Cytoplasmic $[Ca^{2+}]$ and Intracellular pH in Lymphocytes

Role of Membrane Potential and Volume-activated Na^+/H^+ Exchange

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ABSTRACT The effect of elevating cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) on the intracellular pH (pH_i) of thymic lymphocytes was investigated. In Na^+-containing media, treatment of the cells with ionomycin, a divalent cation ionophore, induced a moderate cytoplasmic alkalinization. In the presence of amiloride or in Na^+-free media, an acidification was observed. This acidification is at least partly due to H^+ (equivalent) uptake in response to membrane hyperpolarization since: (*a*) it was enhanced by pretreatment with conductive protonophores, (*b*) it could be mimicked by valinomycin, and (*c*) it was decreased by depolarization with K^+ or gramicidin. In addition, activation of metabolic H^+ production also contributes to the acidification. The alkalinization is due to Na^+/H^+ exchange inasmuch as it is Na^+ dependent, amiloride sensitive, and accompanied by H^+ efflux and net Na^+ gain. A shift in the pH_i dependence underlies the activation of the antiport. The effect of $[Ca^{2+}]_i$ on Na^+/H^+ exchange was not associated with redistribution of protein kinase C and was also observed in cells previously depleted of this enzyme. Treatment with ionomycin induced significant cell shrinking. Prevention of shrinking largely eliminated the activation of the antiport. Moreover, a comparable shrinking produced by hypertonic media also activated the antiport. It is concluded that stimulation of Na^+/H^+ exchange by elevation of $[Ca^{2+}]_i$ is due, at least in part, to cell shrinking and does not require stimulation of protein kinase C.

INTRODUCTION

An amiloride-sensitive, electroneutral Na^+/H^+ antiport has been detected in the plasma membranes of most mammalian cells (see Mahnensmith and Aronson, 1985, and Grinstein and Rothstein, 1986, for reviews). This transport system is involved in the regulation of cellular volume (Cala, 1985) and, under certain

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circumstances, cytoplasmic pH (R. C. Thomas, 1984; Mahnensmith and Aronson, 1985). In some epithelia, it is essential for transcellular ion transport (Aronson, 1983). The rate of Na^+/H^+ countertransport is dictated by the concentrations of the transported ions, but it can also be modulated by a wide variety of agents. These include hormones (Moore, 1981; Schuldiner and Rozengurt, 1982), growth factors and other mitogens (Rothenberg et al., 1983; Benos and Sapirstein, 1983; Moolenaar et al., 1983), tumor-promoting agents (Burns and Rozengurt, 1983; Moolenaar et al., 1984), and platelet- and neutrophil-activating factors (Siffert et al., 1984; Molski et al., 1980). The molecular mechanism of action of these agents is not understood.

Serum, growth factors, mitogenic lectins, thrombin, chemotactic factors, and other agents that activate Na^+/H^+ exchange also induce an increase in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). The change in $[Ca^{2+}]_i$ generally precedes the activation of the antiport (e.g., Hesketh et al., 1985), which suggests a causal relationship. The involvement of Ca^{2+} in the activation of Na^+/H^+ exchange is further suggested by the inhibitory effects of "Ca^{2+} antagonists" and of calmodulin blockers (Villereal et al., 1985) and particularly by the finding that A23187, a divalent cation ionophore, can induce activation in human foreskin fibroblasts (Owen and Villereal, 1982; Villereal et al., 1985; Muldoon et al., 1985). However, the latter observation is not universal: amiloride-sensitive Na^+ uptake was not increased by A23187 in mouse neuroblastoma (Moolenaar et al., 1981) or fibroblast cells (Frelin et al., 1983). The ionophore also failed to produce the predicted cytoplasmic alkalinization in human foreskin fibroblasts (Moolenaar et al., 1983). Instead, a significant acidification was observed. In addition, preloading renal microvillus vesicles with various amounts of Ca^{2+} had no significant effect on Na^+/H^+ exchange activity (Aronson et al., 1982).

In thymic lymphocytes (thymocytes), the Na^+/H^+ antiport can be activated by mitogenic lectins (Hesketh et al., 1985). It is not clear whether this activation is due to the associated increase in cytoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) or to the production of diacylglycerol, which is known to stimulate the antiport in these cells (Grinstein et al., 1985*a*). In the present study, we have analyzed the effects of ionomycin and A23187 on cytoplasmic pH (pH_i) in rat thymocytes in order to define the role of Ca^{2+} in the regulation of the Na^+/H^+ antiport. Two independent responses were observed: in normal (Na^+-containing) medium, a small alkalinization was detected; in Na^+-free media and in normal medium containing amiloride, an acidification was recorded. The mechanisms underlying these pH_i changes were investigated.

MATERIALS AND METHODS

Reagents

Valinomycin, gramicidin (from *Bacillus brevis*), quinine, phenylmethylsulfonylfluoride, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 2-[*N*-morpholino]ethanesulfonic acid (MES), phosphatidylserine, albumin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), histone type III-S, and molecular weight standards were purchased from Sigma Chemical Co., St. Louis, MO. Acrylamide, bis-acrylamide, and the silver protein staining kit were from Bio-Rad Laboratories, Cambridge, MA. Nigericin, ionomycin, and ionophore A23187 were

from Calbiochem-Behring Corp., San Diego, CA. Bis-(1,3-diethylthiobarbituric)trimethine oxonol (bis-oxonol), 3,3′-dipropylthiadicarbocyanine [diS-C_3-(5)], quin2 acetoxymethyl ester, and 2′,7′-bis-(carboxyethyl)-5,6-carboxyfluorescein (BCECF) acetoxymethyl ester were from Molecular Probes, Junction City, OR. Medium RPMI 1640 (10-times concentrated without HCO_3^-) and HEPES were from Gibco, Grand Island, NY. [^{32}P]orthophosphate and γ[^{32}P]ATP were from ICN Pharmaceuticals, Irvine, CA. Amiloride was the kind gift of Merck Frosst, Montreal, Quebec.

Solutions

Stock solutions of diS-C_3-(5), bis-oxonol, ionomycin, A23187, gramicidin, and the acetoxymethyl esters of quin2 and BCECF were made in dimethyl sulfoxide. Valinomycin, nigericin, and CCCP stocks were prepared in ethanol. Amiloride was stored as a 10-mM aqueous stock. Na^+ solution contained (in mM): 140 NaCl, 1 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 20 Tris-MES, pH 7.3. *N*-methyl-D-glucamine$^+$ (NMG^+) solution and K^+ solution were prepared by iso-osmotic replacement of Na^+ by NMG^+ or K^+, respectively. Where indicated, $CaCl_2$ was omitted and 1 mM EGTA was added to the media. For external pH (pH_o) recordings, Tris-MES was omitted. All media were adjusted to 290 ± 5 mosM with the major salt.

Cell Isolation and Characterization

Thymocytes were isolated from male Wistar rats (150–200 g) as described earlier (Grinstein et al., 1984*a*). Unless otherwise indicated, the cells were maintained in HEPES-buffered RPMI 1640 at room temperature for up to 8 h. Cell viability was >95% throughout this period. Cell counting and sizing were performed with a Coulter Counter/Channelyzer (Coulter Electronics, Hialeah, FL) as described (Grinstein et al., 1984*a*).

pH_i Measurement and Manipulation

pH_i was measured fluorimetrically with BCECF as described (Grinstein et al., 1985*a*) using Perkin-Elmer (Norwalk, CT) 650-40 or LS-5 fluorescence spectrophotometers attached to Perkin-Elmer R-100 recorders. Calibration was performed using nigericin and K^+ and titrating the medium with concentrated Tris or MES, by the method of J. A. Thomas et al. (1979).

In some experiments, pH_i was manipulated by incubation of the cells in NMG^+ solution with nigericin. The ensuing acidification was monitored in BCECF and terminated by the addition of defatted serum albumin, essentially as described earlier (Grinstein et al., 1984*a*). The cells were then sedimented and resuspended in a small volume of fresh NMG^+ medium without nigericin. Where indicated, this concentrated suspension was then treated with ionomycin and after 3 min added to a cuvette containing prewarmed Na^+ solution to initiate Na^+/H^+ exchange, which was recorded fluorimetrically as an amiloride-sensitive cytoplasmic alkalinization.

Acid extrusion was measured as changes in extracellular pH with an Orion Research, Inc. (Cambridge, MA) 601A digital Ionalyzer connected to an *X* vs. time recorder (Perkin-Elmer R-100) exactly as described (Grinstein et al., 1985*a*). The buffering power of the medium, which was provided largely by the HEPES carried over with the cell suspension, was determined at the end of each experiment by titration with KOH and HCl.

Membrane Potential Determinations

Membrane potential was estimated fluorimetrically using bis-oxonol, with excitation at 540 nm (3-nm slit) and emission at 580 nm (10-nm slit). Aliquots of the cell suspension (10^6 cells/ml, final) were added to the indicated medium containing 0.3 μM of the dye

and the membrane potential was recorded as described by Rink et al. (1980). Calibration was made by adding gramicidin (0.1 μM final) to cells suspended in isotonic media containing various ratios of Na^+ and NMG^+ (which does not permeate through gramicidin). Potential (E_m, in millivolts) was then calculated as $E_m = 60 \log([Na^+]_o/[Na^+ + K^+]_i)$, assuming that Na^+ and K^+ permeation through gramicidin are comparable.

Because valinomycin cannot be used in combination with bis-oxonol, the effect of this ionophore on membrane potential was measured in some experiments with diS-C_3-(5). This cyanine dye was used at 0.6 μM with 3×10^6 cells/ml as previously described (Grinstein et al., 1982). Because valinomycin is normally used to calibrate diS-C_3-(5), the effect of the ionophore is reported as the fractional fluorescence change ($\Delta F/F$), rather than in absolute units, to avoid a circular argument.

Free $[Ca^{2+}]_i$ Determinations

$[Ca^{2+}]_i$ was measured using quin2 essentially as described by Tsien et al. (1982). Thymocyte suspensions (5×10^7 cells/ml) in HEPES-buffered RPMI 1640 were loaded for 30 min at 37°C with 5 μM quin2 acetoxymethyl ester. The cells were then washed and resuspended in the indicated medium at $3–5 \times 10^6$ cells/ml. Fluorescence was measured with excitation at 339 nm (2-nm slit) and emission at 495 nm (15-nm slit). Calibration was made with 1–2 μM ionomycin and 2 mM Mn^{2+} as previously described (Rink et al., 1983).

Protein Kinase C Determination and Manipulation

To assess the migration of protein kinase C, thymocytes (20×10^6/ml) were incubated in medium with or without 10^{-7} M TPA or 10^{-6} M ionomycin for 10 min at 37°C. The cells were then sedimented and resuspended at 10^9 cells/ml in ice-cold medium containing 140 mM Tris-HCl, 7 mM EDTA, pH 7.5. Then the cells were lysed by addition of 6 vol of ice-cold distilled water with phenylmethylsulfonylfluoride (final concentration, 0.5 mM). After stirring for 10 min at 4°C, the cells were sedimented at 48,000 g_{max} for 30 min at 4°C and the supernatant was used for kinase activity determinations. The activity of the particulate fraction was determined after extraction of the pellet with 0.1% Triton X-100 for 10 min at 4°C followed by centrifugation. The protein kinase assay mixture contained 20 mM Tris-HCl, 20 mM magnesium acetate, 1 mM $CaCl_2$, 100 μM ATP, 0.5–0.1 μCi $\gamma[^{32}P]$ATP, and 50 μg histone (type III-S) in a final volume of 250 μl. To define protein kinase C–mediated phosphorylation, the determinations were performed in triplicate in the presence and absence of TPA (10^{-7} M, final) and phosphatidylserine (24 μg/sample). The reaction was started by the addition of 5–20 μg of thymocyte protein and, after a 3-min incubation at 30°C, terminated by the addition of 1 ml of ice-cold 25% trichloroacetic acid. The precipitated histone was then separated by filtration on 0.45-μm HA Millipore filters, followed by four washes, each with 3 ml of 5% trichloroacetic acid. The filters were then counted by liquid scintillation.

Depletion of intracellular protein kinase C was accomplished as described (Grinstein et al., 1986*b*). Briefly, 2×10^7 cells/ml were incubated for 24 h at 37°C in HEPES-buffered RPMI 1640 containing antibiotics and $2–5 \times 10^{-7}$ M TPA. The cells were then washed twice and used for pH_i determinations. This procedure has been shown to reduce the protein kinase C activity of fibroblasts (Rodriguez-Peña and Rozengurt, 1984) and thymocytes (Grinstein et al., 1986*b*) by >90%. Loading with BCECF was performed in the absence of TPA.

Other Methods

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate and autoradiography of the dried gels were performed as described elsewhere (Grinstein et al., 1986*a*). Protein

determination was made by the method of Lowry et al. (1951). Intracellular Na^+ and K^+ contents were measured by flame photometry using Li^+ as an internal standard, as described (Grinstein et al., 1982). Unless otherwise specified, all the experiments were carried out at 37°C. The results are presented either as representative traces (e.g., Fig. 1) of at least three experiments or as the mean ± SE of the number of determinations specified. Straight lines were calculated by least squares.

RESULTS

Effect of Ionomycin on pH_i

To determine the effect of intracellular Ca^{2+} on pH_i, we treated rat thymocytes with various concentrations of the divalent cation ionophore ionomycin. This nonfluorescent ionophore was chosen because it does not interfere with the fluorimetric determinations of pH_i, $[Ca^{2+}]_i$, or membrane potential, and because it is more specific for Ca^{2+} over Mg^{2+} than the more commonly used ionophore A23187 (Kauffman et al., 1980). The effects of low (e.g., 0.4 μM) ionomycin concentrations are illustrated in Fig. 1, *A–C*. In normal (Na^+-containing) medium, the ionophore induced a cytoplasmic alkalinization (Fig. 1 *A*). In 23 experiments using 0.2–0.5 μM ionomycin, pH_i increased by a maximum of 0.095 ± 0.002 units. The alkalinization was absent when low concentrations of the ionophore were added to cells in Na^+-free NMG^+ solution. Instead, a significant cytoplasmic acidification was recorded (Fig. 1 *B*). That this reversal in the direction of the pH_i change is probably due to impairment of Na^+/H^+ exchange is demonstrated by experiments using amiloride. The addition of this inhibitor of the antiport to cells in Na^+ medium also abolished the alkalinization induced by ionomycin (Fig. 1 *C*). As was the case in NMG^+ medium, the ionophore acidified cells suspended in Na^+ solution with amiloride (200 μM). In eight experiments using 0.2–0.5 μM ionomycin, the maximal acidification averaged 0.14 ± 0.012 pH units.

Markedly different results were obtained at high (e.g., 3 μM) ionomycin concentrations (Fig. 1, *D–F*). A rapid alkalinization was observed in both Na^+ and NMG^+ media, in the presence or absence of amiloride. The alkalinization reached a maximum of ~0.25 units within 30 s and relaxed thereafter. This pH_i change probably reflects H^+ efflux through ionomycin,[1] an electroneutral carboxylic ionophore known to operate by exchanging Ca^{2+} for $2H^+$ (Kauffman et al., 1980). Accordingly, the alkalinization was not observed when extracellular Ca^{2+} was omitted (Fig. 1 *F*). The relaxation of the pH_i change may be due to the partial collapse of the inward Ca^{2+} gradient caused by the exchange of extracellular Ca^{2+} for intracellular Mg^{2+} and/or to the onset of acid uptake or production by the cell (see below).

A more detailed study of the concentration dependence of the effects of ionomycin on pH_i is presented in Fig. 2, *A* and *B*. The maximal pH_i change

[1] In cells loaded with ~2 mM quin2, 3 μM ionomycin sufficed to saturate the Ca^{2+}-sensitive probe. Since, in resting cells, quin2 is less than half-saturated ($[Ca^{2+}]_i \leq 100$ nM and $K_d = 115$ nM), this implies that the ionophore catalyzed the uptake of at least 1 mmol Ca^{2+}/liter cells. Considering a buffering power of 25 mmol·liter $cells^{-1}$·pH $unit^{-1}$ and an H^+:Ca^{2+} stoichiometry of 2, the efflux of H^+ through ionomycin would produce a minimum ΔpH of ~0.1 unit, which is consistent with the results of Fig. 1, *D–F*.

recorded in Na^+ medium in the presence or absence of amiloride is shown in Fig. 2*A*. No detectable effects were recorded with concentrations ≤2 nM. In the absence of amiloride, higher concentrations induced a progressively increasing alkalinization, which reached a plateau of ~0.1 unit. A further alkalinization of nearly 0.1 unit was observed at 3 μM, presumably resulting from $[Ca^{2+}]_o/[2H^+]_i$ exchange. In amiloride-containing medium, a gradual acidification was observed at ≥2 nM ionomycin, which reached a maximum at 0.5 μM. At higher concentrations, where $[Ca^{2+}]_o/[2H^+]_i$ exchange contributes significantly to pH_i, the acidification was reduced or even reversed, which is consistent with the data in Fig. 1*F*.

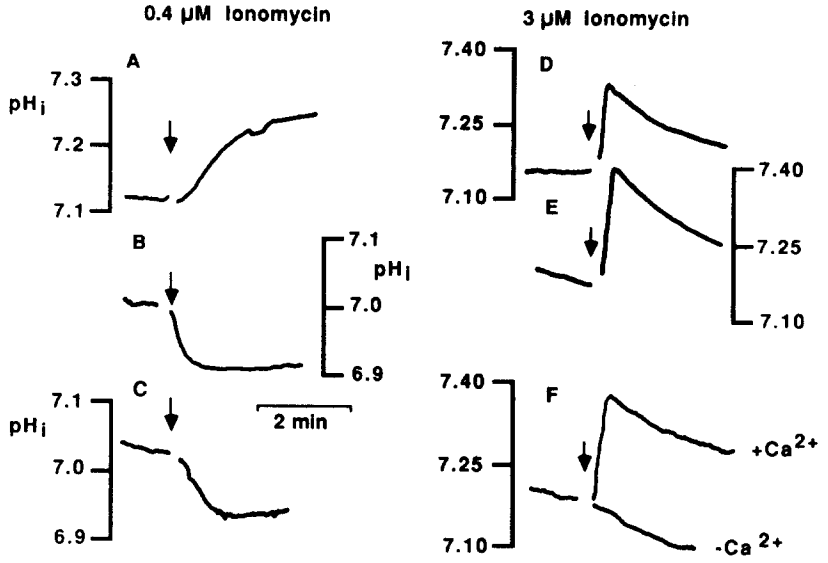

FIGURE 1. Effects of ionomycin on pH_i in rat thymic lymphocytes. BCECF-loaded cells were resuspended in either Na^+ solution (*A* and *D*), NMG^+ solution (*B* and *E*), or Na^+ solution containing 200 μM amiloride at 37°C. Where indicated by the arrows, either 0.4 (*A–C*) or 3 (*D–F*) μM ionomycin was added. Cytoplasmic pH (pH_i) was measured fluorimetrically as described in the Methods. Calibration was accomplished by the K^+/nigericin method of J. A. Thomas et al. (1979). The traces are representative of at least three similar experiments.

Even though the final pH_i attained with 10 nM to 1 μM ionomycin was similar (Fig. 2*A*), the rate of alkalinization increased along with the concentration of ionophore (Fig. 2*B*). In this concentration range, the maximum rate of alkalinization reached 0.11 pH units/min and could be completely inhibited by 200 μM amiloride. A much higher rate (1.3 pH units/min) was attained at 3 μM ionomycin, but this was amiloride insensitive.

To correlate the different concentrations of ionomycin with the level of $[Ca^{2+}]_i$, cells were loaded with quin2 and treated with the ionophore under the same conditions used in Fig. 2, *A* and *B*. As shown in Fig. 2*C*, the resting $[Ca^{2+}]_i$ was ~90 nM. This level was nearly doubled by 2 nM ionomycin, but it

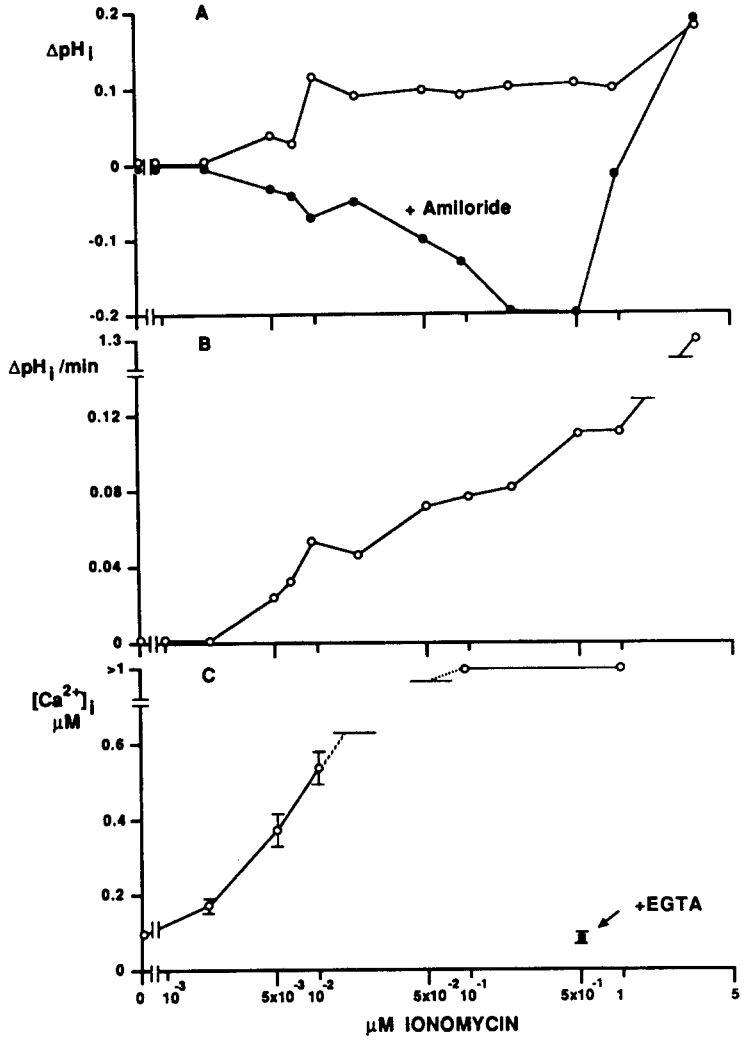

FIGURE 2. Concentration dependence of the effects of ionomycin on cytoplasmic pH (pH_i) and cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$). (*A*) Concentration dependence of the effects of ionomycin on the maximal ΔpH_i obtained in Na^+ solution in the presence (filled symbols) or absence (open symbols) of 200 μM amiloride. BCECF-loaded cells suspended in the indicated media were treated with the concentration of ionomycin specified in the abscissa. The maximal ΔpH_i attained was recorded fluorimetrically as described for Fig. 1. A positive ΔpH_i indicates alkalinization. The points are means of two experiments. (*B*) Effect of ionomycin on the maximal rate of ΔpH_i. Cells were suspended in Na^+ solution (without amiloride) and pH_i was measured after the addition of the indicated concentration of the ionophore. The rate of ΔpH_i was calculated from the maximal slope of the fluorescence recording. Notice that the ordinate is discontinuous above 0.12 pH/min. The data are means of the two experiments reported in *A*. (*C*) Effect of ionomycin on $[Ca^{2+}]_i$. Quin2-loaded cells were suspended in normal Na^+ solution (open symbols) or in Ca^{2+}-free Na^+ solution containing 1 mM EGTA (filled symbols). After the baseline $[Ca^{2+}]_i$ was recorded, the indicated concentration of ionomycin was added. The maximal $[Ca^{2+}]_i$ attained after the addition of the ionophore is reported. The data are means ± SE of three experiments. Notice that at high ionomycin concentrations, $[Ca^{2+}]_i$ cannot be determined accurately, because the indicator dye approaches saturation.

failed to affect pH_i. As expected, higher levels were obtained with increasing concentrations of the ionophore. Accurate measurements could not be obtained at concentrations >10 nM, which elevated $[Ca^{2+}]_i$ well above the K_d for quin2, a range where this dye becomes rather insensitive.

Taken together, the data in Figs. 1 and 2 suggest that the elevation of $[Ca^{2+}]_i$ above 200 nM results in alterations of pH_i. That the effects on pH_i are due to the change in $[Ca^{2+}]_i$, rather than to the presence of ionomycin itself, is shown by the experiments in Fig. 3. The addition of the ionophore (0.5 μM) to cells suspended in Na^+ medium devoid of extracellular Ca^{2+} and containing 1 mM EGTA produced a reduced acidification. Under these conditions, ionomycin produced only a small and transient increase in $[Ca^{2+}]_i$ (Fig. 2*C*; also see below). The reintroduction of Ca^{2+} to this medium resulted in a rapid alkalinization (Fig. 3*A*). Moreover, replacement of extracellular Ca^{2+} by Mg^{2+}, Mn^{2+}, Cd^{2+}, or Ba^{2+}

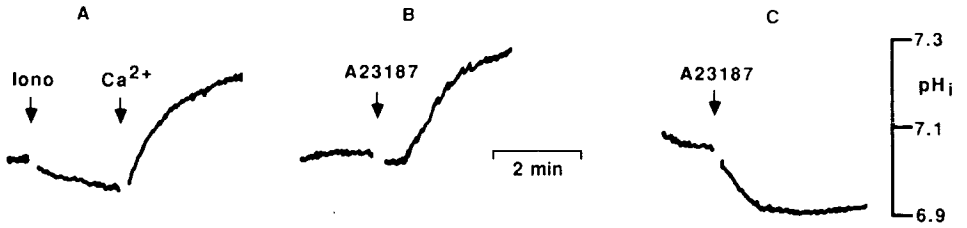

FIGURE 3. Evidence for the Ca^{2+} requirement of the pH_i changes. (*A*) BCECF-loaded thymocytes were suspended in Ca^{2+}-free Na^+ solution and pH_i was recorded as in Fig. 1. Where indicated, 0.4 μM (final) ionomycin (Iono) was added to the cuvette. After ~2 min, 2 mM Ca^{2+} was added and recording was continued. (*B*) Cells were suspended in Ca^{2+}-containing (normal) Na^+ solution and 50 nM A23187 (final) was added where indicated. (*C*) Cells were suspended in Ca^{2+}-containing NMG^+ solution. 50 nM A23187 was added at the arrow. The traces are representative of at least three similar experiments. pH_i was recorded and calibrated as described for Fig. 1.

also prevented the responses to ionomycin. Finally, the actions of ionomycin on pH_i can be mimicked by another, chemically unrelated divalent cation ionophore, A23187. As before, A23187 was observed to alkalinize cells suspended in Na^+ medium (Fig. 3*B*). In the absence of extracellular Na^+ (Fig. 3*C*), or in the presence of amiloride (not illustrated), this ionophore similarly induced a cytoplasmic acidification.

Mechanism of Cytoplasmic Acidification

The possible mechanisms underlying the alkalinization and acidification observed in the presence and absence of extracellular Na^+, respectively, will be considered separately.

A cytoplasmic acidification could, in principle, be produced by (*a*) increased metabolic acid generation, (*b*) release of acid equivalents from intracellular stores such as lysosomes, or (*c*) entry of acid equivalents from the external medium. Although *a* and *b* contribute to a measurable extent to the acidification (see Discussion), the evidence presented below indicates that most of the observed effect can be accounted for by uptake of extracellular H^+ (equivalents).

In resting thymocytes suspended in a medium of pH 7.3, protons are not at electrochemical equilibrium, since $pH_i \approx 7.2$ and the membrane potential is close to −50 mV (see below). Thus, H^+ uptake is a thermodynamically spontaneous process and can be increased either by elevating the conductance of the membrane to H^+ or by hyperpolarizing the membrane. Because $[Ca^{2+}]_i$ is a determinant of K^+ permeability, and therefore of membrane potential in a variety of cells, we determined whether addition of ionomycin could hyperpolarize rat thymocytes. The results of one such experiment are illustrated in Fig. 4. The addition of the ionophore to cells in Ca^{2+}-containing medium resulted in a rapid, marked hyperpolarization of >20 mV (Fig. 4*A*).[2] In nominally Ca^{2+}-free medium containing EGTA, the ionophore induced a much smaller and transient potential change (Fig. 4*B*). This hyperpolarization is probably also due to Ca^{2+}-induced K^+ channels, inasmuch as it is associated with a transient increase in $[Ca^{2+}]_i$ (Fig. 4*E*), which presumably originates from intracellular stores.[3] This short-lived change in $[Ca^{2+}]_i$ contrasts with the sustained increase recorded in the presence of extracellular Ca^{2+} (Fig. 4*D*). The results in Fig. 4*C* further suggest that the ionomycin-induced hyperpolarization is generated by an increased K^+ conductance. In this experiment, the cells were suspended in K^+ solution so that the K^+ equilibrium potential, E_{K^+}, was near zero. Since at high $[K^+]_o$ this ion has a transference number near unity (Grinstein et al., 1984*b*), the resting membrane potential approaches E_{K^+}, and further increasing the K^+ conductance is expected to have little effect on potential. Accordingly, ionomycin produced no significant change in membrane potential. Experiments using quin2 (not illustrated) demonstrated that, under these conditions, the change in $[Ca^{2+}]_i$ induced by the ionophore is comparable to that in Fig. 4*D*. In summary, these experiments demonstrate that increasing $[Ca^{2+}]_i$ by means of ionomycin results in a significant hyperpolarization of rat thymic lymphocytes. This membrane potential change could be responsible for the acidification induced by the ionophore.

Two predictions can be made based on the hypothesis that membrane hyperpolarization underlies the change in pH_i: (*a*) the effect should be mimicked by other agents or conditions that produce hyperpolarization, and (*b*) the acidification should be enhanced by increasing the H^+ (OH^-) conductance. Fig. 5 shows a test of the former prediction. Valinomycin, a K^+-selective ionophore, can hyperpolarize thymocytes suspended in Na^+ medium (Fig. 5*B*). A similar concentration of this ionophore also induced a significant cytoplasmic acidification in cells suspended in amiloride-containing Na^+ medium (Fig. 5*E*) or in NMG^+ medium (not shown). The effect of valinomycin on pH_i is probably due to the associated membrane potential change, since it is enhanced by CCCP, a conductive protonophore (Fig. 5*F*). The protonophore, which has only minor effects

[2] The hyperpolarization in Ca^{2+}-containing media was not always sustained. Frequently, a rapid, transient, >20-mV hyperpolarization partially subsided within 1 min, stabilizing at a level only 10–15 mV more negative than the resting potential. This variability may reflect differences in the ability of individual cell batches to regulate $[Ca^{2+}]_i$ after a challenge with the ionophore.

[3] It must be borne in mind that, in quin2-loaded cells, the magnitude of the $[Ca^{2+}]_i$ transient is dampened by the buffering power of the probe. Under similar conditions, we have observed larger transients in cells loaded with 10-fold-lower concentrations of indo-1, a probe with a higher absorption coefficient and quantum yield.

on membrane potential (Fig. 5*D*), induced by itself a significant influx of H^+ equivalents, probably driven by the resting potential of 50–60 mV (negative inside). Further evidence that H ions are moving conductively in response to the potential was obtained with gramicidin. This channel-former, which readily

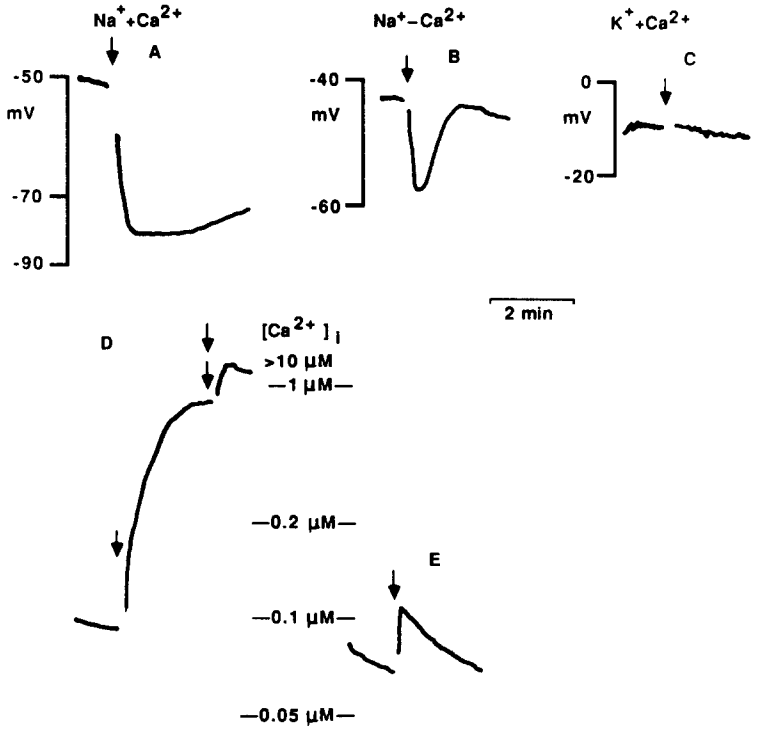

FIGURE 4. Effect of ionomycin on membrane potential and $[Ca^{2+}]_i$ in thymic lymphocytes. (*A–C*) Membrane potential measurements using the anionic fluorophore bis-oxonol. The cells were pre-equilibrated with the fluorescent probe in either normal Na^+ solution (*A*), Ca^{2+}-free Na^+ solution containing 1 mM EGTA (*B*), or K^+ solution with Ca^{2+} (*C*). Where indicated by the arrow, 100 nM ionomycin was added. Calibration of fluorescence vs. membrane potential was accomplished using gramicidin and mixtures of Na^+ and NMG^+ solutions as described in the Methods. The individual traces are representative of at least four experiments.[2] (*D* and *E*) Measurements of $[Ca^{2+}]_i$. Quin2-loaded cells were suspended in either normal Na^+ solution (*D*) or in Ca^{2+}-free Na^+ solution with 1 mM EGTA (*E*). Where indicated by the single arrow, 100 nM ionomycin was added. A second addition of ionomycin (1 μM) is indicated by the two arrows in *D*. The traces are representative of at least three experiments. $[Ca^{2+}]_i$ was calibrated with Mn^{2+} as described in the Methods. The same calibration applies to *D* and *E*.

depolarizes cells in Na^+ medium (Fig. 5, *B* and *C*), partially reversed the CCCP-induced acidification (Fig. 5*G*). Moreover, in cells suspended in K^+ medium, the CCCP-induced acidification was greatly reduced and reversed by the subsequent addition of valinomycin (Fig. 5*H*). The observation that CCCP still produced a

significant though reduced acidification in depolarized cells is suggestive of an increased metabolic production of H^+, secondary to mitochondrial uncoupling (see Discussion).

The prediction that increasing the H^+ conductance should enhance the ionomycin-induced acidification was tested in Fig. 6. It was found that, in cells treated

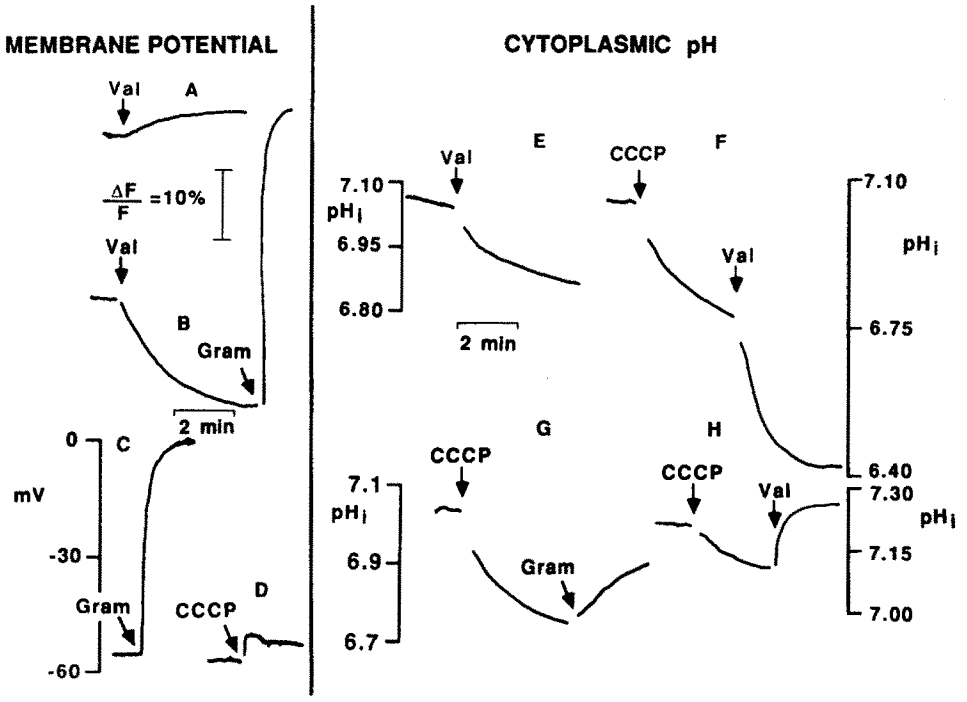

FIGURE 5. Effects of monovalent cation ionophores on membrane potential (left panel) and pH_i (right panel). Membrane potential was measured with either diS-C_3-(5) (*A* and *B*) or bis-oxonol (*C* and *D*). The former dye was used because bis-oxonol is incompatible with valinomycin. (*A*) Cells were suspended in K^+ solution. Where indicated, 1 μM valinomycin (Val) was added. (*B*) Cells were suspended in Na^+ solution. Valinomycin (1 μM) was added where indicated, followed by 50 nM gramicidin (Gram). The fractional fluorescence change ($\Delta F/F$) is given relative to the fluorescence of the dye before the addition of the cells, and applies only to *A* and *B*. (*C*) Cells were pre-equilibrated with bis-oxonol in Na^+ solution. Where indicated, 50 nM gramicidin was added. (*D*) Cells were suspended in Na^+ solution. Where indicated, 1 μM CCCP was added. The membrane potential calibration (in millivolts, negative inside), obtained as described for Fig. 4, applies to *C* and *D*. (*E–H*) pH_i measurements using BCECF. (*E*) Cells were suspended in Na^+ medium containing 200 μM amiloride. Where indicated, 1 μM valinomycin was added. (*F*) Cells in Na^+ medium plus amiloride. First, 1 μM CCCP was added and then 1 μM valinomycin. (*G*) Cells in Na^+ medium plus amiloride. CCCP (1 μM) and valinomycin (1 μM) were added where noted. The traces are representative of at least three similar experiments.

with CCCP, the effect of ionomycin was faster and more pronounced (compare *A* and *C*). It must be taken into account that in cells treated with CCCP, ionomycin was added when pH_i was significantly lower than in the untreated cells. This would tend to diminish the apparent effect of ionomycin for two reasons: (*a*) the driving force for net conductive H^+ uptake has been reduced, and (*b*) the rate of $[Ca^{2+}]_o/[2H^+]_i$ exchange, which tends to counteract the acidification, must have increased in response to the increased $[H^+]_i$. Nevertheless, the observed effect was larger than in untreated cells. Two other observations are consistent with

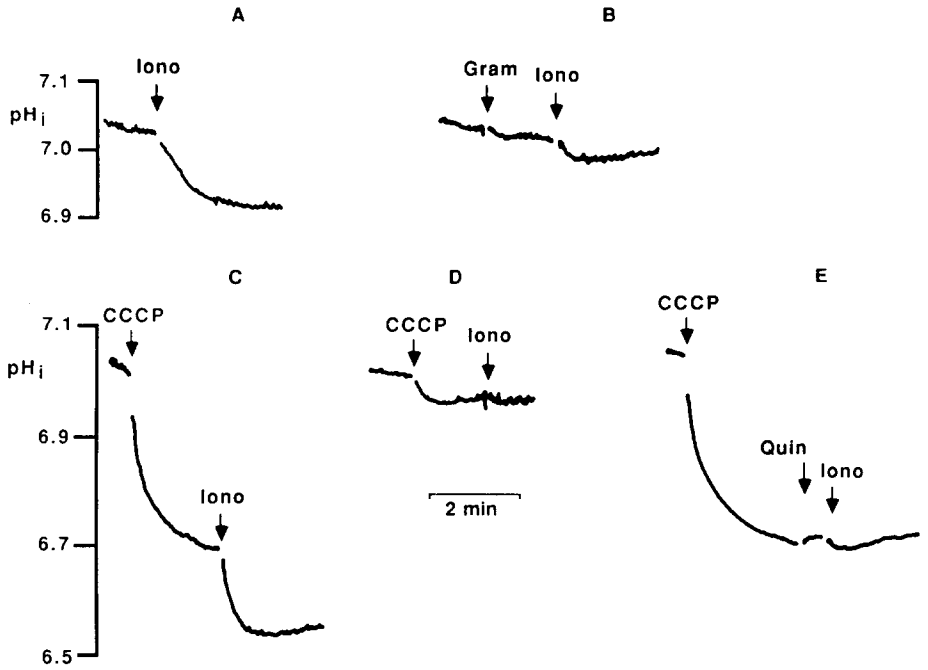

FIGURE 6. Changes in cation conductance affect the ionomycin-induced pH_i changes. BCECF-loaded cells were suspended in Na^+ solution containing 200 μM amiloride (*A*, *B*, *C*, and *E*) or in K^+ solution (*D*). pH_i was measured and calibrated as described for Fig. 1. (*A*) Addition of 0.4 μM ionomycin. (*B*) Sequential addition of gramicidin (100 nM) and ionomycin (0.4 μM). (*C*) Sequential addition of CCCP (1 μM) and ionomycin (0.4 μM). (*D*) Sequential addition of CCCP (1 μM) and ionomycin (0.4 μM). (*E*) Sequential addition of CCCP (1 μM), quinine (100 μM), and ionomycin (0.4 μM). The traces are representative of two or more similar experiments.

the notion that the acidification is associated with hyperpolarization. First, the effect is markedly reduced when the cells are depolarized by either gramicidin (in Na^+ medium) or high $[K^+]_o$ (Fig. 6, *B* and *D*). This observation applies whether CCCP is absent (*B*) or present (*D*). Second, the effect of ionomycin was blocked by concentrations of quinine that were expected to inhibit Ca^{2+}-sensitive K^+ channels (Fig. 6*E*). Taken together, these observations suggest that a significant component of the acidification generated by ionomycin is due to increased conductive H^+ uptake in response to membrane hyperpolarization.

Mechanism of Cytoplasmic Alkalinization

Role of Na^+/H^+ exchange. Measurements of extracellular pH were undertaken to determine whether the net alkalinization observed in Na^+ medium is due to transmembrane H^+ transport. As shown in Fig. 7, the intracellular alkalinization in response to ionomycin was accompanied by the appearance of proton equivalents in the extracellular space, measurable as an acidification in poorly buffered Na^+ medium. This acidification was not observed when ionomycin was added to cells suspended in amiloride-containing or Na^+-free media (not illustrated). These data indicate that the alkalinization induced by the ionophore results from the outward transmembrane transport of internal proton equivalents.

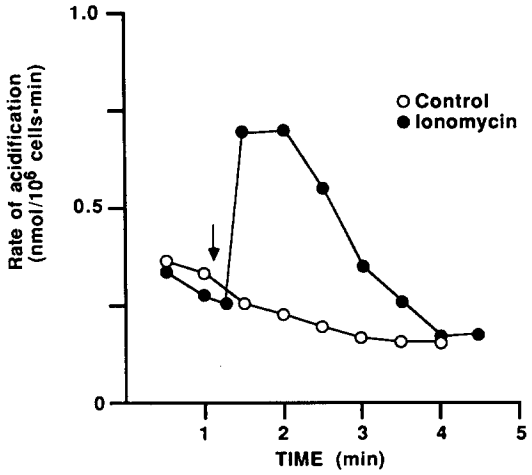

FIGURE 7. Ionomycin-induced extracellular acidification. Thymocytes were suspended in lightly buffered Na^+ solution. The pH_i of this suspension, buffered only by the HEPES carried over with the pellet, was measured with a combination probe as described in the Methods. Where indicated by the arrow, ionomycin was added to one of the samples (filled symbols). The buffering capacity of the medium, used to estimate the rate of acidification (ordinate), was determined at the end of each experiment by titration with KOH and HCl. Extracellular pH was maintained between 7.3 and 7.0 by manual addition of KOH. Representative of four experiments.

The Na^+ dependence and amiloride sensitivity of the transmembrane H^+ flux strongly suggest mediation by the Na^+/H^+ antiport. Additional evidence for the involvement of the antiport was obtained by measuring the net uptake of Na^+. This was accomplished by determination of the cellular Na^+ content before and after treatment with the Ca^{2+} ionophore. In all cases, ouabain (2.5 mM) was added to the media to prevent backflux of Na^+ through the Na^+/H^+ pump. The results are summarized in Table I. The Na^+ content of control thymocytes after a 10-min incubation with ouabain averaged 2.79 ± 0.11 nmol/10^6 cells (n = 9). This figure was not affected when amiloride (200 μM) was also present in the medium, which indicates that the contribution of the Na^+/H^+ antiport to the net uptake of Na^+ in resting cells is negligible, consistent with its reported pH_i

sensitivity (Grinstein et al., 1984*a*). In the presence of ionomycin, Na^+ content increased to 3.91 ± 0.08 nmol/10^6 cells (n = 9) and this effect was virtually eliminated by amiloride. Considering a median cellular volume of 114 μm^3/cell, the amiloride-sensitive net Na^+ gain induced by ionomycin is equivalent to 9.8 mmol·liter cells^{-1}. Because the buffering power of thymocytes in nominally HCO_3^--free media approximates 25 mmol·liter cells^{-1}·pH unit^{-1} (Grinstein et al., 1984*a*), the change in Na^+ is sufficient to account for the observed change in pH_i, assuming a 1:1 Na^+:H^+ stoichiometry (Aronson, 1985). These data strongly support the participation of the Na^+/H^+ antiport in the alkalinizing response to ionomycin.

External Na^+ concentration dependence. The mechanism of activation of the antiport by Ca^{2+} was analyzed next. This question was addressed by comparing the kinetic parameters of transport in untreated and ionomycin-activated cells.

TABLE I

Effect of Ionomycin on Intracellular Na^+ Content

Condition	Amiloride	Na^+ content	n
		nmol/10^6 cells	
Control	−	2.79±0.11	9
	+	2.77±0.09	9
Ionomycin	−	3.91±0.08	9
	+	2.82±0.09	9

Thymic lymphocytes were incubated in Na^+ solution containing 2.5 mM ouabain for 10 min at 37°C. Where indicated, amiloride (200 μM) and/or ionomycin (0.5 μM) was added at the beginning of the incubation. Na^+ content was determined by flame photometry. The results are means ± 1 SE of the number of determinations (n) indicated.

The antiport was stimulated in control cells by manipulating their pH_i as described earlier (Grinstein et al., 1984*a*).

One conceivable mechanism of activation is an increased extracellular affinity for Na^+. This possibility was analyzed in the experiment shown in Fig. 8. The rate of alkalinization in response to ionomycin (0.5 μM) was measured in BCECF-loaded cells as a function of $[Na^+]_o$. As reported for both resting (acid-activated) and osmotically stimulated thymocytes (Grinstein et al., 1984*a*), the activation of H^+ efflux by Na^+ in ionophore-treated cells follows Michaelis-Menten kinetics. A Lineweaver-Burk–type linearization of data from two experiments is illustrated in Fig. 8. The apparent K_m, calculated from the intercept of this line on the abscissa, was 66.7 mM. This number is somewhat higher than those obtained earlier for control cells by a similar fluorescence method (59 mM; Grinstein et al., 1984*a*) or by measurement of unidirectional Na^+ uptake (51 mM; Grinstein et al., 1984*c*). Therefore, a change in the affinity for extracellular Na^+ cannot account for the observed stimulation of Na^+/H^+ exchange.

Similarity to responses evoked osmotically and by phorbol esters. The Na^+/H^+ antiport of thymocytes can also be activated by phorbol esters such as TPA, or by osmotically shrinking the cells (Grinstein and Rothstein, 1986). The osmotic

and TPA-induced effects share some pharmacological properties with the response to ionomycin: the pH_i change can be inhibited in all cases by trifluoperazine (half-maximal inhibition at ~20 μM) and by pretreatment with *N*-ethylmaleimide (0.25 mM). It was therefore conceivable that the effect of ionomycin was mediated by the same pathways used by the osmotic or TPA responses. If this hypothesis is correct, then maximal stimulation by either TPA or by cell shrinking should preclude further alkalinization by the Ca^{2+} ionophore, i.e., the responses should not be linearly additive. Conversely, additivity would be expected if independent processes are involved. The additivity of the responses was determined in the experiments of Fig. 9. As reported above, the addition of ionomycin

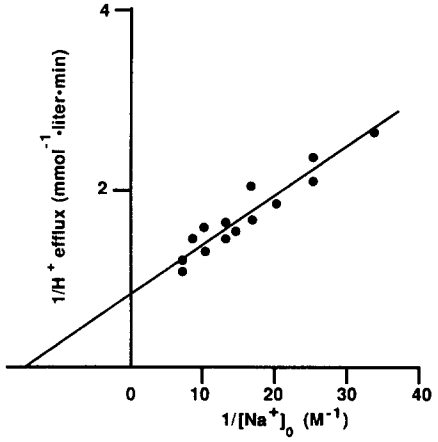

FIGURE 8. Na^+ concentration dependence of the ionomycin-induced cytoplasmic alkalinization. Thymocytes were loaded with BCECF and suspended in media with the indicated concentration of Na^+. Iso-osmolarity was maintained by substitution with NMG^+. After the baseline pH_i was established, 0.5 μM ionomycin was added. The rate of H^+ (equivalent) efflux was calculated as the product of the maximal rate of alkalinization times the buffering power (25 mmol·liter cells^{-1}·pH unit^{-1}; Grinstein et al., 1984). Data from two experiments are presented as a double-reciprocal (Lineweaver-Burk) plot. The line was calculated by least squares and had a correlation coefficient of 0.95.

to cells in Na^+ medium resulted in a pH_i increase of nearly 0.1 unit (Fig. 9*A*). A larger alkalinization (~0.2 units) was obtained with TPA alone (Fig. 9*B*). The addition of TPA to cells pretreated with ionomycin induced only a small additional alkalinization (Fig. 9*A*), reaching a pH_i comparable to that obtained with TPA alone. In contrast, when the ionophore was added to TPA-treated cells, a slight acidification was consistently recorded (Fig. 9*B*). Essentially identical results were obtained when ionomycin and hypertonicity were combined: the osmotic response was not linearly additive with that of the ionophore (Fig. 9*C*). Moreover, the addition of the ionophore in fact slightly acidified cells pretreated with hypertonic medium.

Is the effect of ionomycin due to cell shrinking? We next analyzed the possibility

that the ionomycin-induced alkalinization was mediated by cellular shrinking. This was considered because the responses to ionomycin and to osmotic shrinking are not additive and have a common mechanism, i.e., a shift in the pH_i dependence of transport (see below). Moreover, the addition of Ca^{2+} ionophores has been shown to induce cellular shrinking in other cell types (Hoffmann, 1985; Cala, 1985), which results from the loss of KCl and osmotically obliged water.

Measurements of thymocyte volume by electronic sizing indicated that shrinking was indeed occurring when cells suspended in Na^+ solution were exposed to ionomycin (Fig. 10). In 12 experiments, the cell volume decreased by 12.1 ±

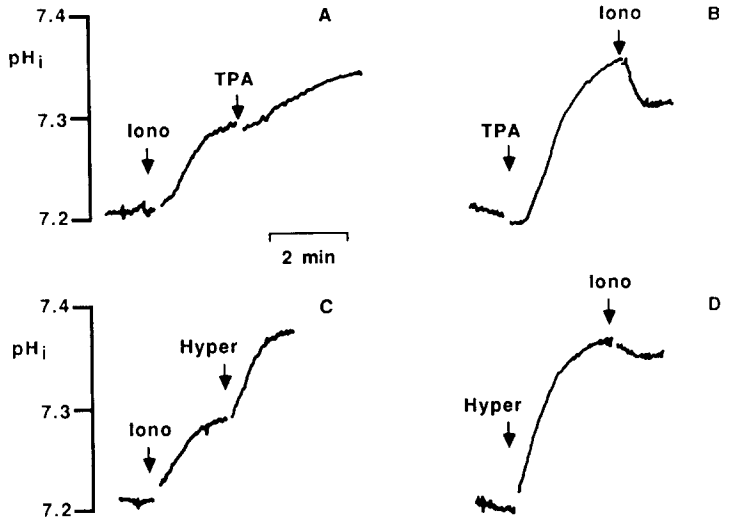

FIGURE 9. Additivity of the ionomycin-induced alkalinization with the TPA- and hypertonicity-induced effects. Thymocytes were loaded with BCECF and resuspended in Na^+ solution at 37°C, and their pH_i was monitored as described for Fig. 1. (*A*) Sequential addition of ionomycin and TPA. (*B*) Sequential addition of TPA and ionomycin. (*C*) Addition of ionomycin, followed by addition of concentrated NMG-Cl to raise the osmolarity to 550 mosM. (*D*) Sequential hypertonic stress (as in *C*), followed by addition of ionomycin. The concentrations of ionomycin and TPA were 0.5 μM and 2×10^{-8} M, respectively. Typical traces representative of three experiments.

0.4% after 10 min. No significant change was noted during this period in untreated cells. Consistent with these observations, the K^+ content of ionomycin-treated cells decreased by 14.4 ± 1.2% after 10 min. Therefore, the activation of Na^+/H^+ exchange by the ionophore could conceivably be secondary to cell shrinking. To test this possibility, cells were exposed to media made hypertonic by addition of 25 mM NMG-Cl, which was empirically found to produce a degree of shrinking comparable to that attained with ionomycin in isotonic Na^+ medium (Fig. 10). The pH_i of these cells was then monitored and compared with that of cells shrunken by the addition of the Ca^{2+} ionophore. The results of a typical experiment are shown in Fig. 11. In agreement with earlier reports (Grinstein et al., 1985*b*), shrinking of thymocytes induced a cytoplasmic alkalinization (Fig.

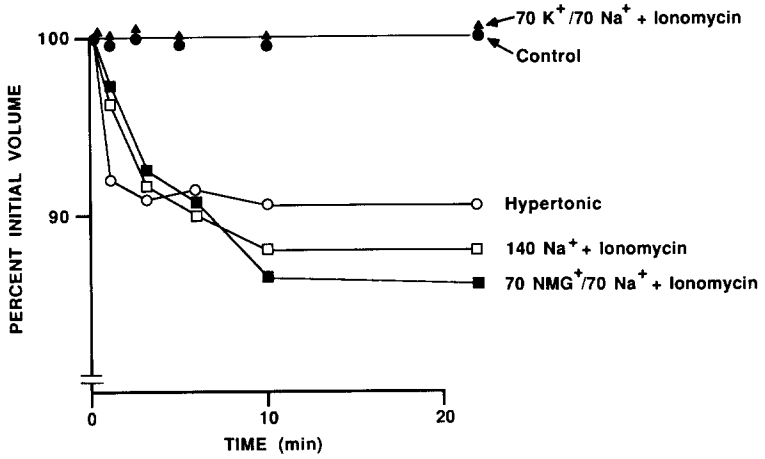

FIGURE 10. Volume changes in ionomycin-treated thymocytes. The cells were suspended in: isotonic Na^+ solution (140 mM Na^+; filled circles and open squares); isotonic mixtures containing 1 vol Na^+ solution and 1 vol of either NMG^+ solution (70 NMG^+/70 Na^+; filled squares) or K^+ solution (70 K^+/70 Na^+; triangles); or in Na^+ solution made hypertonic by the addition of 25 mM NMG-Cl (open circles). Where indicated, ionomycin (0.4 μM) was added at time zero. Cell volume was determined electronically at the indicated intervals using the Coulter Counter/ Channelyzer combination. Representative of five similar experiments.

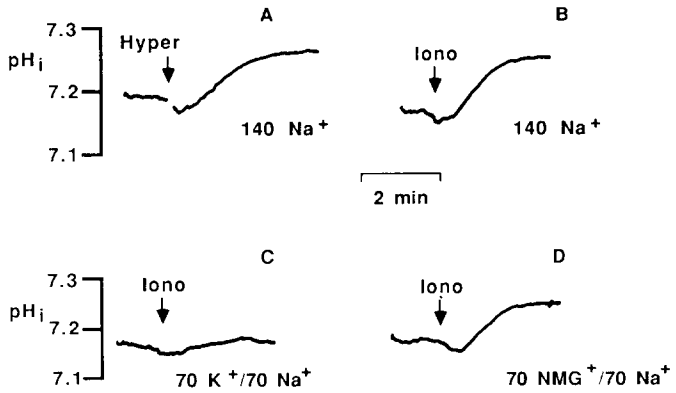

FIGURE 11. Similarity of the ionomycin-induced and osmotically induced activations of the Na^+/H^+ antiport. Thymocytes were loaded with BCECF and pH_i was measured and calibrated as described for Fig. 1. (*A*) Cells were suspended in isotonic Na^+ solution. Where indicated, the medium was made hypertonic by the addition of 25 mM NMG-Cl. (*B*) Cells were suspended in Na^+ solution and ionomycin was added at the arrow. (*C*) Cells were suspended in a medium containing 1 vol of K^+ solution and 1 vol of Na^+ solution (70 K^+/70 Na^+). Ionomycin was added where indicated. (*D*) Cells suspended in a medium containing 1 vol of NMG^+ solution and 1 vol of Na^+ solution (70 NMG^+/70 Na^+). Ionomycin was added at the arrow. The ionophore concentration in *B–D* was 0.4 μM. The traces are representative of at least three experiments.

11 *A*) that was dependent on the presence of extracellular Na^+ and was sensitive to amiloride (not shown). The magnitude of the alkalinization induced by hypertonic medium (isotonic Na^+ medium plus 25 mM NMG-Cl) was comparable to that produced by ionomycin (compare *A* and *B* of Fig. 11), the former developing somewhat more slowly. These results suggest that cellular shrinking can at least partially account for the activation of the antiport observed in ionomycin-treated cells.

To confirm the relationship between cell shrinking and the ionophore-induced activation of the antiport, cells were treated with ionomycin under conditions where shrinking does not occur. This was achieved by manipulating the combined electrochemical K-Cl gradient, which provides the driving force for the volume changes. As reported earlier for blood lymphocytes and other cells (Grinstein et al., 1982; Hoffmann, 1985), complete substitution of extracellular Na^+ by K^+ reverses the direction of the ionophore-induced volume change, because of uptake of extracellular KCl. Therefore, there must exist an intermediate range of concentrations of extracellular K^+ where the volume changes are minimal. In rat thymocytes, virtually no volume change was produced by ionomycin when the medium contained 70 mM KCl and 70 mM NaCl (Fig. 10). We therefore tested the effects of the ionophore on pH_i in this medium. As shown in Fig. 11 *C*, the alkalinization was largely eliminated under these conditions, which suggests a correlation between the activation of the antiport and the change in cell volume. It could be argued that the rate and extent of the alkalinization were reduced as a consequence of the reduction in the concentration of extracellular Na^+ (from 140 to 70 mM). This possibility was tested in Figs. 10 and 11 *D*. When a solution was prepared in which 50% of the Na^+ was replaced with the impermeant cation NMG^+, ionomycin induced a cellular shrinking comparable to that recorded in Na^+ (140 mM) solution. Under these conditions, the ionophore produced a sizable alkalinization, which was only slightly smaller than that observed in Na^+ solution. A similar result was obtained when the antiport was activated by osmotic shrinking in media containing only 70 mM Na^+ (not shown). These results are consistent with the reported K_m for extracellular Na^+ (see above) and are also thermodynamically sound: based on an intracellular Na^+ concentration of ~15 mM (Grinstein et al., 1984*a*) and a pH_o of 7.3, the operation of an electroneutral 1:1 Na^+/H^+ exchange can potentially drive the cytoplasmic pH to ≥7.9 with 70 mM external Na^+, provided the Na^+/K^+ pump keeps $[Na^+]_i$ constant, and assuming that the external medium is adequately buffered.

Intracellular pH dependence. One of the most important determinants of the rate of Na^+/H^+ exchange is pH_i. The antiport appears to have two distinct sites, the substrate and modifier sites (Aronson, 1985), which bind and are affected by intracellular H^+. Hence, $[H^+]_i$ plays a central role in regulating the rate of countertransport. For this reason, we compared the pH_i dependence of the rate of H^+ extrusion in control and ionomycin-treated cells. For these experiments, pH_o and $[Na^+]_o$ were kept constant at 7.3 and 140 mM, respectively. Internal pH was set at various levels by pretreatment with nigericin in NMG^+ solution, while the pH_i was monitored with BCECF. This was followed by removal of the antibiotic with albumin and centrifugation and resuspension of the cells in NMG^+ medium with or without ionomycin. The latter step was essential to

allow development of the ionomycin response while maintaining the acid load. Finally, the cells were suspended in Na^+ medium for the measurement of the rate of Na^+/H^+ exchange, determined as the product of the rate of amiloride-sensitive alkalinization times the buffering power (which is relatively constant in the pH_i range analyzed; Grinstein et al., 1984*a*). A typical experiment is illustrated in Fig. 12. As reported earlier for thymocytes and other cells (Moolenaar et al., 1983; Grinstein and Rothstein, 1986), the rate of Na^+/H^+ exchange is almost negligible at $pH_i \geq 7.2$, but increases sharply as the cytoplasm is acidified, following an approximately linear relationship. In ionomycin-treated cells, Na^+-induced H^+ efflux is still observable at a pH_i of 7.2 and becomes negligible only

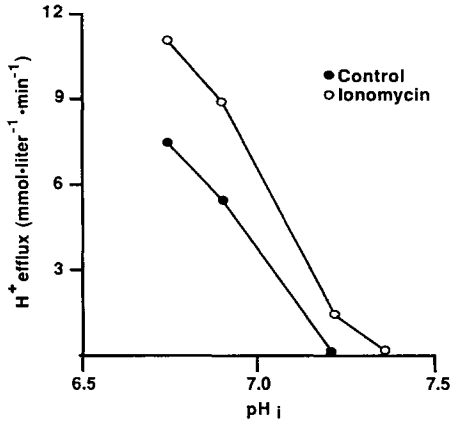

FIGURE 12. Comparison of the cytoplasmic pH (pH_i) dependence of the rate of H^+ extrusion in control (filled symbols) and ionomycin-treated (open symbols) cells. Cells stained with BCECF were acid-loaded to the pH_i levels indicated on the abscissa by incubation in NMG^+ solution with nigericin (0.2 μg/ml). Acid loading was terminated by addition of albumin (5 mg/ml, final) and centrifugation. The cells were then suspended in 100 μl of NMG^+ solution with or without ionomycin and incubated for 3 min at 37°C. This suspension was finally injected into a cuvette containing 2 ml of Na^+ solution. H^+ efflux rates were calculated from the rate of ΔpH_i as described for Fig. 8. Representative of three similar experiments.

at $pH_i \geq 7.3$, which is consistent with the final pH_i attained after addition of the ionophore to otherwise untreated cells. In the pH_i range studied, ionomycin treatment appears to have resulted in an alkaline shift of the pH_i dependence curve of the antiport. In three similar experiments, the intercept at the abscissa averaged 7.18 ± 0.02 in control cells and 7.30 ± 0.04 after ionomycin treatment. These data are consistent with the idea that cell shrinking mediates the effects of the ionophore, inasmuch as the osmotic activation of Na^+/H^+ exchange is also associated with an alkaline displacement in the pH_i dependence of the antiport (Grinstein et al., 1985*b*).

Is the effect of ionomycin mediated by protein kinase C? A shift in the pH_i sensitivity of the antiport is also characteristic of the stimulation of exchange induced by TPA and other phorbol esters (Grinstein et al., 1985*a*). The main, and perhaps the sole, cellular target for phorbol esters is the phospholipid- and

Ca^{2+}-dependent kinase, protein kinase C (Nishizuka, 1984). In vitro, this enzyme is directly activated not only by the addition of phorbol esters, but also by increasing the concentration of Ca^{2+} (Nishizuka, 1984). In some cell types, Ca^{2+} can also induce the translocation of soluble (cytoplasmic) enzyme to a particulate (membrane-bound) fraction (Kraft and Anderson, 1983). Moreover, increasing $[Ca^{2+}]_i$ can also activate cellular phospholipases, with potential release of intrinsic diacylglycerol, the putative physiological activator of protein kinase C. Because stimulation of protein kinase C results in activation of the Na^+/H^+ antiport, it is conceivable that, in addition to causing cell volume changes, ionomycin also stimulates this enzyme. To test this hypothesis, we carried out experiments to determine whether the ionophore activated phosphorylation in intact cells. For this purpose, cells were preloaded with [^{32}P]orthophosphate to label the nucleotide pool and then exposed to ionomycin for 5 min, a period that suffices for full activation of the transport response. The cells were then sedimented and lysed, and a membrane-rich fraction was isolated as described in the Methods. The membrane polypeptides were analyzed by polyacrylamide gel electrophoresis and, after silver-staining, the gels were dried and used for autoradiography. For comparison, untreated samples and samples from cells stimulated by TPA were also analyzed. Typical results are illustrated in Fig. 13. As reported earlier, TPA increased the phosphorylation of several membrane polypeptides, notably those of M_r ~65,000 and 60,000 (arrows in Fig. 13*F*) and a group of smaller bands of M_r 50,000–55,000. In contrast, only slight changes in phosphorylation were found in membranes from ionomycin-treated cells (Fig. 13*D*). A small increase in the M_r 60,000 and 65,000 polypeptides was the only significant change observed. These data indicate that, in intact thymocytes, the concentration of ionomycin required to change pH_i stimulated protein kinase C only marginally.

The possible activation of protein kinase C by ionomycin was also investigated by measuring the subcellular distribution of the enzyme in control and stimulated cells. When most resting cells are homogenized and fractionated, protein kinase activity is recovered predominantly in the soluble fraction. However, if the cells are activated by phorbol esters (Kraft and Anderson, 1983), or by physiological stimuli believed to increase diacylglycerol levels (Guy et al., 1986) before homogenization, the distribution of the kinase is altered, with most of the activity being recovered in the particulate fraction. This apparent translocation of protein kinase C, currently interpreted as binding to membranes, provides an additional means of determining whether the Ca^{2+} ionophore activated this enzyme. For this purpose, cells were homogenized before and 5 min after the addition of 0.5 μM ionomycin or 0.1 μM TPA. The latter was used to ascertain that migration of the kinase would be detectable under our experimental conditions. Protein kinase activity was then determined in the soluble and particulate fractions, after solubilization of the latter with Triton X-100. As reported earlier for several cell types (Kraft and Anderson, 1983; Guy et al., 1986; Melloni et al., 1986), treatment of rat thymocytes with TPA drastically reduced the total and particularly the phospholipid-dependent protein kinase (C kinase) activity of the soluble fraction (Table II). In contrast, no significant changes in the soluble kinase activity were observed in ionomycin-treated cells, irrespective of the presence or

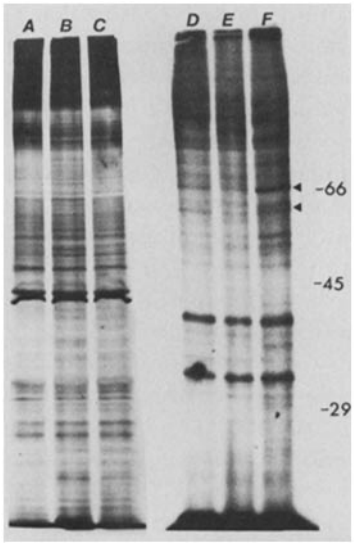

FIGURE 13. Effects of ionomycin and TPA on membrane protein phosphorylation. Thymocytes were preloaded with [^{32}P]orthophosphate for 30 min at 37°C. The cells were then incubated for 5 min in Na^+ solution with the following additions: none (lanes *B* and *E*), ionomycin (lanes *A* and *D*), and TPA (lanes *C* and *F*). The reaction was terminated by centrifugation and homogenization of the cells in ice-cold lysis buffer. A membrane-rich fraction, obtained by differential centrifugation, was analyzed by polyacrylamide (10%) gel electrophoresis. The gel was silver-stained, photographed (lanes *A–C*), and then dried and used for autoradiography (lanes *D–F*). Molecular weights, in kilodaltons, were calculated by comparison with standards (SDS-6H, Sigma Chemical Co.). The arrowheads indicate polypeptides of ~60 and 66 kD with increased labeling (see text). The gel is representative of three similar experiments.

TABLE II

Effects of TPA and Ionomycin on Soluble Protein Kinase C

	− PS	+ PS	ΔPS
	nmol $P_i \cdot min^{-1} \cdot mg\ protein^{-1}$		
Control	1.47±0.09	2.06±0.04	0.59
TPA	0.52±0.09	0.54±0.17	0.02
Ionomycin	1.36±0.05	2.15±0.10	0.79

Thymocytes were incubated with or without TPA (2×10^{-7} M) or ionomycin (0.5 μM) for 5 min at 37°C. The cells were then homogenized and sedimented, and the histone-kinase activity of the soluble fraction was assayed in the presence (+ PS) and absence (− PS) of phosphatidylserine as described in the Methods. ΔPS refers to the difference between these conditions. The data are means ± 1 SE of at least three experiments, each with triplicate determinations. Phosphotransferase activity is reported as nanomoles of inorganic phosphate incorporated into the trichloroacetic acid–insoluble fraction per milligram of cellular protein per minute.

absence of phospholipids during the assay. That the decrease in soluble kinase C induced by TPA reflects migration to the membrane is suggested by measurements of activity in the particulate fraction. The kinase activity of the particulate fraction was substantially increased in phorbol ester–treated samples (0.738 ± 0.092 nmol·min^{-1}·mg protein^{-1}) compared with the controls (0.319 ± 0.046 nmol·min^{-1}·mg protein^{-1}). In agreement with the data in Table II, the kinase activity of the particulate fraction from ionomycin-treated cells (0.391 ± 0.051 nmol·min^{-1}·mg protein^{-1}) was not significantly different from the control. Taken together, these results indicate that the activation of protein kinase C, as measured by in situ phosphorylation and by its migration from the soluble to the particulate fraction, was significant when TPA, but not ionomycin, was added.

The lack of activation of protein kinase C by ionomycin suggests that this enzyme is not involved in the Ca^{2+}-induced stimulation of the Na^+/H^+ antiport.

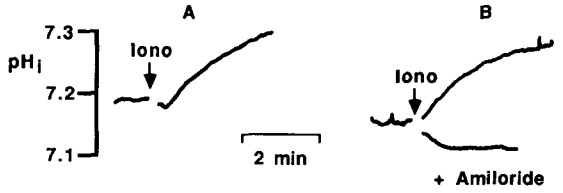

FIGURE 14. Effect of ionomycin on pH_i in control and protein kinase C–depleted thymocytes. Cells were preincubated in HEPES-buffered medium RPMI 1640 for 24 h at 37°C in the absence (*A*) or presence (*B*) of 2×10^{-7} M TPA. The thymocytes were then washed, resuspended, and loaded with BCECF as described above. After one additional wash and resuspension, the cells were used for pH_i determination in Na^+ solution. Where indicated, 0.1 μM ionomycin was added. In one instance (bottom trace in *B*), amiloride (200 μM) was present throughout the pH_i recording period. Representative of three experiments.

More conclusive evidence confirming this view was obtained using protein kinase C–depleted cells. It has been reported that prolonged incubation of fibroblasts with high concentrations of phorbol esters causes a decrease in the number of phorbol ester–binding sites (Collins and Rozengurt, 1984) and in the activity of protein kinase C (Rodriguez-Peña and Rozengurt, 1984). In addition, the cells become unresponsive to the effects normally elicited by phorbol esters in untreated cells, such as the increased ^{86}Rb uptake and pH_i (Vara and Rozengurt, 1985). A similar depletion has been reported in the case of rat thymocytes: overnight incubation with 2×10^{-7} M TPA decreased protein kinase C activity by ~90% and rendered the cells functionally unresponsive to the phorbol ester (Grinstein et al., 1986*b*). These protein kinase C–depleted cells provide a useful model to test the involvement of this enzyme in the ionomycin-induced response of the Na^+/H^+ antiport. A typical experiment is shown in Fig. 14. Cells incubated in the absence of TPA for 24 h respond normally to the addition of ionomycin (Fig. 14*A*). In fact, for reasons that are presently not clear, lower concentrations of the ionophore were usually required to elicit the response in these cells. As reported earlier, the pH_i of thymocytes treated with TPA was ~0.1 unit lower than that of untreated controls, which indicates that the phorbol ester–induced

alkalinization observed in acute experiments did not persist after 24 h. This observation is consistent with the disappearance of protein kinase C. The addition of ionomycin to protein kinase C–depleted cells produced an alkalinization that was indistinguishable from that observed in control cells. The alkalinization was abolished by amiloride (Fig. 14*B*) or by the omission of Na^+ from the medium (not shown), which indicates mediation by the antiport. These results indicate that the stimulation of the Na^+/H^+ exchanger by ionomycin can be accomplished in cells largely devoid of protein kinase C, which is therefore unlikely to play a major role in the Ca^{2+}-dependent activation process. We have previously reported (Grinstein et al., 1986*b*) that down-regulation of protein kinase C does not impair the osmotically induced stimulation of Na^+/H^+ exchange. Therefore, the findings presented here are compatible with a role of cell shrinking in the mediation of the response to ionomycin, which also appears to be independent of the kinase.

DISCUSSION

The relationship between $[Ca^{2+}]_i$ and pH_i has been studied in a variety of biological systems by several techniques (reviewed by Busa and Nuccitelli, 1984). In most systems, including snail neurons, Purkinje fibers, and whole rat hearts, increasing $[Ca^{2+}]_i$ lowered pH_i by 0.1–0.3 units. A similar effect was found by Moolenaar et al. (1983) in human foreskin fibroblasts treated with the ionophore A23187. However, Muldoon et al. (1985) and Hesketh et al. (1985) reported that A23187 induced an alkalinization in the same cells. Thus, it is possible that increased $[Ca^{2+}]_i$ elicits multiple responses and that the predominant effect observed depends on the particular conditions used.

Hesketh et al. (1985) reported a small biphasic pH_i change in response to A23187 in murine thymic lymphocytes. In contrast, two earlier reports (Rink et al., 1982; Grinstein et al., 1984*a*) did not detect any effect of Ca^{2+} ionophores on pH_i in other lymphoid cells. In these cases, the failure to detect pH_i changes may have been due to the offsetting influences of acidifying and alkalinizing processes, or to the low ionophore concentrations and temperatures used, respectively (see below). The existence of at least two antagonistic processes was evidenced in the present studies: in Na^+-containing media, a small but distinct alkalinization was observed. In the presence of amiloride or in solutions devoid of Na^+, a significant intracellular acidification was unmasked (Fig. 1). In addition, a trivial cytoplasmic alkalinization was observed when high concentrations of the ionophore were employed. This cytoplasmic alkalinization was not blocked by amiloride and probably reflects $Ca^{2+}/2H^+$ exchange through the ionophore (Kauffman et al., 1980).

Mechanism of Cytoplasmic Acidification

Three possible mechanisms for cytoplasmic acidification can be envisaged: release of H^+ equivalents from intracellular stores, production of metabolic acid, and uptake of extracellular H^+ equivalents. The release of H^+ from intracellular membrane-bound compartments into the cytoplasm is unlikely to account for the observed drop in pH_i, inasmuch as the acidification was largely dependent on extracellular Ca^{2+}. Increased metabolic acidification can result from stimula-

tion of the Ca^{2+} ATPase, because of elevated $[Ca^{2+}]_i$, or from mitochondrial uncoupling. Some contribution of the latter pathway is suggested by the moderate cytoplasmic acidification observed in depolarized cells upon addition of CCCP, valinomycin,[4] or the Ca^{2+} ionophore (Figs. 5 and 6). However, mitochondrial uncoupling by the divalent cation ionophore cannot entirely account for the observed results, since ionomycin produced an even larger acidification in cells previously uncoupled with CCCP (Fig. 6), which indicates the existence of an additional process.

A major fraction of the ionomycin effect is sensitive to the plasma membrane potential (Fig. 6) and must therefore be associated with transmembrane H^+ fluxes. The likely mechanism underlying most of the acidification is an increased H^+ influx in response to the hyperpolarization resulting from the opening of Ca^{2+}-induced K^+ channels. This interpretation is supported by the following evidence: (*a*) ionomycin induced a Ca^{2+}-dependent hyperpolarization (Fig. 4); (*b*) cytoplasmic acidification was also recorded upon addition of valinomycin, a hyperpolarizing K^+ ionophore (Fig. 5); (*c*) the effects of both ionomycin and valinomycin were magnified by pretreatment with CCCP, a conductive protonophore; and (*d*) the acidification induced by ionomycin and valinomycin, in the presence or absence of CCCP, was markedly reduced by depolarizing the cells with high-K^+ solutions and also by means of gramicidin, an ionophore that nonselectively increases Na^+ and K^+ conductance (Figs. 5 and 6).

These results are consistent with an increased H^+ influx driven by the hyperpolarization and imply that the resting cell has a finite conductance to H^+ (equivalents). Moreover, in addition to hyperpolarizing the cells, the elevation of $[Ca^{2+}]_i$ could also conceivably increase the conductive permeability to H^+. However, this possibility could be ruled out using valinomycin. Cells pretreated with this K^+-specific ionophore failed to acidify further when subsequently treated with ionomycin. In contrast, the addition of CCCP after valinomycin resulted in a pronounced secondary acidification (not illustrated).

In summary, these results indicate that the acidification induced by divalent cation ionophores has at least two components: an increase in the rate of metabolic acid production and a potential-driven uptake of extracellular H^+. The simultaneous existence of these components explains why only small and variable changes in extracellular pH were recorded when cells were treated with ionomycin in Na^+-free media or in the presence of amiloride (not illustrated).

Mechanism of Cytoplasmic Alkalinization

Several lines of evidence indicated that the increase in pH_i recorded at submicromolar concentrations of ionomycin is due to Na^+/H^+ exchange: (*a*) the effect is accompanied by extracellular acidification; (*b*) it requires the presence of extracellular Na^+ and is associated with a gain in cellular Na^+ content; and (*c*) all of these manifestations are prevented by concentrations of amiloride ≤ 200 μM.

The consequences of increasing $[Ca^{2+}]_i$ on the rate of operation of the antiport

[4] The residual acidifying effect of CCCP or valinomycin in K^+ solution could also be due to incomplete depolarization of the cells. This could result from activation of an electrogenic Na^+-K^+ pump or from the contribution of E_{Cl} to the membrane potential.

have been studied before in intact cells and in isolated membranes. No effects were seen in the case of renal brush border vesicles, where the activity of the antiport was assayed by measuring Na^+ fluxes (Aronson et al., 1982). In intact cells, pH_i measurements suggested little or no effect in some cell types (Moolenaar et al., 1981; Frelin et al., 1983), but clear stimulation was reported in others (Muldoon et al., 1985; Villereal et al., 1985). Because pH_i is affected by a variety of factors, it is conceivable that activation (whether direct or indirectly caused by cell shrinking) occurred in every instance, but was in some cases obscured by a concomitant acidifying process, such as the one described above. Alternatively, differences in the experimental conditions may have been responsible for the discrepancies.

Stimulation of protein kinase C has been shown to activate Na^+/H^+ exchange in thymic lymphocytes (Grinstein et al., 1985*a*). Because this enzyme can be activated in vitro by raising [Ca^{2+}] (Nishizuka, 1984), we considered the possibility that ionomycin was stimulating the antiport via a Ca^{2+}-mediated activation of protein kinase C. Indeed, the effects of the ionophore and of TPA were not additive, which is consistent with a common pathway of activation. However, the following evidence suggests that the stimulation of protein kinase C plays a minor role, if any, in the $[Ca^{2+}]_i$-induced Na^+/H^+ exchange: (*a*) phosphorylation, measured in situ, was stimulated markedly by phorbol ester, but only marginally by ionomycin (Fig. 13), and (*b*) the migration of soluble C kinase to the membrane, an independent criterion of activation of the enzyme, was negligible in ionomycin-treated cells. By comparison, TPA induced nearly quantitative translocation of the kinase (Table II). Ionomycin stimulated the antiport in cells pretreated with TPA for 24 h. The prolonged treatment with the phorbol ester was shown to result in down-regulation of total protein kinase C, which implies that this enzyme is not essential to obtain the response to elevated $[Ca^{2+}]_i$. However, it cannot be ruled out that, because of synergy with Ca^{2+}, small amounts of residual protein kinase C are nevertheless involved in the response.

The data presented in Figs. 10 and 11 suggest that the stimulation of the Na^+/H^+ antiport observed in response to an elevation of $[Ca^{2+}]_i$ may be secondary to cell shrinking. The evidence can be summarized as follows. First, treatment of thymocytes with ionomycin induced cell shrinking. This is probably the result of a Ca^{2+}-induced increase in K^+ and perhaps also Cl^- permeability, as demonstrated for Ehrlich ascites cells and other types of lymphocytes (Hoffmann et al., 1984; Hoffmann, 1985; Grinstein et al., 1982). Second, shrinking the cells to a comparable degree by subjecting them to mildly hypertonic media resulted in a comparable alkalinization.[5] Third, both osmotic shrinking and the ionophore-induced stimulations of Na^+/H^+ exchange can be accounted for by a shift in the

[5] It is important to point out that activation of Na^+/H^+ exchange in lymphocytes can be obtained by direct cell shrinking in hypertonic media. Under these conditions, relatively little regulatory volume increase is observed, since the antiport rapidly becomes quiescent as pH_i increases and reaches the new "set point." Reswelling under these conditions is minimal, since the amount of H^+ (and therefore of Na^+) required to overcome the buffering power of the cell contributes relatively little to the total cellular osmolarity (see Grinstein et al., 1985*b*, for further discussion).

pH_i dependence of the antiport. Fourth, preventing the volume change by partial replacement of extracellular Na^+ by K^+ largely inhibited the activation of the antiport. This effect was not due to a decreased substrate (Na^+) concentration, since partial replacement with NMG^+, which does not affect the volume change, failed to inhibit the response to ionomycin. Taken together, these results suggest that at least part of the activation of the antiport is the result of cell shrinking. However, the possible contribution of other pathways cannot be entirely ruled out. In this regard, Villereal and co-workers (1985) have suggested that a mechanism involving calmodulin is responsible for the stimulation of the antiport by A23187. This conclusion was based on the inhibitory effect of a variety of calmodulin antagonists. However, it is conceivable that cell shrinking was also occurring in those experiments and that the antagonists prevented the volume change, as they have been reported to do in other cell types (Grinstein et al., 1982; Hoffmann et al., 1984).

We had earlier reported (Grinstein et al., 1984*a*) that little change in pH_i was observed when rat thymocytes were treated with ionomycin at room temperature. The failure to detect any effects in those experiments could be due to the compensating effects of acidifying and alkalinizing components. Alternatively, and perhaps more likely, the responses may have been absent or minimal at room temperature. Cell volume measurements demonstrated that ionomycin-induced shrinking was markedly slowed at 20°C (not illustrated).

In summary, the elevation of $[Ca^{2+}]_i$ in rat thymic lymphocytes by means of ionophores results in elevated metabolic H^+ production and increased H^+ uptake, as a consequence of hyperpolarization. Under normal conditions, these changes are overcome by a stimulated Na^+/H^+ antiport, which renders the cytoplasmic compartment alkaline. When the latter system is eliminated by removal of external Na^+ or by addition of amiloride, the cytoplasm becomes acidic in response to increased H^+ uptake and production. In all likelihood, the relative contributions of the acidifying and alkalinizing components will vary in different cell types, which would account for the reported inconsistency in the pattern of response to the Ca^{2+} ionophores.

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