Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1429–1433, March 1985
Cell Biology

Characterization of the activation of Na^+/H^+ exchange in lymphocytes by phorbol esters: Change in cytoplasmic pH dependence of the antiport

(phorbol 12-myristate 13-acetate/tumor promoter/pH regulation/Na^+ transport)

S. GRINSTEIN*, S. COHEN*, J. D. GOETZ*, A. ROTHSTEIN*, AND E. W. GELFAND†

Departments of *Cell Biology and †Immunology, Research Institute, Hospital for Sick Children and Department of Biochemistry, University of Toronto, 555 University Avenue, Toronto, ON, Canada M5G 1X8

Communicated by Gerhard Giebisch, October 22, 1984

ABSTRACT **Phorbol 12-myristate 13-acetate and other phorbol esters induce an intracellular alkalinization in rat thymic lymphocytes. An extracellular acidification can be recorded concomitantly. This transmembrane H^+ (equivalent) flux is dependent on external Na^+ and is amiloride sensitive. Phorbol esters also stimulate an amiloride-sensitive uptake of $^{22}Na^+$, suggesting activation of Na_o^+/H_i^+ exchange. Only those phorbol derivatives that are tumor promoters and activators of protein kinase C stimulate the antiport. Activation of the Na^+/H^+ exchange is brought about by a change in the cytoplasmic pH sensitivity of the antiport. Activation of the Na^+/H^+ exchanger by phorbol esters results in membrane hyperpolarization, due to indirect stimulation of the electrogenic Na^+/K^+ pump by the increased intracellular Na^+ concentration. Increased Na^+/H^+ exchange also produces cell swelling, which may be one of the earliest manifestations of the growth-promoting properties of the phorbol esters.**

A Na^+/H^+ antiport was recently detected in the plasma membranes of peripheral blood and thymic lymphocytes (1, 2). This exchanger, which is inhibited by amiloride, is thought to play a major role in the regulation of cellular volume (1) and in cytoplasmic pH (pH_i) homeostasis (1, 2). In other cell types, similar antiports are activated by growth factors (3–5), suggesting their role in mitogenesis. Because a number of growth-factor receptors display protein kinase activity upon ligand binding (6, 7), it is conceivable that activation of Na^+/H^+ exchange occurs through phosphorylation of the antiport or of an ancillary protein. That the level of phosphorylation could regulate the rate of Na^+/H^+ exchange is also suggested by the finding that vanadate, an inhibitor of phosphatases, activates Na^+/H^+ countertransport in A431 cells (8).

Phosphorylation of membrane proteins can also result from activation of protein kinase C, a ubiquitous Ca^{2+}- and phospholipid-requiring enzyme (9, 10). Protein kinase C is activated by diacylglycerol, a product of the hydrolysis of inositol phospholipids, or by the tumor-promoting phorbol diesters, which are structural analogs of diacylglycerol (9, 10). Recently, phorbol esters were shown to increase Na^+ uptake and pH_i in cultured cells (11, 12), suggesting stimulation of Na^+/H^+ exchange. The present paper reports the stimulation of the Na^+/H^+ antiport of normal rat thymic lymphocytes by activators of protein kinase C and describes experiments analyzing the underlying mechanism. Alterations in membrane potential and cellular volume were also found to be associated with activation of Na^+/H^+ exchange. The significance of these changes and the Ca^{2+} dependence of the stimulation of the antiport were also investigated.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

EXPERIMENTAL PROCEDURES

Materials. Phorbol and phorbol esters were purchased from Sigma. $^{22}Na^+$ was from Amersham. Bis(1,3-diethylthiobarbituric)trimethine oxonol (bis-oxonol) was from Molecular Probes (Junction City, OR). Amiloride was the gift of Merck Sharp & Dohme. Quin-2 acetoxymethyl ester was the gift of T. J. Rink (Cambridge University). Ionomycin was the gift of Squibb. Trifluoperazine was a gift from Smith Kline & French. The tetracetoxymethyl ester of bis(carboxyethyl)carboxyfluorescein (BCECF) was synthesized by M. Ramjeesingh (Hospital for Sick Children, Toronto). RPMI-1640 medium (with or without HCO_3^-) was purchased from GIBCO. Na^+ solution was 140 mM NaCl/1 mM KCl/1 mM $CaCl_2$/1 mM $MgCl_2$/10 mM glucose/20 mM Tris/2-(*N*-morpholino)ethanesulfonic acid, pH 7.2. Where indicated, $CaCl_2$ was omitted. K^+ solution and *N*-methylglucamine$^+$ solution were prepared by isosmotic replacement of Na^+ by K^+ or *N*-methylglucamine$^+$ but were otherwise identical.

Methods. Rat thymocytes were isolated as described (2) and maintained in nominally HCO_3^--free RPMI-1640 medium buffered with 20 mM Hepes. Cell sizing and counting were done in a Coulter Counter with attached Channelyzer as described (1). pH_i was determined fluorimetrically using BCECF as described (2). Where indicated, pH_i was manipulated by means of nigericin in *N*-methylglucamine$^+$ solution essentially as described earlier (2). Membrane potential was measured with bis-oxonol (0.3 μM final concentration) by the method of Rink *et al.* (13) using 10^6 cells per ml. Free cytosolic Ca^{2+} concentration was estimated using quin-2 as described by Tsien *et al.* (14), calibrating with ionomycin and Mn^{2+} (15). The rate of acidification of lightly buffered medium was measured as described (2). Uptake of ^{22}Na was measured by sedimentation through oil as described (16). All the experiments were performed at 37°C. Data are presented as the mean ± SEM of *n* experiments or as representative traces.

RESULTS

Effect of Phorbol Esters on pH_i. The resting pH_i of thymocytes in Na^+ solution (Na_o^+ = 140 mM) at 37°C was 7.23 ± 0.017 (n = 11). As shown in Fig. 1*A*, addition of phorbol 12-myristate 13-acetate (PMA) to these cells induces a sizable alkalinization, which becomes apparent ≈45 sec after addition of the phorbol ester. In 11 experiments using 10 nM PMA, the final pH_i (measured 6–8 min after addition of the phorbol ester) averaged 7.42 ± 0.03. As shown in Fig. 1*B*, this cytoplasmic alkalinization is accompanied by the appearance of proton equivalents in the external medium, mea-

Abbreviations: PMA, phorbol 12-myristate 13-acetate; BCECF, bis-(carboxyethyl)carboxyfluorescein.

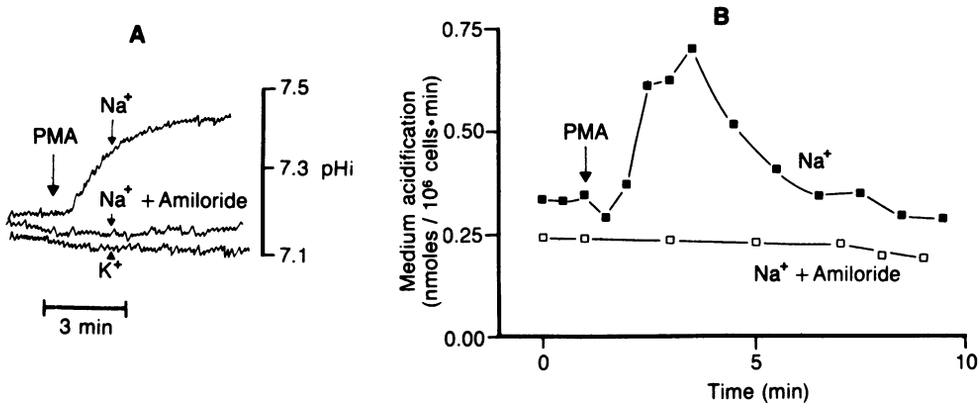

FIG. 1. (*A*) Effect of PMA on cytoplasmic pH (pH_i). BCECF-loaded thymocytes (3×10^6 cells per ml) were suspended in either K^+ solution (Na^+-free) or in Na^+ solution (Na_o = 140 mM) with or without 100 μM amiloride. Where indicated, 10 nM PMA was added to all the samples and recording was resumed. The pH_i values were calculated by calibration with nigericin/K^+ as described (2). The figure is a composite of representative traces from at least four similar experiments. Temperature, 37°C. (*B*) PMA-induced extracellular acidification. Thymocytes (5×10^7 per ml) were resuspended in unbuffered Na^+ medium (Na_o = 140 mM) with or without 100 μM amiloride. The pH_i of this suspension (buffered only by the Hepes carried over with the pellet) was measured under constant magnetic stirring with an Orion 601A Ionalyzer attached to an X vs. time recorder, while keeping the temperature at 37°C in a water-jacketed chamber. Where indicated, 100 nM PMA was added to the samples. The buffering capacity of the medium, used to estimate the rate of medium acidification (ordinate), was determined at the end of each experiment by titration with KOH or HCl. Extracellular pH was maintained between 7.3 and 7.0 by addition of KOH. Representative of three experiments.

surable as an acidification in poorly buffered Na^+ solution. As shown in Fig. 1*A*, a lag period follows addition of PMA and maximal rates of medium acidification are observed after 2–3 min. Moreover, the amount of acid appearing in the medium, calculated as the area under the peak in Fig. 1*B*, is similar to that leaving the cells, calculated as the product of the PMA-induced ΔpH and the cellular buffering power (25 $mmol \cdot liter^{-1} \cdot pH^{-1}$; see ref. 2). In three experiments, the former averaged 0.482 ± 0.02 nmol per 10^6 cells, whereas the latter was 0.542 nmol per 10^6 cells (calculated from the average ΔpH reported above for 11 determinations and using a volume of 114 μm^3 per cell, determined by electronic sizing). These data indicate that the PMA-induced alkalinization results from the outward transmembrane transport of internal proton (H_i^+) equivalents.

The phorbol ester-induced change in pH_i is strictly dependent on the presence of extracellular Na^+ (Na_o^+). PMA had no significant effect on pH_i in cells suspended in K^+ solution (Fig. 1*A*) or *N*-methylglucamine$^+$ solution (not illustrated). Similarly, the acidification of the external medium was absent in these media. The Na^+ dependence of the PMA-induced alkalinization suggests that exchange of Na_o^+ for H_i^+ is involved. Amiloride, an inhibitor of Na^+/H^+ exchange in thymocytes (2) and other cells (17), was used to test this hypothesis. As shown in Fig. 1*A*, a concentration of the diuretic known to inhibit Na^+/H^+ exchange in these cells (2) completely abolished the effect of the phorbol ester on pH_i. The acidification of the external medium was similarly inhibited (Fig. 1*B*).

Effect of Phorbol Esters on Na^+ Uptake. Additional evidence for the involvement of Na_o^+/H_i^+ exchange was obtained by measuring the effects of PMA on isotopic Na^+ uptake. As reported elsewhere (16), the fluxes of $^{22}Na^+$ in resting (unstimulated) thymocytes are comparatively rapid (see below). To prevent equilibration of the isotope during the lag period required for expression of the maximal PMA effect, the cells were incubated with the phorbol ester in Na^+-free K^+ solution for 5 min at 37°C. This was followed by a brief (1 min) incubation in $^{22}Na^+$-containing (Na_o^+ = 14 mM) solution in the presence or absence of amiloride (100 μM). The results of these experiments are shown in Table 1. Previous treatment with PMA increased the rate of $^{22}Na^+$ uptake nearly 4-fold. As expected, the phorbol ester-induced stimulation was almost entirely prevented by amiloride. These data strongly support the hypothesis that PMA activates Na_o^+/H_i^+ exchange in thymocytes.

Concentration Dependence and Specificity of Phorbol Ester Action. The concentration dependence of the effect of PMA was analyzed in the experiments summarized in Fig. 2. The maximal rate of cytoplasmic alkalinization was determined in BCECF-loaded cells in Na^+ solution at various concentrations of the phorbol ester (circles). Half-maximal effects were obtained at 0.4 nM PMA, which is similar to the apparent binding constant of this ester in blood lymphocytes (19) and other cells (20) and to the concentration producing half-maximal activation of protein kinase C (for review, see ref. 9). As shown in Fig. 2 (triangle), even supramaximal concentrations of PMA failed to affect pH_i when amiloride (100 μM) was also present.

In addition to PMA, a number of other 4β-phorbol diesters have also been reported to activate protein kinase C *in vitro* and to promote tumor growth *in vivo* (20). These include 4β-phorbol dibenzoate, 4β-phorbol didecanoate, and 4β-phorbol dibutyrate. As shown in Table 2, all of these diesters elicited a cytoplasmic alkalinization, which at the appropriate concentrations was comparable to the maximal PMA effect in terms of both rate and extent. Half-maximal effects were observed at concentrations that closely resemble the binding constants of these diesters to their receptor, which is presumably protein kinase C (20). In all cases, amiloride inhibited the change in pH_i. In contrast to the 4β diesters, un-

Table 1. Effect of PMA on $^{22}Na^+$ uptake in rat thymic lymphocytes

Condition	Amiloride, μM	Na^+ uptake, pmol per 10^6 cells·min^{-1}	*n*
Control	0	83.6 ± 5.8	10
	100	23.5 ± 1.1	10
PMA	0	321.6 ± 7.4	10
	100	38.6 ± 2.9	9

Thymocytes ($20–25 \times 10^6$ per ml) were preincubated in K^+ solution with or without 100 nM PMA for 5 min at 37°C. After sedimentation, the initial rate of $^{22}Na^+$ uptake was measured in a medium containing 9 vol of K^+ solution and 1 vol of Na^+ solution (Na_o^+ = 14 mM) and 10–20 μCi of $^{22}Na^+$ per ml (1 Ci = 37 GBq), with or without 100 μM amiloride. Data are means ± SEM of the number of determinations (*n*) indicated.

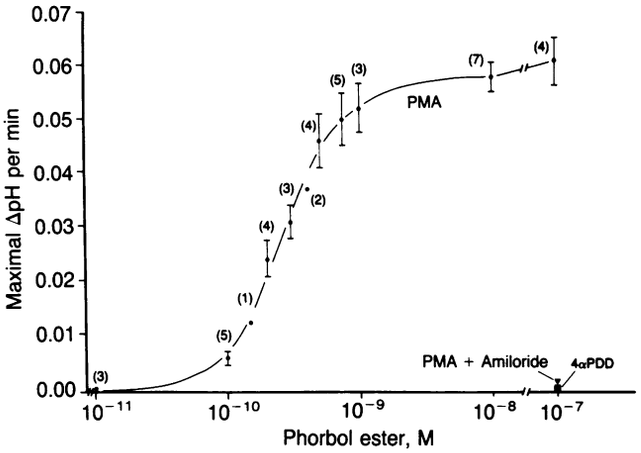

FIG. 2. Concentration dependence of the effect of phorbol esters on pH_i. BCECF-loaded thymocytes (3×10^6 per ml) were suspended in Na^+ medium and pH_i was measured fluorimetrically as described (2). The maximal rate of change of pH_i (ordinate) is plotted as a function of the concentration of phorbol ester added (in mol/liter). ●, PMA; ▼, PMA added in the presence of 100 μM amiloride; ■, 4α-phorbol 12,13-didecanoate (4αPDD). Bars represent SEM of the number of experiments indicated in parentheses.

esterified 4β- and 4α-phorbol as well as 4α-phorbol didecanoate had no effect on pH_i even at high concentrations (Fig. 2; Table 2). These analogs are not activators of protein kinase C (18), have no tumor-promoting activity (20), and do not affect the binding of biologically active diesters to their receptor(s) (20). These results suggest that activation of protein kinase C may be involved in the stimulation of Na^+/H^+ exchange by phorbol esters.

Mechanism of Activation of Na^+/H^+ Exchange. The mechanism of activation of Na^+/H^+ exchange by PMA was studied in BCECF-loaded cells. Because the K_m for Na_o^+ in the absence of PMA is ≈51 mM (2), it is unlikely that phorbol esters activate exchange in Na^+ solution (140 mM Na_o^+) by increasing the affinity for this cation. Indeed, preliminary experiments indicate that this parameter is not affected by PMA (unpublished observations). Instead, as shown in Fig. 3, the phorbol esters seem to alter the pH_i sensitivity of the antiport. In these experiments, pH_i was clamped at various levels by pretreatment with nigericin in *N*-methylglucamine$^+$ solution, followed by removal of the antibiotic with albumin and centrifugation (see ref. 2 for details). The activity of the Na^+/H^+ antiport was then assessed upon resuspension of the cells in Na^+-containing solution as the product of the amiloride-sensitive rate of change of pH_i times the buffering power (which is relatively constant in the pH_i range analyzed; ref. 2). A typical experiment is illustrated in Fig. 3. As reported earlier (2), the rate of H^+ extrusion is critically dependent on pH_i. In untreated cells at 37°C, amiloride-sensitive H^+ efflux is undetectable at $pH_i \geq 7.2$, but it increases sharply at lower pH_i. The increase is entirely amiloride sensitive (Fig. 3). In PMA-treated cells, Na^+-induced H^+ efflux is still observable at pH_i 7.2 and becomes negligible only at $pH_i \geq 7.35$, consistent with the final pH_i attained after addition of the phorbol ester (see above and Fig. 1). In the pH_i range studied, PMA treatment appears to have resulted in an alkaline shift of the pH_i-dependence curve of the antiport. Amiloride also completely inhibited exchange in acid-loaded PMA-treated cells (Fig. 3).

Effect of Phorbol Esters on Cell Volume. In osmotically shrunken mammalian lymphocytes (1) and amphibian red cells (21), activation of Na^+/H^+ exchange results in cell swelling. This is partly due to the buffering power of the cytoplasm, which replaces extruded H^+ with consequent osmotic (Na^+) gain. Swelling also occurs as a result of Cl^- uptake, which is accumulated in exchange for HCO_3^-. The intracellular concentration of the latter increases with cytoplasmic alkalinization. Even though the anion exchange *per se* is osmotically inactive, the extruded HCO_3^- is rapidly regenerated by diffusion of CO_2, formation of H_2CO_3, and efflux of H^+ through the cation antiport, resulting in net Cl^- gain. Accordingly, it can be predicted that activation of the Na^+/H^+ antiport by PMA ought to increase cellular volume and that swelling should be more noticeable in HCO_3^--containing medium. A summary of experiments designed to test this prediction is shown in Fig. 4. Addition of PMA to cells in HCO_3^--containing medium consistently produced cell swelling, detectable 2–5 min after addition of the ester. Maximal swelling (12%–14% of starting volume) was achieved after 8–10 min, and cell volume remained constant thereafter for up to 3 hr. As shown in Fig. 4, swelling was considerably diminished in nominally HCO_3^--free solution and was largely eliminated by amiloride, which by itself produced marginal swell-

Table 2. Effect of phorbol derivatives on pH_i and correlation with tumor-promoting and protein kinase C-stimulating activity

Analog	Concentration, M	Maximal rate of alkalinization, $\Delta pH \cdot min^{-1}$	Tumor-producing activity, relative units*	Protein kinase C activation, %†
PMA	10^{-8}	0.058 ± 0.003 (7) [100%]	+ + +	100
4β-Phorbol 12,13-didecanoate	5×10^{-8}	0.051 ± 0.005 (3) [88%]	+ +	81
4β-Phorbol 12,13-dibutyrate	10^{-7}	0.052 ± 0.003 (3) [90%]	+ +	88
	10^{-8}	0.015 ± 0.009 (3) [26%]		
4β-Phorbol 12,13-dibenzoate	10^{-6}	0.052 ± 0.004 (3) [90%]	+	NA
4β-Phorbol	2.5×10^{-6}	0	Nonpromoter	0
4α-Phorbol	2.5×10^{-6}	0	Nonpromoter	0
4α-Phorbol 12,13-didecanoate	10^{-6}	0	Nonpromoter	0

pH_i was measured in BCECF-loaded cells suspended in Na^+ solution at 37°C. Maximal rate of alkalinization was recorded after addition of indicated concentration of the phorbol analogs. Data are means ± SEM of the number of determinations shown in parenthesis. The activity, calculated as percent of that of PMA, is indicated in brackets. NA, not available.

*Taken from ref. 20.

†Taken from ref. 18, in which the protein kinase C-stimulating activity was compared using 5 μM phorbol derivatives.

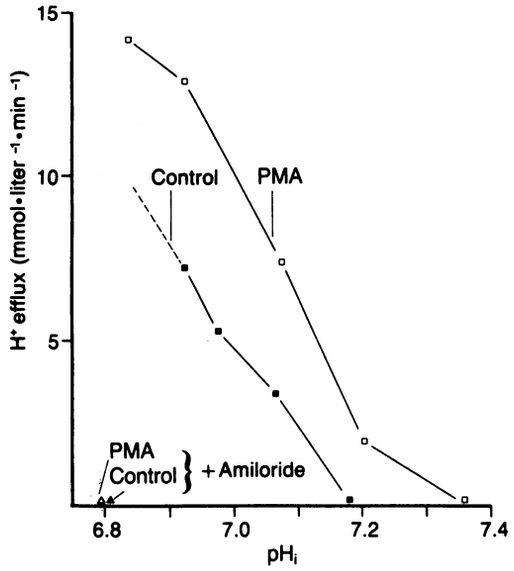

FIG. 3. Cytoplasmic pH (pH_i) dependence of the rate of H^+ (equivalent) efflux in control and PMA-treated thymocytes. Cells stained with BCECF were acid-loaded to the pH_i levels indicated on the abscissa by incubation in *N*-methylglucamine$^+$ solution with nigericin (0.2 μg/ml). Acid loading was terminated with albumin (5 mg/ml, final concentration) and centrifugation. The cells were then resuspended in 100 μl of *N*-methylglucamine$^+$ solution with or without 10 nM PMA for 3 min at 37°C and finally injected to a fluorescence cuvette containing Na^+ solution with (triangles) or without (squares) 100 μM amiloride. H^+ extrusion rates were calculated as the product of the rate of ΔpH_i, measured over the first minute after resuspension in Na^+ medium, and the buffering power (determined earlier to be 25 mmol·liter^{-1}·pH^{-1}; see ref 2). Solid symbols, control; open symbols, PMA-treated. Representative of four similar experiments.

ing. Swelling was also absent in Na^+-free medium as well as in Cl^--free, gluconate$^-$ solution (not illustrated). These data are consistent with activation of Na_o^+/H_i^+ exchange by PMA and with a secondary inward Cl^- shift, resulting from cytoplasmic alkalinization and HCO_3^- accumulation.

Effect of Phorbol Esters on Membrane Potential. It was reported earlier that activation of Na_o^+/H_i^+ exchange in acid-loaded thymocytes induces a small secondary hyperpolarization (2). This membrane potential change likely results from stimulation of the electrogenic Na^+/K^+ pump after an increase in $[Na^+]_i$, inasmuch as it is ouabain sensitive and K_o^+ dependent. Interestingly, Tsien *et al.* (22) reported a similar hyperpolarization in murine thymocytes treated with PMA. To test whether activation of Na^+/H^+ countertransport underlies this effect, the membrane potential of rat thymic lymphocytes was measured before and after treatment with PMA. Bis-oxonol, a nontoxic fluorescent probe (13) was used for these determinations. As shown in Fig. 5, PMA also hyperpolarized rat thymic cells, with a time course that closely resembles that of activation of Na^+/H^+ exchange (Fig. 1). That activation of the Na^+/K^+ pump underlies this hyperpolarization is indicated by experiments using ouabain. The glycoside virtually eliminated the response to the phorbol ester (Fig. 5). The stimulation of the pump is due, at least in part, to increased Na^+ uptake through the Na^+/H^+ antiport. This is suggested by the inhibitory effect of amiloride, which significantly decreased the hyperpolarization (Fig. 5). Moreover, addition of PMA to cells in Na^+-free *N*-methylglucamine$^+$ solution failed to produce the hyperpolarization (not illustrated). Taken together, these results indicate that the phorbol ester-induced hyperpolarization is secondary to activation of Na^+/H^+ exchange and that it results from stimulation of the pump in response to increased $[Na^+]_i$.

Role of Ca^{2+}. In other systems (23), it has been suggested that activation of Na^+/H^+ exchange by serum growth factors is mediated by an increase in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$). We used the fluorescent $[Ca^{2+}]_i$ indicator quin-2 to determine whether a similar mechanism underlies the effect of PMA. As illustrated in Fig. 6*A*, addition of the phorbol ester to cells in normal Na^+ medium resulted in a substantial increase in $[Ca^{2+}]_i$. In seven experiments using 20 nM PMA, Ca_i^{2+} concentration increased from a resting value of 84 ± 6 nM to 135 ± 16 nM after 8 min. The PMA concentration dependence of this effect correlates well with that found for activation of Na^+/H^+ exchange (not illustrated). On the other hand, no increase in $[Ca^{2+}]_i$ was found when comparable concentrations of PMA were added to cells in nominally Ca^{2+}-free Na^+ solution (Fig. 6*A*). In fact, a slight decrease in Ca_i^{2+} concentration was usually observed (from 47 ± 4 nM to 41 ± 3 nM, n = 4). In these experiments, the cells were suspended in Ca^{2+}-free solutions exclusively during the course of the measurement, so depletion of internal stores is unlikely. Moreover, addition of Ca^{2+} to cells treated with PMA in Ca^{2+}-free solutions resulted in a rapid increase in $[Ca^{2+}]_i$ (not shown). Taken together, these results suggest that influx of external Ca^{2+} underlies the increase recorded in Ca^{2+}-containing medium. PMA induced cytoplasmic alkalinization also in Ca^{2+}-free medium (Fig. 6*B*). The magnitude of the ΔpH_i was in fact slightly larger in the nominal absence than in the presence of Ca_o^{2+}. The results indicate that the

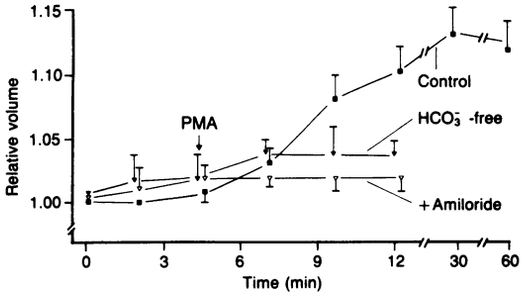

FIG. 4. Effect of PMA on cellular volume. Thymocytes were suspended in HCO_3^--containing RPMI-1640 medium with (▽) or without (■) 200 μM amiloride or in Hepes-buffered nominally HCO_3^--free RPMI-1640 medium (▼). Cell volume was measured electronically at the indicated intervals with the Coulter Counter-Channelyzer combination. The relative median volume is given (ordinate). Where indicated, 10 nM PMA was added to all samples. Data are means ± SEM of four experiments. Where missing, the SEM was smaller than the symbol.

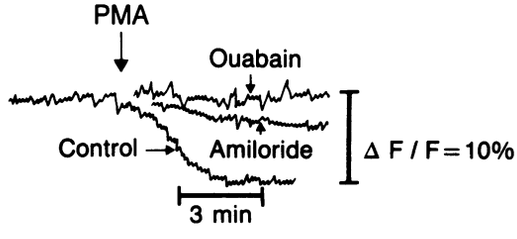

FIG. 5. Effect of PMA on membrane potential. Thymocytes (10^6 cells per ml) were preequilibrated in Na^+ solution containing 0.3 μM bis-oxonol, a fluorescent membrane-potential indicator. When indicated, amiloride (100 μM) or ouabain (2 mM) was also present in the medium. Where indicated by the arrow, PMA (20 nM, final concentration) was added to all the samples. The figure is a composite of three representative traces. Ouabain and amiloride produced only insignificant changes of the initial fluorescence. $\Delta F/F$ is the fractional fluorescence change, where F is the fluorescence of the cell suspension prior to addition of PMA. A downward deflection indicates hyperpolarization. Traces are representative of five experiments. Temperature, 37°C.

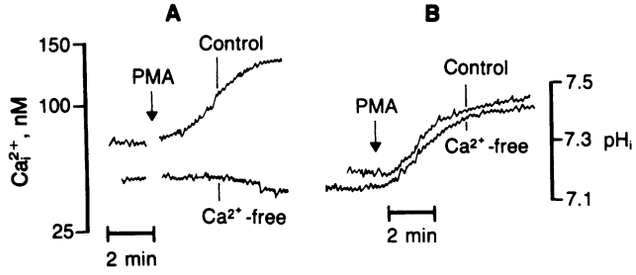

FIG. 6. Correlation between changes in cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) and cytoplasmic pH (pH_i). (*A*) Cells were loaded with the fluorescent indicator quin-2 and suspended in normal Na^+ medium or in Na^+ medium without Ca^{2+}. $[Ca^{2+}]_i$ was measured fluorimetrically (14), using ionomycin and Mn^{2+} for calibration as described (15). Where indicated, 20 nM PMA was added to both samples. Tracings are representative of five similar experiments. (*B*) Cells were loaded with BCECF, and pH_i was measured in Ca^{2+}-containing and in nominally Ca^{2+}-free Na^+ solution as described for Fig. 1*A*, using 20 nM PMA. Tracings are representative of at least three experiments.

activation of Na^+/H^+ exchange is not mediated by an increased $[Ca^{2+}]_i$. Instead, it is conceivable that the increased uptake of Ca^{2+}_o is secondary to the change in pH_i, in $[Na^+]_i$, or in membrane potential. This idea is consistent with the somewhat slower kinetics of $[Ca^{2+}]_i$ increase (Fig. 6*A*) compared to the course of alkalinization (Figs. 1 and 6*B*). However, the $[Ca^{2+}]_i$ response may have been spuriously slowed by the Ca^{2+}-buffering properties of the indicator quin-2.

DISCUSSION

Specific high-affinity receptors for biologically active phorbol esters have been detected in the cytosol and membrane fractions of several cell types, including lymphoid cells (19, 20). It is now generally accepted that the main, and perhaps the sole, target of the phorbol esters is the Ca^{2+}- and phospholipid-dependent protein kinase C (9, 10, 18). This enzyme has widespread occurrence in various tissues of most animals (24) and has been detected in lymphocytes from thymus and other sources (24).

Two lines of evidence suggest that activation of Na^+/H^+ exchange by phorbol esters is mediated by stimulation of the kinase. First, only those phorbol derivatives that accelerate kinase activity had an effect on Na^+/H^+ countertransport, detected as a cytoplasmic alkalinization (Table 2). Moreover, similar concentrations are required to elicit both effects. Second, the activation of Na^+/H^+ exchange could be blocked by trifluoperazine (unpublished observations), which is known to prevent the stimulation of protein kinase C by diacylglycerol or phorbol esters (25).

As shown in Fig. 3, the mechanism underlying activation of Na^+/H^+ exchange seems to be a shift in the pH_i dependence of the antiport. The pH_i sensitivity of the exchange system appears to be largely determined by an allosteric modifier site (2, 16), situated on the cytoplasmic face of the membrane. This site, originally described by Aronson *et al.* (26) for renal membranes, controls the rate of transport of the antiport, rendering it virtually quiescent at physiological pH_i [≈7.2 at 37°C (this value is somewhat more alkaline than that reported for the same cells at 20°C–22°C; see ref. 2)]. The exchanger is minimally active at this pH_i, in spite of the prevalent chemical Na^+ gradient, which is energetically capable of driving pH_i to more alkaline levels (see refs. 2, 16, and 26 for details). Upon addition of the phorbol ester, activation of the antiport is likely to reflect an alkaline shift in the pH_i responsiveness of the modifier. A similar conclusion was reached earlier by Moolenaar *et al.* (4) when analyzing the effects of growth factors on fibroblasts. In the case of phorbol esters, the change in pH_i responsiveness could conceivably result from protein kinase C-mediated phosphorylation at or near the modifier site.

The significance of the activation of Na^+/H^+ exchange by phorbol esters and growth factors has not been definitely established. The resulting cytoplasmic alkalinization could favor the operation of enzyme pathways involved in cellular proliferation. This is suggested by reports that mitogenic activation of quiescent cells can in some cases be accomplished by a brief incubation in alkaline medium (27). Alternatively, the increase in $[Na^+]_i$ may provide the signal for proliferation (11) or differentiation, as suggested in the case of 70Z/3 cells, a pre-B lymphoblastic line (28). Cell swelling, a frequently overlooked consequence of Na^+/H^+ exchange, may also participate in mitogenic signalling and, in addition, may represent a step in mitogen-induced growth. Thus, increased cellular volume appears to precede *de novo* synthesis of organelles.

This work was supported by the National Cancer Institute (Canada) and the Medical Research Council (Canada). S.G. is a Medical Research Council Scientist.

1. Grinstein, S., Clarke, C. A. & Rothstein, A. (1983) *J. Gen. Physiol.* **82**, 619–637.
2. Grinstein, S., Cohen, S. & Rothstein, A. (1984) *J. Gen. Physiol.* **83**, 341–369.
3. Schuldiner, S. & Rozengurt, E. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7778–7782.
4. Moolenaar, W. H., Tsien, R. Y., Vander Saag, P. T. & deLaat, S. W. (1983) *Nature (London)* **304**, 645–648.
5. Rothenberg, P., Glaser, L., Schlesinger, P. & Cassel, D. (1983) *J. Biol. Chem.* **258**, 12644–12653.
6. Nilsen-Hamilton, M. & Hamilton, R. T. (1979) *Nature (London)* **279**, 444–446.
7. Cohen, S., Carpenter, G. & King, L. (1980) *J. Biol. Chem.* **255**, 4834–4842.
8. Cassel, D., Zhuang, Y. & Glaser, L. (1984) *Biochem. Biophys. Res. Commun.* **118**, 675–681.
9. Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698.
10. Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360.
11. Dicker, P. & Rozengurt, E. (1981) *Biochem. Biophys. Res. Commun.* **100**, 433–441.
12. Rosoff, P. M., Stein, L. F. & Cantley, L. C. (1984) *J. Biol. Chem.* **259**, 7056–7060.
13. Rink, T. J., Monterucco, C., Hesketh, T. R. & Tsien, R. Y. (1980) *Biochim. Biophys. Acta* **595**, 15–30.
14. Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334.
15. Rink, T. J., Sanchez, A., Grinstein, S. & Rothstein, A. (1983) *Biochim. Biophys. Acta* **726**, 593–596.
16. Grinstein, S., Goetz, J. D. & Rothstein, A. (1984) *J. Gen. Physiol.* **84**, 585–600.
17. Benos, D. J. (1982) *Am. J. Physiol.* **242**, C131–C145.
18. Kikkawa, Y., Takai, W., Tanaka, Y., Miyake, R. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 11442–11445.
19. Estensen, R. D., Deltoogh, D. K. & Cole, C. F. (1980) *Cancer Res.* **40**, 1119–1124.
20. Shoyab, M. & Todaro, G. J. (1980) *Nature (London)* **288**, 451–455.
21. Cala, P. M. (1980) *J. Gen. Physiol.* **76**, 683–708.
22. Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *Nature (London)* **295**, 68–71.
23. Owen, N. E. & Villereal, M. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3537–3541.
24. Kuo, J. F., Andersson, R. G. G., Wise, B. C., Salomonsson, I., Brackett, N. L., Katoh, N., Shoji, M. & Wrenn, R. W. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7039–7043.
25. Mori, T., Takai, Y., Minakuchi, R., Yu, B. & Nishizuka, Y. (1980) *J. Biol. Chem.* **255**, 8378–8380.
26. Aronson, P. S., Nee, J. & Suhm, M. A. (1982) *Nature (London)* **299**, 161–163.
27. Zetterberg, A. & Engström, W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 4334–4338.
28. Rosoff, P. M. & Cantley, L. C. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7547–7550.