetric experiments of at least three recordings or as means ± S.E.M. Curves were fitted by a non-linear regression method and initial rates were determined by the slope of the linear fit of the initial recorded curve (30 s at 25–37 °C and 2 min at 17–21 °C). Comparisons were made using Student's unpaired *t* test.

RESULTS

RCJ bone cells were exposed to a solution made hyperosmotic by the addition of sucrose to a HEPES-buffered HCO_3^--free solution. An initial acidification followed by a lag period, an alkalinization and an overshoot were regularly observed at various temperatures (Fig. 3*A*, *B*, and *C*). Replacement of sucrose by sorbitol or mannitol at different osmolarities yielded a comparable pH_i response to the hypertonic stress. A similar response to hyperosmotic stress composed of an acidification, lag period and alkalinization has been described in a human epidermoid carcinoma cell line (A431 cells) (Cassel *et al.* 1985). The dilution or concentration effects on intracellular dye due to cell volume changes are probably minimized by the double excitation methodology employed. Increasing medium osmolarity from 300 to 500 mosM by sucrose addition causes an increase in the initial rate of cellular alkalinization. Above 500 mosM a saturation of the rate change is observed (Fig. 4). The intracellular buffering power at different media osmolarities can be estimated assuming that the cells behaved as perfect osmometers. Accordingly, the proton efflux ($J_{H^+} = (dpH/dt) \times \beta_i$) was calculated, yielding a continuous increase of the flux from 300 to 700 mosM (Fig. 4). A parallel dependence of the alkalinization efflux and medium osmolarity was demonstrated in lymphocytes (Grinstein *et al.* 1985). Accordingly, all ensuing experiments were performed by adding 200 mosM of sucrose to cells suspended in an isotonic HEPES-buffered medium yielding a maximal rate of activation.

A hyperosmotic stress activates the Na^+–H^+ exchanger

The hyperosmotically induced pH_i increase was completely abolished by 0·33 mM amiloride at physiological or lower temperatures (Fig. 5*A*). Amiloride induces an immediate drop of the fluorescence followed by a gradual decrease of the pH_i. The initial decrease of the fluorescence ratio upon the addition of amiloride is attributed

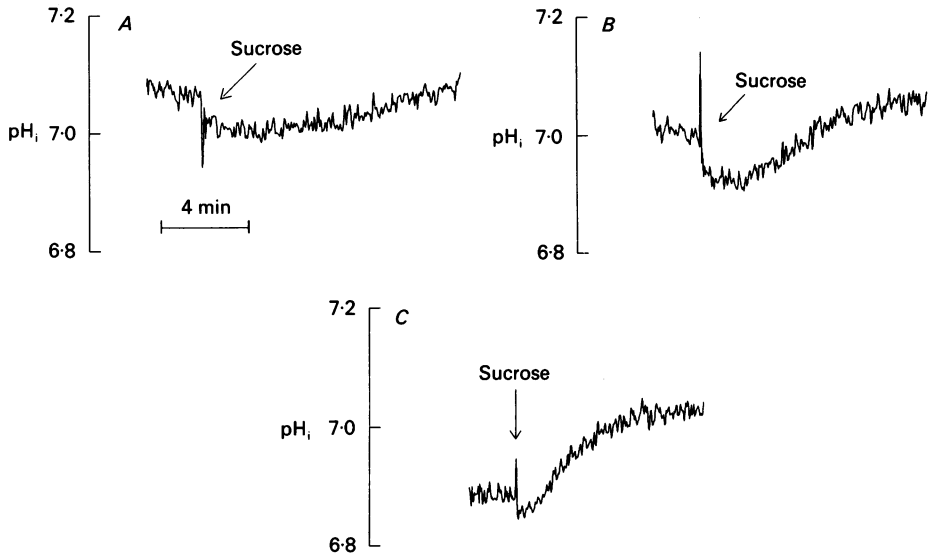

Fig. 3. The temperature effect on the time course of intracellular pH changes following a hyperosmotic stimulus. RCJ bone cells were BCECF loaded and the pH_i was recorded as described in Methods. Where indicated, a hyperosmotic stress (200 mosM sucrose) was administered to cells kept in an isotonic Na^+ medium buffered at $pH_o = 7{\cdot}3$ at 21 °C (*A*), 25 °C (*B*) and 27 °C (*C*). Cells suspended in the isotonic solution were pre-equilibrated for 4 min at the desired temperature prior to the osmotic stimulus.

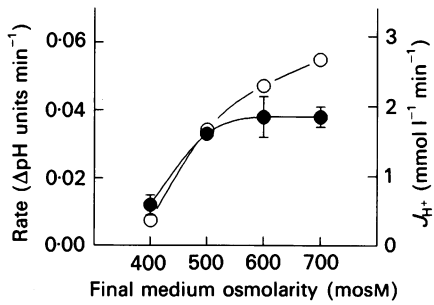

Fig. 4. Relation between the hyperosmotically induced alkalinization and medium osmolarity. BCECF-loaded cells were kept in an isotonic HEPES medium. Different hyperosmotic stimuli were applied by the addition of 100–400 mosM sucrose (from a concentrated 1·6 M stock) at 37 °C. Values represent the mean, and vertical bars the S.E.M. where they exceed the size of the symbols ($n = 4$). The alkalinization rates (●) beyond 500 mosM did not differ statistically. Proton efflux (○) was estimated assuming the cells behaved as perfect osmometers.

to a known quenching effect of amiloride at the selected excitation wavelengths (Paradiso *et al.* 1986). Most probably, the quenching effect is dominant at the higher excitation wavelength and thereby causes a decrease of the excitation ratio. Equimolar replacement of the extracellular sodium by choline (Fig. 5*B*) also

inhibited the hyperosmotically induced alkalinization (K_m = 67·1 mM for $[Na^+]_o$ was obtained under isosmotic conditions, data not shown). The hyperosmotic response was not affected by pre-incubation with 50 μM DIDS, 0·5 mM furosemide, 0·1 mM ouabain (inhibitors of chloride–bicarbonate exchanger, Na^+–K^+–$2Cl^-$ co-transporter

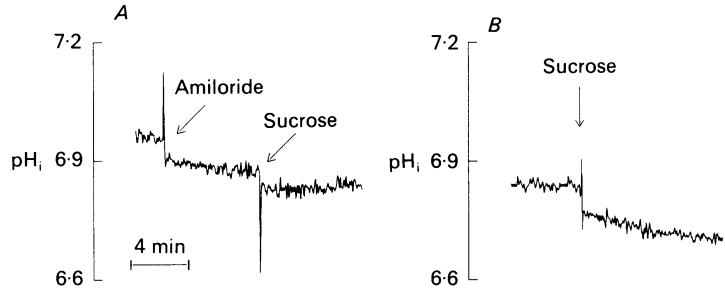

Fig. 5. Inhibition of the hyperosmotically induced alkalinization. Cells were loaded with BCECF and then suspended in an isosmotic Na^+ medium at 25 °C (pH_o = 7·3). *A*, the pH_i was recorded in cells pre-incubated for 2–3 min in the presence of 0·33 mM amiloride. *B*, sucrose (200 mosM) was added where indicated. Extracellular sodium was replaced by equimolar choline and the cells challenged hyperosmotically by sucrose.

and Na^+–K^+ ATPase, respectively) or by replacement of the external chloride with gluconate. Substituting Na^+ by Li^+ in the external medium maintained the pH_i increase at a lower rate, whereas a similar substitution by K^+ and Rb^+ abolished the hypertonic response (data not shown). These results suggest that under our experimental conditions an amiloride-sensitive and sodium-dependent transporter may be responsible for the hyperosmotic response. If it is assumed that shrinkage-induced changes in the pH_i reflect changes in the activity of this transporter, a sodium–proton exchanger is the main transporter complying with the previous findings and underlies the pH_i response to the hyperosmotic stress.

Temperature effects on the Na^+–H^+ exchanger activation

An observed lag period in the pH_i response to the hyperosmotic stimulus preceding alkalinization can be resolved at temperatures lower than physiological (Fig. 3*A* and *B*). In order to characterize this lag time we studied its dependence on temperature. Experiments were carried out in the temperature range of 17–37 °C. All the relevant experiments were performed after the cells were pre-equilibrated for a period of 5 min at the desired temperature. The lack of temperature equilibration between cells kept at 22–23 °C and a solution of 37 °C biases the baseline pH_i by 0·056 ± 0·005 pH units (n = 9). The later hyperosmotic alkalinization following a stimulus is altered due to a temperature-induced acidification (initial rate of 0·022 ± 0·003 pH units min^{-1}, n = 6) as can be seen from the lower hyperosmotically induced alkalinization rates in cells deprived of the pre-equilibration period (Fig. 6A) as compared to well-equilibrated cells (Fig. 6*B*). The shift in the temperature of the incubated cells from 37 to 17 °C increases the basal pH_i of RCJ cells by 0·13 ± 0·01 pH units (n = 3). From the decreased rate of proton extrusion by the exchanger, upon lowering temperature, we

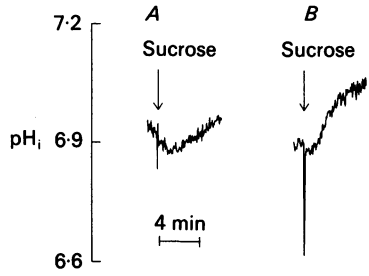

Fig. 6. Effect of the pre-equilibration period on the hyperosmotic response. The pH_i response to sucrose of cells kept at room temperature was recorded following 1 min (*A*) and 10 min (*B*) of pre-equilibration at 37 °C.

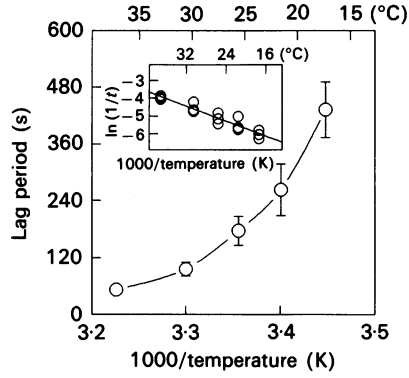

Fig. 7. Temperature dependence of the lag time following a hyperosmotic stimulus. Cells suspended in an isotonic Na^+ medium at $pH_o = 7{\cdot}3$ were osmotically activated by 200 mosM sucrose at different temperatures. The duration of the lag time of the pH_i response is plotted *versus* the temperature reciprocal and the corresponding Arrhenius plot is shown in the inset. The calculated apparent activation energy of the lag time is 19 kcal mol^{-1}. Data are presented as means ± S.E.M. ($n = 3$). The Arrhenius plot was fitted by least-squares linear regression.

would expect to obtain a parallel decrease of the intracellular pH. However, a pH_i increase is observed. This apparent discrepancy may be resolved assuming that the lower intracellular metabolic activity at low temperature is a dominant factor in increasing the pH_i.

The hyperosmotic activation of the Na^+–H^+ exchange reveals an exponential decrease of the lag time period with the increase of temperature (Fig. 7). This observation suggests the existence of a temperature-sensitive rate-limiting step in the hyperosmotic activation of the exchanger. The apparent activation energy of the process underlying the lag time can be calculated from an Arrhenius plot. Since the slope of the plot depends on the ratio of the rate constants, their absolute values are not needed. Thus we could use the inverse of the lag time as an apparent rate which

is proportional to the unknown rate constant underlying the activation process. An apparent activation energy of 19 kcal mol^{-1} for this process was calculated (Fig. 7, inset). The dependence of the proton efflux (J_{H^+}) on temperature is shown in Fig. 8. The hyperosmotically induced proton efflux was used in order to account for the

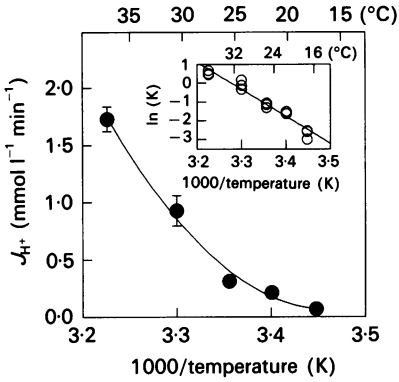

Fig. 8. Temperature dependence of the initial efflux following hyperosmotic stress. The experimental conditions were identical to those of Fig. 7. The initial rate of alkalinization is plotted *versus* the reciprocal of temperature and the corresponding Arrhenius plot is given in the inset. A calculated apparent activation energy of 29 kcal mol^{-1} is derived from the inset plot. Data are means ± S.E.M. ($n = 3$) and the Arrhenius plot is fitted by least-squares linear regression.

change of β_i with temperature, as shown in Fig. 2*B* (β_i decreases significantly when the temperature is lowered). The corresponding Arrhenius plot (Fig. 8, inset) yields an apparent activation energy of 29 kcal mol^{-1} for the sodium–proton exchange process. Thus, at least two processes appear to be involved in the hyperosmotic activation and underlie the lag period and the activation of the transporter, respectively.

Intracellular pathways of isosmotic activation

In order to examine the antiporter activation pathways in isosmotic conditions, the influence of different agents on the basal pH_i was examined. The possible modulation of the transporter by cyclic nucleotides was studied by elevating their intracellular concentrations with membrane permeable derivatives, 8-Br-cAMP (0·5 mM) and mono-butyryl cGMP (0·5 mM). Short-time pre-incubation (15 min) with these compounds had no effect on the basal pH_i. Forskolin (15 μM), an activator of adenylate cyclase, had no effect either.

In different cell types the sodium–proton exchanger is activated by growth factors and phorbol esters (Grinstein *et al.* 1989), suggesting the involvement of tyrosine kinase and protein kinase C in the activation process (Grinstein & Rothstein, 1986; Green & Muallem, 1989). Comparably RCJ cells are activated by addition of 25 nM PMA whereas its inactive derivative, 4α-phorbol, shows no effect (Fig. 9*A* and *B*). This alkalinization of 0·06 ± 0·01 pH units ($n = 6$) is completely inhibited by the

addition of 0·33 mM amiloride. Addition of 0·1 mM-vanadate, a potent inhibitor of phosphotyrosine phosphatase (Swarup, Cohen & Garbers, 1982), shown to activate the exchanger through a pertussis toxin-sensitive pathway (Paris, Chambard & Pouysse'gur, 1987), induced an analogous alkalinization of 0·08 ± 0·01 ($n = 4$) pH

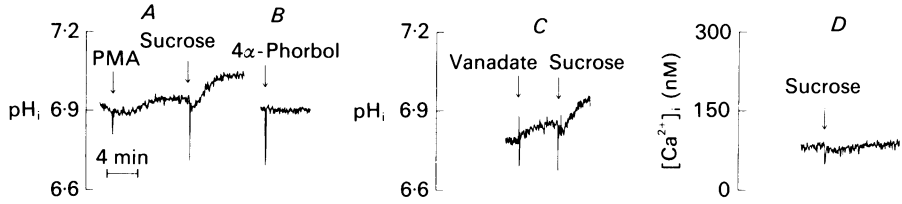

Fig. 9. Relationship of PMA, vanadate and intracellular Ca^{2+} to the hyperosmotic stimulus. PMA (25 nM), its inactive derivative, 4α-phorbol (25 nM) and vanadate (100 μM) were added to BCECF-loaded bone cells (*A*, *B* and *C*, respectively) suspended in an isosmotic Na^+-buffered medium ($pH_o = 7·3$) at 37 °C. A hyperosmotic stimulus (200 mosM sucrose) followed the PMA or the vanadate addition, as indicated. The intracellular Ca^{2+} of Fura-2-loaded cells was measured following a hyperosmotic stimulus at 37 °C (*D*) as described under Methods.

units of the bone cells (Fig. 9*C*). These observations suggest similar activation pathways for the RCJ osteoblasts and the previously described cell types regarding protein kinase C and tyrosine kinase activation.

The influence of intracellular Ca^{2+} on the pH_i was studied by the addition of ionomycin (1 μM) and A23187 (100 nM) ionophores. The ionophores' induced $[Ca^{2+}]_i$ elevation led to a pH response consisting of a transient intracellular acidification (0·1 pH units), followed by an alkalinization lasting for 1–2 min, which is similar to previous reports (Zavoico, Cragoe & Feinstein, 1986; Mitsuhashi & Ives, 1988).

Intracellular pathways of hyperosmotic activation

The hyperosmotic activation of the exchanger in RCJ bone cells shows additivity to phorbol and vanadate stimulations (Fig. 9*A* and *C*), suggesting independent activation pathways for the hyperosmotic stress. Furthermore, the depletion of protein kinase C following incubation with PMA (200 μM) for 24 h did not affect the response to the osmotic stress. Similarly, pre-incubation for 10 min with H-7 (100 μM), an inhibitor of protein kinase C, was without any effect upon the lag period or the overshoot rate. Hence, protein kinase C and tyrosine kinase do not appear to be involved in the transduction of the hyperosmotic stimulus.

The different agents used to investigate a possible modulation of the exchanger response to a hyperosmotic stress are presented in Table 1. Amiloride completely inhibits the hyperosmotic response. Membrane-permeable cyclic nucleotides and forskolin had no effect on the hyperosmotic activation. Indomethacin, an inhibitor of prostaglandin E_2 synthesis, which was shown to modulate the response to a direct mechanical load (Binderman, Zor, Kaye, Shimshoni, Harrell & Sómjen, 1988), had no effect. The intracellular calcium was not affected by the hyperosmotic stress (Fig. 9*D*). Neither the hyperosmotically induced alkalinization nor the lag period were influenced by incubation of the cells in a medium containing different calcium

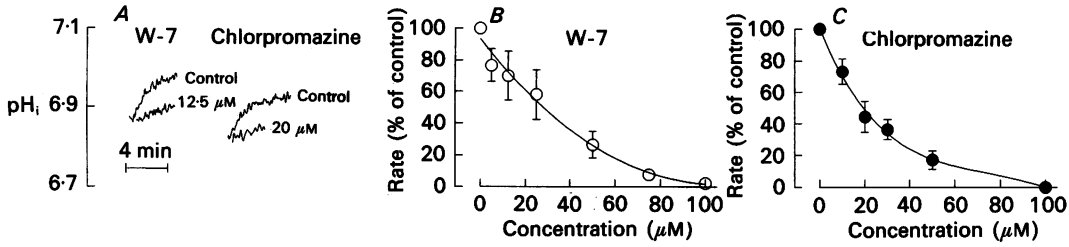

Fig. 10. The inhibition of the hyperosmotically induced alkalinization by W-7 and chlorpromazine. Bone cells were suspended in an isosmotic Na^+ solution (37 °C) containing W-7 or chlorpromazine at different concentrations for 5 min and then stimulated by addition of 200 mM sucrose. The inhibition of the hyperosmotically induced pH_i response was derived from the initial rate of alkalinization (*A*). Under these conditions a K_i of 28·4 μM and 19·9 μM was obtained for W-7 and chlorpromazine, respectively (Fig. 10*B* and *C*). The figure shows the means ± S.E.M. of four experiments. When absent, the error was smaller than the symbol.

TABLE 1. Influence of various effectors on the hyperosmotic response*

Effector	Initial rate† (%)
Amiloride (0·33 mM)	0 (6)
8-Br-cAMP (0·5 mM)	92·2 ± 3·4 (9)
Mono-butyryl cGMP (0·5 mM)	94·7 ± 5·4 (4)
Forskolin (15 μM)	93·1 ± 2·7 (4)
A23187 (100 μM)	95·1 ± 6·4 (9)
Ionomycin (1 μM)	109·1 ± 9·1 (3)
EGTA (2 mM)	93·3 ± 6·7 (4)
$[Ca^{2+}]_o$(0–5 mM)	90·8 ± 6·0 (8)
Indomethacin (10 μM)	91·7 ± 8·3 (3)
H-7 (100 μM)	85·4 ± 16·9 (4)

* The number of experiments (*n*) is given in parentheses.

† Besides amiloride, none of the initial rates achieved a statistically significant difference from control.

concentrations in the range of 0–5 mM. Modification of the intracellular calcium levels by applying ionomycin (1 μM) or A23187 (100 μM) in the presence of various extracellular concentrations of Ca^{2+} (0–5 mM) or by pre-incubating the cells for 5 min with 2 mM EGTA did not change the lag period or the alkalinization rate (data not shown). Therefore, the extra and/or intracellular calcium levels do not seem to affect the hyperosmotic response of these cells.

Abolishment of the hyperosmotic activation by calmodulin inhibitors and cytochalasin B

The initial rate of alkalinization induced by the hyperosmotic stress was inhibited by chlorpromazine and W-7 (Fig. 10*A*), two phenothiazines known for their calmodulin antagonist activity. Chlorpromazine is also known for its membrane stabilizing effects. This inhibition is concentration dependent with a K_i of 28·4 μM and 19·9 μM for W-7 and chlorpromazine, respectively (Fig. 10B and *C*). Chlorpromazine sulphoxide, an inactive derivative of chlorpromazine with local anaesthetic effects, did not inhibit the response, suggesting that the inhibitory effect of chlorpromazine is not due to its local anaesthetic effects on the membrane.

The hyperosmotically induced changes occurred concomitantly with a 25% decrease of the mean cellular volume of the cell population. Since cell shrinkage accompanies the stimulation of the exchanger, it may be proposed that the cytoskeletal elements are involved in the primary sensing of volume decrease. In

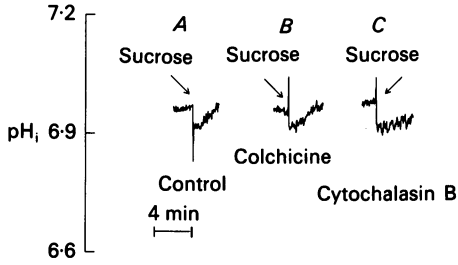

Fig. 11. Cytochalasin B inhibition of the osmotically induced alkalinization. Osteoblasts were pre-incubated for 45 min in the absence (*A*) or the presence of 10 μM colchicine (*B*) and 20 μM cytochalasin (*C*). The cells were loaded with BCECF during the pre-incubation period for 30 min. The bone cells were suspended in an isotonic Na^+ medium and a hyperosmotic sucrose stimulus (200 mosM) was applied at 37 °C. The intracellular initial rate of alkalinization was recorded fluorimetrically. Each experiment shown is representative of at least three similar experiments.

order to examine the involvement of actin and tubulin in the regulatory response to an osmotic stress we employed cytochalasin B and colchicine, respectively (Fig. 11). The regulatory pH_i increase following the hyperosmotic load was abolished when the cells were pre-incubated for 45 min with 20 μM cytochalasin B (decrease of the initial exchange rate down to $10{\cdot}0 \pm 5{\cdot}1\%$, $P < 0{\cdot}001$), but was insensitive to 10 μM colchicine ($78{\cdot}3 \pm 7{\cdot}5\%$, not significant). This inhibition of the pH_i recovery following the hyperosmotic stimulus in the presence of cytochalasin B may be attributed either to a direct effect on the exchanger itself or be due to an impairment of the hyperosmotic transduction sequence. In order to distinguish between these two alternatives the activity of the antiporter was examined in the presence of cytochalasin B by loading RCJ cells with 20 mM NH_4Cl (see Methods) followed by resuspension in an isosmotic HEPES solution. The pH_i recovery rate was unaltered in cells exposed to cytochalasin B as compared with control cells. Similarly, the amiloride-sensitive rate of the recovery was identical in controls and in cytochalasin B-treated cells (respectively, $89{\cdot}2 \pm 1{\cdot}8\%$, $n = 3$, and $90{\cdot}7 \pm 0{\cdot}8\%$, $n = 3$, not significant, where the data refer to the percentage inhibition of the rate of pH_i increase by amiloride). It is concluded that in the presence of cytochalasin B the Na^+–H^+ exchange activity is unimpaired, whereas the hyperosmotically induced pH_i recovery is inhibited.

DISCUSSION

The present study demonstrates that the hyperosmotically induced intracellular alkalinization of RCJ cells requires the activation of the sodium–proton exchanger. This is supported by the complete abolishment of the pH_i increase in the presence of amiloride or in the absence of extracellular sodium. The dominant involvement of the

sodium–proton exchanger in the induced pH changes is also suggested by the insensitivity of the response either to DIDS, furosemide, ouabain, or chloride replacement in the medium. This is true only if we assume that shrinkage-induced changes in the pH_i reflect changes in the activity of the antiporter. The employed cell suspension methodology in monitoring pH_i alterations due to cell shrinkage limits the interpretation of data, e.g. the observed pH_i changes require a Na^+–H^+ exchanger, but may not reflect changes in the rate of Na^+–H^+ exchange.

A lag time in the activation of the Na^+–H^+ exchanger is observed in RCJ cells following the hyperosmotic stimulus. A similar lag time has been observed previously in A431 cells and lymphocytes (Cassel, Whiteley, Zhuang & Glaser, 1985; Grinstein *et al.* 1985). The appearance of a lag period in the time course of a biochemical process reflects the rate-limiting step of the process. Thus, the existence of a lag time in the hyperosmotic activation of the sodium–proton exchange in the different cell types suggests a common rate-limiting step in the activation of the exchanger. The length of the lag time is temperature sensitive, increasing with the lowering of the temperature. The lag time has a Q_{10} value of $\approx 2{\cdot}8$ (the corresponding apparent activation energy of the process is 19 kcal mol^{-1}) sustaining the biochemical nature of the underlying reaction. The sodium–proton exchange process, reflected by the pH elevation following the lag time, is itself temperature dependent possessing a higher Q_{10} value of $\approx 4{\cdot}6$ (the corresponding apparent activation energy of the process is 29 kcal mol^{-1}). The effect of temperature on the initial rate via the temperature shift of the steady-state pH should be considered. When we lower the temperature from 37 to 17 °C, the basal pH_i increases by 0·13 pH units. In order to distinguish between the direct temperature effect and the indirect effect due to the induced change of pH_i, the exchange rate should be examined by varying the pH_i from 6·94 to 7·07 while maintaining a constant pH_o (at 37 °C). It was shown recently by us (Dascalu *et al.* 1991) that increasing pH_i from 6·94 to 7·07 (under a constant $pH_o = 7{\cdot}3$) shifts the rate of Na^+–H^+ exchange from 0·22 to 0·17 pH units min^{-1}. This shift of the rate is not statistically significant ($P = 0{\cdot}44$). Apparently, the mere change of pH_i when lowering the temperature is not expected to affect our measurements of the initial rate. These results may imply that the biochemical reaction involved in the activation of the exchanger, underlying the lag period, is less temperature dependent than the rate of proton extrusion itself. This suggestion could be an oversimplification of the process, considering additional factors, e.g. a temperature-induced change of buffering power of pK variation of different titrable groups on the exchanger with temperature, influencing the pH_i dependence at different temperatures. It should be stressed that thermal equilibration alone, following the addition of a very small volume of concentrated cells (kept at room temperature) to a large volume of the medium at 37 °C is completed within a few seconds. However, Fig. 6*A* and *B* demonstrates that the transporter reaches an equilibrium state after a period of several minutes, possibly reflecting the time needed for chemical reactions to attain a new equilibrium state. It may be concluded that the process underlying the lag period is a biochemical process which is rate limiting for the subsequent Na^+–H^+ exchange.

The exchanger is stimulated under isosmotic conditions by protein kinase C activators, like PMA and vanadate, as in previous studies (Grinstein & Rothstein,

1986). However, the hyperosmotic stress triggers a PMA, vanadate, cAMP and cGMP independent response of the RCJ cells. The hyperosmotic response is $[Ca^{2+}]_i$ independent, which is similar to findings in smooth muscle cells (Mitsuhashi & Ives, 1988) but contrary to a $[Ca^{2+}]_i$ increase observed in lymphocytes (Grinstein & Goetz, 1985). Thus, it appears that the classical pathways of signal transduction are not involved in the hyperosmotic stress response of these cells.

The hyperosmotic stimulus leads to an instantaneous cell shrinkage. This process should affect the cell cytoskeleton. Therefore, actin, tubulin and other structural elements of the cytoskeletal network can be viewed as possible candidates to serve as sensors of the hyperosmotically induced volume change. Cytochalasin B, a known disruptor of the filamentous actin, was found to inhibit the hyperosmotic activation of the antiporter. The possible non-specific inhibition of the Na^+–H^+ exchange by cytochalasin B should be disregarded. Indeed, in response to acidification, the amiloride-sensitive component in the pH_i recovery in control and cytochalasin B-treated cells was similar. Therefore, it may be suggested that the actin network is involved in the transduction of hyperosmotic stimuli and leads to the activation of the Na^+–H^+ antiporter.

Inhibition of the exchange activity was achieved by addition of two calmodulin inhibitors, chlorpromazine and W-7. The local anaesthetic-like effects of chlorpromazine on the plasma membrane can be disregarded in connection with its effect on the exchanger in view of the lack of inhibition of its inactive disulphoxide derivative. Comparably, the exchanger activation by a hyperosmotic stress is inhibited by W-7 in renal epithelial cells (Burns, Homma & Harris, 1989). These results are in line with previous findings describing the inhibition of the hyperosmotic activation by trifluoperazine (Grinstein, Cohen, Goetz & Rothstein, 1985), a calmodulin inhibitor. The K_i of trifluoperazine (15 μM) found in a previous report is within a similar concentration range to the two calmodulin inhibitors used in this study. Our results lead to an apparent discrepancy of a $[Ca^{2+}]_i$-independent hyperosmotic response modulated by inhibitors of calmodulin. It was shown recently that calmodulin in yeast can function during growth without binding Ca^{2+} (Geiser, van Tuinen, Brockerhoff, Neff & Davis, 1991). We suggest that the hyperosmotic transduction mechanism involves calmodulin as a structural element of the cytoskeleton, as in the 110 kD protein–calmodulin complex of the intestinal microvillus (Mooseker & Coleman, 1989). The regulation of this calmodulin-linked myosin I by Ca^{2+} at physiological concentration is uncertain (Pollard, Doberstein & Zot, 1991).

The exact mechanism of regulation of the sodium–proton exchanger by hyperosmotic shrinkage of cells is still unknown. The present work characterizes the activation energies of the processes underlying the lag period and the exchange process of the Na^+–H^+ exchanger. The direct participation of actin in the transduction of the hyperosmotic stimulus and the possible involvement of calmodulin inhibitors are described.

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