Mechanisms of activation of Na^+/H^+ exchange in human osteoblast-like SaOS-2 cells

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Because of the importance of pH homeostasis in bone and the current uncertainty about the mechanisms by which intracellular pH (pH_i) is regulated in this tissue, we have investigated the roles of cytosolic free Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) and protein kinase C on the activation of Na^+/H^+ exchange in human osteoblast-like SaOS-2 cells. $[\text{Ca}^{2+}]_i$ and pH_i were measured using Fura-2 and 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) respectively. The basal pH_i in HCO_3^- -free buffer was 7.36 ± 0.04 units (mean $\pm$ S.D.). Addition of ionomycin in Ca^{2+} -containing buffer did not cause a rise in basal pH_i ; however, addition of the phorbol ester phorbol 12-myristate 13-acetate (PMA) did cause a slowly developing rise in resting pH_i of 0.14 ± 0.02 unit over 4–5 min. Nigericin, a K^+/H^+ ionophore, caused an abrupt fall in pH_i to 6.70 ± 0.07 units. In nigericin-pretreated cells, PMA caused a rapid rise in pH_i without changing the $[\text{Ca}^{2+}]_i$. In acidified cells, ionomycin increased $[\text{Ca}^{2+}]_i$ and pH_i in a parallel concentration-dependent (30–500 nM) manner. This action of ionomycin occurred in both the presence and the nominal absence of extracellular Ca^{2+} . Ionomycin-induced alkalization depended on extracellular Na^+ and was inhibited in cells incubated with hexamethylene amiloride. When the incremental increase in $[\text{Ca}^{2+}]_i$ induced by ionomycin was blocked by preincubation with bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid (BAPTA)/AM, the effect on pH_i was inhibited. Staurosporine, a protein kinase C inhibitor, blocked the action of PMA on pH_i , but it had no effect on the ionomycin-induced increase in pH_i . The action of ionomycin was not due to osmotic shock. We conclude that SaOS-2 cells have a protein kinase C-activatable Na^+/H^+ antiporter that is also stimulated, in acidified cells, in a concentration-dependent fashion by transients in $[\text{Ca}^{2+}]_i$ which act via a non-protein kinase C pathway.

INTRODUCTION

An electroneutral Na^+/H^+ exchange mechanism has been studied in a wide variety of mammalian cells (Aronson, 1985). A functioning Na^+/H^+ exchanger is important for intracellular pH (pH_i) homeostasis, especially under acidic conditions. Activation of Na^+/H^+ exchange, which increases the pH_i above resting levels, has been shown to contribute to the action of mitogens, and may play a role in signal-modulating transduction pathways (Busa & Nuccitelli, 1984; Seifter & Aronson, 1986).

Regulation of pH_i in osteoblasts is of interest for several reasons. The processes of bone formation and reabsorption result in local fluctuations of pH in the bone environment that need to be controlled in order to maintain homeostasis. Ion fluxes, especially Ca^{2+} and H^+ , have been shown to contribute to the process of bone resorption (Vaes, 1988). Disruption of intracellular Na^+ and H^+ levels with the ionophore nigericin has been shown to inhibit parathyroid hormone-stimulated bone resorption in mouse calvaria (Krieger & Tashjian, 1980). An acidic extracellular pH induces Ca^{2+} efflux from calvaria, while at the same time promoting a proton influx into bone cells (Bushinsky *et al.*, 1983). Rat calvarial osteoblasts have been shown to possess a functional Na^+/H^+ exchanger that participates in recovery from an acid load (Redhead & Baker, 1988). A functional Na^+/H^+ exchanger has also been identified in the rat osteoblast-like cell line UMR 106 (Green *et al.*, 1988a,b, 1990), but does not appear to be activatable by hormones or phorbol esters (Green & Muallem, 1989; Green *et al.*, 1990).

We have been investigating the activation of Na^+/H^+ exchange in the human osteoblast-like cell line SaOS-2, both under basal conditions and after challenge with an acid load. Such studies are important because activation of Na^+/H^+ exchange clearly plays a role in hormone-stimulated bone remodelling. In this paper we have investigated the roles of activation of protein kinase C (PKC) and transient increases in the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in the stimulation of Na^+/H^+ exchange in SaOS-2 cells. In a HCO_3^- -free buffer, addition of phorbol 12-myristate 13-acetate (PMA) (to activate PKC) induced a gradual increase in pH_i from resting levels. Ionomycin, a calcium ionophore, had no detectable effect on pH_i under resting conditions. Acidification of the cytosol with the K^+/H^+ ionophore nigericin allowed analysis of the role of Ca^{2+} transients in activation of Na^+/H^+ exchange as well as the relationship between the PKC and $[\text{Ca}^{2+}]_i$ -mediated pathways.

EXPERIMENTAL

Materials

Media and sera were purchased from Gibco (Grand Island, NY, U.S.A.). Tissue culture plasticware was obtained from Corning (Corning, NY, U.S.A.). 2',7'-Bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)/AM, Fura-2/AM and bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid (BAPTA) were purchased from Molecular Probes (Eugene, OR, U.S.A.). PMA was obtained from LC Services (Woburn, MA, U.S.A.). Ionomycin was purchased from Calbiochem (San Diego, CA,

Abbreviations used: $[\text{Ca}^{2+}]_i$, cytosolic free Ca^{2+} concentration; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; pH_i , intracellular pH; HMA, hexamethylene amiloride; BCECF, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; BAPTA, bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid.

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U.S.A.). Hexamethylene amiloride (HMA) was purchased from Research Biochemicals Inc. (Natick, MA, U.S.A.). Digitonin and nigericin were from Sigma (St. Louis, MO, U.S.A.). Staurosporine was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.).

Cell culture

Human osteoblast-like osteosarcoma cells (Rodan *et al.*, 1987), SaOS-2, were grown in monolayer culture in Eagle's minimum essential medium supplemented with 5% (v/v) fetal bovine serum and 5% (v/v) horse serum at 37°C in a humidified atmosphere of 95% air/5% CO₂. Cells were subcultured with 0.05% (w/v) trypsin/0.02% (w/v) EDTA in 100 mm dishes and grown for 4 days. Medium was changed on the day before each experiment.

Measurement of pH_i

SaOS-2 cells were harvested with 0.02% EDTA in HBSS buffer (118 mM-NaCl, 4.6 mM-KCl, 10 mM-D-glucose, 20 mM-Hepes, pH 7.2), washed twice with Ca²⁺/HBSS (HBSS containing 1.5 mM-CaCl₂), and resuspended in 1.5 ml of Ca²⁺/HBSS. Cells were incubated with 5 μM-BCECF/AM for 40 min at 37°C. After washing the cells twice with Ca²⁺/HBSS, cells were washed two additional times with the appropriate experimental buffer: Ca²⁺/HBSS, low-Ca²⁺ HBSS (without CaCl₂), or Na⁺-free HBSS (HBSS with choline chloride, 118 mM, replacing NaCl). The cells were then suspended in the appropriate buffer. pH_i was determined fluorometrically with a Spex Fluorolog F111A spectrofluorimeter using an excitation wavelength of 500 nm and an emission wavelength of 530 nm. To calibrate, the cells were lysed with digitonin at the end of each run to release BCECF into the buffer. Known pH values were then imposed, and the fluorescence of BCECF was measured. To correct for the red-shift in the spectrum of BCECF inside cells as compared with the spectrum obtained for BCECF in solution, a calibration curve was constructed by equilibrating internal and external pH with 10 μg of nigericin/ml in high-K⁺ buffer (Thomas *et al.*, 1979). This calibration was compared to a calibration of BCECF after cells were lysed with digitonin. Both curves were linear over the pH range used (6.4–7.4), but were not parallel. In six separate determinations, the relationship between BCECF fluorescence inside and outside SaOS-2 cells was described by the equation: pH_i = [pH_{out} × 1.108] – 0.518, and all measured pH_i values were corrected accordingly.

Measurement of [Ca²⁺]_i

SaOS-2 cells were harvested as described for the measurement of pH_i. Cells were incubated with 1 μM-Fura-2/AM for 40 min at 37°C, and washed as described for pH_i measurements. Fluorescence was measured in the same Spex spectrofluorimeter using an excitation wavelength of 342 nm and an emission wavelength of 492 nm. To calibrate the Fura-2 fluorescence, cells were lysed with digitonin at the end of each run. Minimum and maximum Fura-2 fluorescence values were measured using an excess of EGTA and CaCl₂ respectively. [Ca²⁺]_i was calculated using a K_d of 224 nM for Fura-2 (Grynkiewicz *et al.*, 1985). All drugs used were tested for their effect on Fura-2 fluorescence: HMA decreased the intensity, nigericin increased the signal, and all other drugs had no effect; these effects are shown in the Results section.

Statistical analysis

All results are given as the mean value ± s.d., with *n* equal to the number of independent measurements made. When appropriate, Student's *t* test was used for comparison between two means.

RESULTS

Actions of PMA and ionomycin on basal pH_i

The basal pH_i in SaOS-2 cells loaded in BCECF in a HCO₃⁻-free Hepes-buffered solution containing 1.5 mM-CaCl₂ was 7.36 ± 0.04 units (mean ± s.d., *n* = 40). (Numerical data given in the text for [Ca²⁺]_i or pH_i are the mean values ± s.d. for *n* independent measurements; therefore they may not correspond exactly to the numerical value in a single representative experiment, as illustrated by the Figures.) To determine whether Na⁺/H⁺ exchange could be activated at resting pH_i, we added stimuli which have been shown to induce alkalization in other cell types. Addition of 200 nM-PMA caused a gradual rise in pH_i of 0.14 ± 0.02 unit (*n* = 5; Fig. 1a). However, addition of 500 nM-ionomycin did not have a detectable effect on resting pH_i (*n* = 4; Fig. 1b); the small decrease was not statistically significant.

Effect of nigericin-induced acidification on SaOS-2 cell pH_i and [Ca²⁺]_i

To allow an examination of the mechanisms by which ionomycin could activate Na⁺/H⁺ exchange in SaOS-2 cells challenged with an acid load, and to compare the ionomycin- and PMA-induced alkalizations, we acidified the cytosol with the K⁺/H⁺ ionophore nigericin. In Fig. 2(a), addition of nigericin (1 μg/ml) caused a rapid lowering of pH_i (about 2.5 min for the nadir to be reached) followed by a gradual recovery. The mean maximum acidification obtained using nigericin (1 μg/ml) in

Fig. 1. Actions of PMA and ionomycin on resting pH_i in SaOS-2 cells

Cells were harvested and loaded with BCECF as described in the Experimental section. (a) Addition of PMA (200 nM); (b) addition of ionomycin (Iono; 500 nM).

Fig. 2. Actions of nigericin on pH_i and [Ca²⁺]_i in SaOS-2 cells

(a) Cells were harvested and loaded with BCECF as described in the Experimental section. Nigericin (Nig, 1 μg/ml) was added. (b) Cells were harvested and loaded with Fura-2 as described in the Experimental section. Nigericin (1 μg/ml) was added.

Fig. 3. Actions of PMA on acidified SaOS-2 cells

(a) Cells were harvested and loaded with BCECF as described in the Experimental section. After acidifying the cells with nigericin (Nig, 1 μ g/ml), PMA (200 nM) was added. (b) Cells were harvested and loaded with Fura-2 as described in the Experimental section. Nigericin (Nig, 1 μ g/ml) and PMA (200 nM) were added as indicated. The significance of the small reproducible decrease in $[Ca^{2+}]_i$ seen after PMA is not known.

Fig. 4. Actions of ionomycin on pH_i and $[Ca^{2+}]_i$ in acidified SaOS-2 cells

Cells were harvested and loaded with BCECF (a) or Fura-2 (b) as described in the Experimental section. The cells were acidified with nigericin (Nig, 1 μ g/ml), and then ionomycin (Iono, 500 nM) was added.

Ca^{2+} /HBSS was 6.70 ± 0.07 units ($n = 29$). These results were essentially the same in cells suspended in low- Ca^{2+} HBSS (6.72 ± 0.06 units, $n = 12$).

To determine the effect of nigericin on $[Ca^{2+}]_i$, we added nigericin (1 μ g/ml) to Fura-2-loaded cells. Basal $[Ca^{2+}]_i$ was 91 ± 26 nM ($n = 34$) in Ca^{2+} /HBSS. As shown in Fig. 2(b), nigericin induced a gradual increase in $[Ca^{2+}]_i$ which reached a plateau in about 2 min. The mean incremental increase in $[Ca^{2+}]_i$ seen with nigericin (1 μ g/ml) in Ca^{2+} /HBSS was 76 ± 36 nM ($n = 21$). The increase in $[Ca^{2+}]_i$ induced by nigericin also occurred in SaOS-2 cells in low- Ca^{2+} HBSS to which 300 μ M-EGTA was added (increase in $[Ca^{2+}]_i$ above baseline was 66 ± 47 nM, $n = 13$). Thus the increases in $[Ca^{2+}]_i$ induced by

nigericin in the presence and absence of extracellular calcium were not significantly different, indicating that the calcium was derived from intracellular sources. At this time we have not identified the source of intracellular calcium which is mobilized by the addition of nigericin; however, this effect has also been noted in other cell types (Nasmith & Grinstein, 1987).

Action of PMA on pH_i and $[Ca^{2+}]_i$ in acidified SaOS-2 cells

In many cell types, activation of PKC enhances Na^+/H^+ exchange activity. We have previously reported that addition of PMA caused rapid translocation of PKC from the cytosol to the membrane fraction of SaOS-2 cells (Krug & Tashjian, 1987). The data in Fig. 3(a) show the effect of PMA (200 nM) on pH_i in SaOS-2 cells previously acidified with nigericin. There was a lag of about 45 s followed by a marked alkalization (0.34 ± 0.04 units, $n = 4$).

In UMR 106 osteoblast-like cells, PMA causes an increase in $[Ca^{2+}]_i$ (Green *et al.*, 1990). To determine whether an increase in $[Ca^{2+}]_i$ is involved in PMA-induced alkalization in SaOS-2 cells, we measured $[Ca^{2+}]_i$ under the same conditions as used to measure pH_i . As shown in Fig. 3(b) ($n = 4$), addition of PMA to acidified SaOS-2 cells caused no increase in $[Ca^{2+}]_i$.

Actions of ionomycin on pH_i and $[Ca^{2+}]_i$ in acidified SaOS-2 cells

Many hormones which activate Na^+/H^+ exchange and increase pH_i also increase $[Ca^{2+}]_i$. To determine the effect of a Ca^{2+} transient on pH_i in SaOS-2 cells, we used the ionophore ionomycin. Fig. 4(a) shows the action of ionomycin (500 nM), on pH_i in acidified SaOS-2 cells. There was a delay of 8–10 s and then a rapid alkalization ensued, which reached a plateau in about 60 s. The change in pH_i induced by ionomycin in this and subsequent experiments was calculated as the difference between the pH_i at the time at which ionomycin was added and the pH_i at the beginning of the resultant plateau. For 500 nM-ionomycin the change in pH_i was 0.24 ± 0.04 unit ($n = 6$). The corresponding effect on $[Ca^{2+}]_i$ is shown in Fig. 4(b). The incremental increase in $[Ca^{2+}]_i$ seen with 500 nM-ionomycin was 381 ± 20 nM ($n = 5$). The change in $[Ca^{2+}]_i$ in this and subsequent experiments was calculated as the difference between the peak of the ionomycin-induced Ca^{2+} transient and the preceding nigericin-induced plateau in $[Ca^{2+}]_i$.

Relationship between changes in pH_i and $[Ca^{2+}]_i$ induced by increasing concentrations of ionomycin

To determine whether there was a direct relationship between the amount of Ca^{2+} released by various concentrations of ionomycin and the corresponding change in pH_i , we examined the dose-response relationships for both pH_i and $[Ca^{2+}]_i$. Fig. 5(a) shows the effects of addition of 30, 50, 100, 250 and 500 nM-ionomycin on pH_i and $[Ca^{2+}]_i$ in Ca^{2+} /HBSS. There was a clear correlation between increases in peak $[Ca^{2+}]_i$ and increases in the induced change in pH_i . Similar results are shown in Fig. 5(b) with 50, 100 and 500 nM-ionomycin in low- Ca^{2+} HBSS to which 300 μ M-EDTA had been added. Note that the $\Delta[Ca^{2+}]_i$ induced by 100 and 500 nM-ionomycin in the nominal absence of extracellular Ca^{2+} caused increases in pH_i that were similar to the changes in $[Ca^{2+}]_i$ and pH_i induced by 30 nM- and 50–100 nM-ionomycin respectively in the presence of 1.5 mM extracellular Ca^{2+} . The results suggest that it is the absolute $[Ca^{2+}]_i$ reached that is causally related to the magnitude of the change in pH_i .

Effects of HMA and Na^+ -free buffer on ionomycin-induced changes in pH_i and $[Ca^{2+}]_i$ in acidified SaOS-2 cells

A trivial explanation for the alkalization induced by ionomycin is that the ionophore induces an exchange of

Fig. 5. Relationships between the changes in [Ca²⁺]_i and pH_i with increasing concentrations of ionomycin in acidified SaOS-2 cells

Shown are the incremental changes in pH_i (●) and [Ca²⁺]_i (○) in HBSS containing 1.5 mM-Ca²⁺ (a) and in low-Ca²⁺ HBSS plus 300 μM-EGTA (b). Each point is the mean value and the bars give the S.D. of 4–6 separate measurements.

Fig. 6. Effect of HMA on pH_i and [Ca²⁺]_i in acidified SaOS-2 cells treated with ionomycin

Cells were harvested and loaded with BCECF (a) or Fura-2 (b) as described in the Experimental section. Addition of HMA (5 μM) occurred prior to acidification of the cells with nigericin (Nig, 1 μg/ml) and addition of ionomycin (Iono, 500 nM).

Ca²⁺_{in}/2H⁺_{out}, leading to the non-specific extrusion of protons (Grinstein & Cohen, 1987). To determine whether the ionomycin-induced increase in pH_i occurred via activation of Na⁺/H⁺ exchange, we incubated cells with the Na⁺/H⁺ exchange inhibitor HMA. As shown in Fig. 6(a) (*n* = 4), HMA abolished the ionomycin-induced increase in pH_i. Higher concentrations of ionomycin (1 μM) caused a slight change in the recovery slope of the pH_i curve in acidified cells incubated with HMA, indicating possible non-specific efflux of protons (results not shown); therefore 500 nM-ionomycin or less was used in subsequent experiments. When extracellular Na⁺ was replaced by choline⁺, the ionomycin-induced increase in pH_i was also completely inhibited (results not shown; *n* = 4). To determine whether ionomycin still elicited a Ca²⁺ transient in the presence of HMA, we incubated cells with HMA, acidified the cells with nigericin, and then added ionomycin. As shown in Fig. 6(b), the Ca²⁺

Fig. 7. Effect of the intracellular Ca²⁺ chelator BAPTA on the actions of ionomycin on pH_i and [Ca²⁺]_i in acidified SaOS-2 cells

Cells were harvested as described in the Experimental section and were loaded with 35 μM-BAPTA/AM for 1 h at 37 °C. BCECF (a) was added for the last 45 min of the incubation or Fura-2 (b) was added for the last 40 min of the incubation in low-Ca²⁺ HBSS. About 3 min before acidification with nigericin (Nig, 1 μg/ml), EGTA (300 μM) was added. Note elimination of the [Ca²⁺]_i transient induced by ionomycin (Iono, 500 nM) and a lack of effect of ionomycin on pH_i. The effects of ionomycin on [Ca²⁺]_i and pH_i were not eliminated in low-Ca²⁺ HBSS plus EGTA alone (see text).

response to ionomycin was attenuated. In three experiments the incremental increase in [Ca²⁺]_i was 132 ± 42 nM, which was a 65% reduction as compared with control cells not treated with HMA. Similarly, when the increase in [Ca²⁺]_i induced by ionomycin was determined in the absence of extracellular Na⁺, the [Ca²⁺]_i transient was reduced to 137 ± 22 nM (*n* = 4). However, these increases in [Ca²⁺]_i would have been sufficient to cause an increase in pH_i if the ionomycin-induced alkalinization had occurred via a pathway other than activation of Na⁺/H⁺ exchange (see dose–response relationship). Therefore, we conclude that ionomycin-induced Ca²⁺ transients exert their effect on pH_i via activation of Na⁺/H⁺ exchange.

Effect of BAPTA/AM on ionomycin-induced changes in pH_i and [Ca²⁺]_i in acidified SaOS-2 cells

To demonstrate that the effect of ionomycin on pH_i was due to the induction of a Ca²⁺ transient as opposed to some other possible effect of ionomycin, we incubated SaOS-2 cells for 1 h in the presence of BAPTA/AM, an intracellular Ca²⁺ chelator, in order to prevent the ionomycin-induced [Ca²⁺]_i transient. Cells were then suspended in low-Ca²⁺ HBSS which, in cells not preincubated with BAPTA, reduced the ionomycin-induced alkalinization to 0.14 ± 0.02 unit (*n* = 5) and the incremental [Ca²⁺]_i transient to 144 ± 14 nM (*n* = 5). As shown in Fig. 7(a), in cells that had been preincubated with BAPTA, the effect of ionomycin on pH_i was abolished. To insure that the Ca²⁺ transient had been eliminated, [Ca²⁺]_i was measured. A representative example of five such experiments using 35–50 μM-BAPTA/AM shows little or no increase in [Ca²⁺]_i with ionomycin (Fig. 7b). The apparent basal [Ca²⁺]_i was higher than that of control cells; it is possible that this finding is an artefact of direct BAPTA/Fura 2 interactions (Muallem *et al.*, 1990).

Action of hyperosmotic shock on pH_i in acidified SaOS-2 cells

One mechanism by which ionomycin has been postulated to activate Na⁺/H⁺ exchange is by inducing hyperosmotic shock via loss of intracellular K⁺ followed by water (Hoffmann, 1985; Grinstein & Cohen, 1987). To examine this possibility in acidified

Fig. 8. Lack of effect of an acute hyperosmotic shock on pH_i in acidified SaOS-2 cells

Cells were harvested and loaded with BCECF as described in the Experimental section. After acidifying the cells with nigericin (Nig, $1 \mu\text{g/ml}$), sucrose (80 mM final concentration) was added.

Fig. 9. Interactions of PMA and ionomycin on pH_i in acidified SaOS-2 cells

Cells were harvested and loaded with BCECF as described in the Experimental section. After acidifying the cells with nigericin (Nig, $1 \mu\text{g/ml}$), cells were exposed to 10 nM-PMA alone (a) or PMA and then 500 nM-ionomycin (Iono; b). The effect of 500 nM-ionomycin alone is shown in Fig. 4.

SaOS-2 cells, we induced an acute hyperosmotic shock by adding 80 mM-sucrose to iso-osmotic buffer. The lack of an acute effect of sucrose on pH_i in SaOS-2 cells is shown in Fig. 8. From this result we concluded that the effect of ionomycin on pH_i is not mediated by osmotic shock in SaOS-2 cells, but that the Ca^{2+} transient induced by ionomycin is involved, in a signal-transducing manner, in the activation of the Na^+/H^+ exchanger.

Addition of ionomycin to acidified SaOS-2 cells pretreated with PMA

To investigate the relationship between the ionomycin-induced increase in pH_i and the PMA-induced increase in pH_i , we added a low concentration of PMA (10 nM) followed by 500 nM-ionomycin. As shown in Fig. 9(a), addition of 10 nM-PMA alone produced a slow rise in pH_i ; subsequent addition of 500 nM-ionomycin elicited an additional acute increase in pH_i (Fig. 9b). The total change in pH_i from the addition of PMA to the end of the ionomycin-induced response was 0.28 ± 0.05 unit ($n = 5$).

Fig. 10. Actions of staurosporine on pH_i in acidified SaOS-2 cells treated with ionomycin or PMA

Cells were harvested and loaded with BCECF as described in the Experimental section. Staurosporine (Stauro, 100 ng/ml) was added prior to acidification of the cells with nigericin (Nig, $1 \mu\text{g/ml}$). (a) Addition of ionomycin (Iono, 50 nM); (b) addition of PMA (200 nM).

During a comparable period of time the effect of PMA alone on pH_i was 0.17 ± 0.02 unit ($n = 4$). Thus the change in pH_i resulting from the action of ionomycin after PMA treatment was approx. 0.11 unit, which was significantly less than that seen with ionomycin alone (0.24 ± 0.04 unit). Therefore ionomycin had an effect on pH_i in the presence of PMA, but the effect was not additive to that of PMA.

Action of staurosporine on ionomycin and PMA-induced activation of Na^+/H^+ exchange in acidified SaOS-2 cells

Because the effect of ionomycin on pH_i appeared to be qualitatively different from that of PMA, we postulated that these two agents activated Na^+/H^+ exchange via different mechanisms. To test whether ionomycin-induced Ca^{2+} transients activated Na^+/H^+ exchange via a non-PKC-mediated pathway in acidified SaOS-2 cells, we used the PKC inhibitor staurosporine (Tamaoki *et al.*, 1986). Fig. 10(a) shows that staurosporine did not block the effect of 50 nM-ionomycin on pH_i . In four such experiments the change in pH_i induced by 50 nM-ionomycin after staurosporine was not significantly different ($P > 0.05$) from that in cells treated with 50 nM-ionomycin alone (0.11 ± 0.03 unit versus 0.13 ± 0.02 unit). The $\Delta[\text{Ca}^{2+}]_i$ induced by 50 nM-ionomycin was also unaffected by staurosporine (results not shown). On the other hand, cells incubated with staurosporine and acidified with nigericin showed no alkalinization in response to 200 nM-PMA (Fig. 10b, representative of three such experiments). Therefore we conclude that the effect of ionomycin on Na^+/H^+ exchange is not mediated by a PKC-dependent pathway.

DISCUSSION

A number of regulatory mechanisms have been implicated in the activation of Na^+/H^+ exchange in diverse cell types; these include pH_i , osmotically induced cell shrinkage, PKC activation and Ca^{2+} /calmodulin-dependent protein kinase-mediated pathways (Frelin *et al.*, 1988). However, little is known about

mechanisms of activation of Na^+/H^+ exchange in bone cells. In this report we present evidence that human osteoblast-like SaOS-2 cells possess a functional Na^+/H^+ exchange mechanism. The Na^+/H^+ exchanger can be activated at resting pH_i by addition of PMA, indicating a role for a PKC-mediated pathway in these cells. PMA did not cause an increase in $[\text{Ca}^{2+}]_i$ in SaOS-2 cells, and its effect on pH_i was present in a low- Ca^{2+} buffer (results not shown); therefore the action of PMA on Na^+/H^+ exchange operates at resting $[\text{Ca}^{2+}]_i$ levels and does not depend on transient changes in $[\text{Ca}^{2+}]_i$.

However, our results also demonstrate that Ca^{2+} transients can activate Na^+/H^+ exchange and induce alkalization in acidified SaOS-2 cells. The effect of raising the $[\text{Ca}^{2+}]_i$ on pH_i is sensitive to HMA and dependent on extracellular Na^+ , indicating a role for Na^+/H^+ exchange. When the ionomycin-induced transient in $[\text{Ca}^{2+}]_i$ was prevented by incubating cells with BAPTA/AM, ionomycin had no effect on pH_i , indicating that the effect was not due to some non- Ca^{2+} -dependent action of ionomycin.

In thymocytes, ionomycin induces alkalization that is due to activation of Na^+/H^+ exchange, but this effect appears to be secondary to the efflux of K^+ and Cl^- , along with osmotically obligated water (Grinstein & Cohen, 1987). Grinstein & Cohen (1987) showed that addition of 25 mM-*N*-methyl-D-glucamine chloride to thymocytes in iso-osmotic buffer produced cell shrinkage and alkalization comparable to that with 400 nM ionomycin. We tested whether a similar effect occurred in SaOS-2 cells by adding 80 mM-sucrose to acidified cells. Because this treatment resulted in no change in pH_i , it appears that: (1) ionomycin is not inducing alkalization secondary to cell shrinkage, and (2) the Na^+/H^+ exchange mechanism is not activated by the hyperosmotic shock induced by 80 mM-sucrose.

Grinstein and co-workers also showed a concentration-dependence of ionomycin-induced Ca^{2+} elevations on pH_i (Grinstein & Cohen, 1987). However, in thymocytes, increases in ionomycin caused an increased rate of alkalization, but the final pH_i reached was similar. In SaOS-2 cells the time to reach final pH_i was about 60 s for all concentrations of ionomycin used, but the magnitude of the change in pH_i increased with increasing concentrations of ionomycin.

Compared with the ionomycin-induced increase in pH_i , which showed an 8–10 s delay followed by a rapid alkalization over about 60 s, PMA-induced alkalization was initiated after a 45 s delay and took about 5 min before returning to the control rate of alkalization. When ionomycin was added to cells pretreated with PMA, the rapid alkalization was still seen upon addition of ionomycin, but the final pH_i reached was not additive to that seen in cells treated with PMA alone. There are several possibilities to explain these findings: (1) ionomycin only affects the Na^+/H^+ exchanger at an acidic pH_i , and this mechanism is independent of the action of PMA at more alkaline pH levels; (2) ionomycin and PMA act via separate biochemical pathways, but have a final common mechanism of activation of the Na^+/H^+ exchanger; or (3) ionomycin and PMA act via the same biochemical pathway, but differences in diffusion rates between the two agents account for the differences in time courses of alkalization.

Evidence that PMA and ionomycin act via separate biochemical pathways to activate Na^+/H^+ exchange comes from data using the PKC inhibitor staurosporine. In cells incubated with staurosporine, PMA had no effect on pH_i , but the action of ionomycin on pH_i was unaffected. Other cells appear to have PKC-independent pathways for Na^+/H^+ exchange activation (Muldoon *et al.*, 1987; Huang *et al.*, 1987; Manganel & Turner, 1990), and one of the additional pathways that has been implicated involves Ca^{2+} /calmodulin-dependent protein kinase

(Owen & Villereal, 1982; Ober & Pardee, 1987; Little *et al.*, 1988; Negishi *et al.*, 1990). We attempted to address this possibility in SaOS-2 cells by using the putative calmodulin inhibitors calmidazolium (Van Belle, 1981) and W-7 (Inagaki *et al.*, 1986). However, calmidazolium itself acidified the cytosol in SaOS-2 cells. W-7 (50 μM) inhibited the ionomycin-induced alkalization, but the effect of PMA on pH_i was also abolished; therefore we could not rule out non-specific actions of W-7 on Na^+/H^+ exchange (results not shown).

Green *et al.* (1988a) have examined the Na^+/H^+ exchange mechanism in the osteoblast-like osteosarcoma cell line UMR 106. The Na^+/H^+ exchanger in these cells does not appear to be activated by PMA or ionomycin (Green *et al.*, 1990). However, in a bicarbonate-buffered system, they found that ionomycin induced acidification via action on the Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism (Green *et al.*, 1990). In A10 vascular smooth muscle cells it has been shown that Na^+/H^+ exchange is the major mechanism by which the pH_i is regulated in acidified cells, while Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchange is the predominant regulatory mechanism at basal or alkaline pH_i (Kikeri *et al.*, 1990). It is likely that Na^+ -dependent $\text{Cl}^-/\text{HCO}_3^-$ exchange is also operative in SaOS-2 cells. However, in the present study we have limited our investigation of pH_i regulation to the role of Na^+/H^+ exchange in HCO_3^- -free buffer.

In conclusion, we have identified two distinct mechanisms by which the Na^+/H^+ exchange mechanism is activated in SaOS-2 cells: (1) regulation by PKC and (2) by transients in $[\text{Ca}^{2+}]_i$, which act via a non-PKC pathway. It will be of interest to investigate whether hormones which have been shown to stimulate bone remodelling contribute to pH_i homeostasis via these signal transduction pathways (Ofori-Darko *et al.*, 1990).

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