Osmotic and Phorbol Ester–induced Activation of Na$^+$/H$^+$ Exchange: Possible Role of Protein Phosphorylation in Lymphocyte Volume Regulation

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ABSTRACT   The Na$^+$/H$^+$ antiport is stimulated by 12-O-tetradecanoylphorbol-13,acetate (TPA) and other phorbol esters in rat thymic lymphocytes. Mediation by protein kinase C is suggested by three findings: (a) 1-oleoyl-2-acetylglycerol also activated the antiport; (b) trifluoperazine, an inhibitor of protein kinase C, blocked the stimulation of Na$^+$/H$^+$ exchange; and (c) activation of countertransport was accompanied by increased phosphorylation of specific membrane proteins. The Na$^+$/H$^+$ antiport is also activated by osmotic cell shrinking. The time course, extent, and reversibility of the osmotically induced and phorbol ester–induced responses are similar. Moreover, the responses are not additive and they are equally susceptible to inhibition by trifluoperazine, N-ethylmaleimide, and ATP depletion. The extensive analogies between the TPA and osmotically induced effects suggested a common underlying mechanism, possibly activation of a protein kinase. It is conceivable that osmotic shrinkage initiates the following sequence of events: stimulation of protein kinase(s) followed by activation of the Na$^+$/H$^+$ antiport, resulting in cytoplasmic alkalinization. The Na$^+$ taken up through the antiport, together with the HCO$_3^-$ and Cl$^-$ accumulated in the cells as a result of the cytoplasmic alkalinization, would be followed by osmotically obliged water. This series of events could underlie the phenomenon of regulatory volume increase.

An electroneutral transport system that exchanges Na$^+$ and H$^+$ appears to be a ubiquitous component of the plasma membranes of nucleated mammalian cells (1, 19, 22, 26), including peripheral blood and thymic lymphocytes (12, 15). The exchanger, which is specifically inhibited by the K$^+$-sparing diuretic amiloride, is believed to play a major role in the regulation of cytoplasmic pH (pH$_i$)[1] (12, 22), in the control of cellular volume (7, 8, 23), and in transepithelial ion transport (1, 2, 30). In several cell types, the antiport is activated by a variety of growth factors (19, 26), which suggests a role in the initiation of proliferation.

In fibroblasts (20), A431 cells (an epidermoid carcinoma [reference 31]), HL60 cells (a promyelocytic cell line [reference 5]), 70z/3 cells (a pre-B-lymphoid line [reference 25]),

[1] *Abbreviations used in this paper:* BCECF, bis(carboxyethyl)carboxyfluorescein; [Ca^{2+}]$_i$, free cytoplasmic Ca^{2+} concentration; OAG, 1-oleoyl-2-acetylglycerol; pH$_i$, cytoplasmic pH; RPMI, Roswell Park Memorial Institute; TFP, trifluoperazine; TPA, 12-O-tetradecanoyl-phorbol-13, acetate.

and thymic lymphocytes (10), the exchanger can also be stimulated by phorbol diesters, a family of tumor promoters that are co-mitogenic in a variety of cell systems. The primary target for phorbol diesters is thought to be the Ca^{2+} and phospholipid-dependent protein kinase C (4, 21). This enzyme has widespread occurrence in various tissues of most animals and has been detected in lymphocytes from thymus (18). Two lines of evidence suggest that activation of Na$^+$/H$^+$ exchange by phorbol esters in thymocytes is mediated by stimulation of protein kinase C. First, the concentrations of 12-O-tetradecanoylphorbol-13,acetate (TPA)[1] required for activation of countertransport are similar to those reported to activate the kinase. Second, only those phorbol derivatives that accelerate kinase activity have an effect on Na$^+$/H$^+$ exchange. Moreover, the relative potencies of a variety of diesters as activators of countertransport correlate with their potency as stimuli of protein kinase C (10).

In dog (23) and *Amphiuma* (7) erythrocytes as well as in blood and thymic lymphocytes (11, 13), the Na$^+$/H$^+$ antiport

is also stimulated by osmotic cell shrinking. The ensuing increases in intracellular Na^+ content and pH_i (which in turn drive the accumulation of HCO_3^- and Cl^-) are manifested as cell swelling. This sequence of events is thought to underlie the observed regulatory volume increase (7, 8, 11). In contrast to activation by phorbol esters, the molecular mechanism underlying the osmotic activation of the Na^+/H^+ exchanger is not known.

The purpose of the experiments described in this communication is twofold: (a) to provide further evidence that stimulation of protein kinase C results in activation of the Na^+/H^+ antiport in thymocytes, and (b) to obtain information regarding the molecular nature of the events that trigger the volume-induced activation of the exchanger. The latter was accomplished by comparing the properties of the phorbol ester- and volume-induced countertransport. We found that, though not identical, the stimulations of the antiport by osmotic shrinking and by TPA share a number of properties and are not additive, thus suggesting a common step in the activation process.

MATERIALS AND METHODS

Materials: Roswell Park Memorial Institute (RPMI) 1640 solution (without HCO_3^-) was purchased from Gibco Laboratories, Grand Island, NY; 4β-phorbol 12,13-dibutyrate, TPA, N-ethylmaleimide, antimycin A, 2-deoxyglucose, HEPES, 2-[N-morpholino]ethanesulfonic acid, and monensin were from Sigma Chemical Co., St. Louis, MO; nigericin, 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate HCl, and the luciferin–luciferase ATP determination kit were from Calbiochem-Behring Corp., La Jolla, CA; 1-oleoyl-2-acetyl-glycerol (OAG) was obtained from Molecular Probes (Junction City, OR); Bis(carboxyethyl)carboxyfluorescein BCECF) acetoxymethylester was synthesized by Dr. M. Ramjeesingh of the Hospital for Sick Children Research Development Corporation, Toronto, Canada; quin-2 tetraacetoxymethylester was the gift of Dr. T. J. Rink, Cambridge University, England; amiloride was the gift of Merck, Sharp and Dohme, Montreal, Canada; trifluoperazine (TFP) was a gift from Smith, Kline and French Canada Ltd., Montreal.

Solutions: Stock solutions of OAG, nigericin, and monensin were prepared in ethanol. Stocks of 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate HCl, the phorbol diesters, and the acetoxymethylesters of quin-2 and BCECF were made in dimethylsulfoxide. Amiloride and TFP were kept as 10 mM aqueous solutions. RPMI 1640 (HCO_3^--free) solution was buffered with 20 mM Hepes-Na to pH 7.3. Na^+ solution contained 140 mM NaCl, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM Tris-MES, pH 7.2. N-Methyl-D-glucamine$^+$ solution and K^+ solution were prepared by isotonic replacement of Na^+ by N-methyl-D-glucamine$^+$ and K^+, respectively, but were otherwise identical. Na-propionate solution contained 140 mM Na-propionate, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES-Na, pH 6.7. Where indicated, the media were made hypertonic (550 ± 5 mosM) by addition of concentrated NaCl or N-methyl-D-glucamine chloride. Similar results were obtained using both solutes. For ATP depletion, the cells were incubated in a medium that contained 140 mM KCl, 20 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM Tris-2-[N-morpholino]ethanesulfonic acid, pH 7.2, and 1 μg/ml antimycin A.

Methods: Thymocytes were isolated from male Wistar rats (~200 g) as previously described (12). The cells were maintained in HEPES-buffered RPMI 1640 solution at room temperature for up to 8 h. Cell viability, determined by trypan blue exclusion, was >95% throughout this period and was not affected by any of the procedures or reagents used. Cell counting and sizing were performed with a Coulter Counter/Channelyzer combination (Coulter Electronics Inc., Hialeah, FL). Where indicated, the activity of the Na^+/H^+ antiport was estimated from the rate of swelling of cells suspended in Na-propionate medium.[2] The rationale and details of this method have been published elsewhere (15).

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[2] The method is based on the cytoplasmic acidification that results from permeation of undissociated propionic acid. The activation of the Na^+/H^+ exchanger in response to the acidification extrudes H^+ from the cells and results in cytoplasmic accumulation of Na-propionate. The ensuing influx of osmotically obliged water produces cellular swelling, which is detectable using the Coulter Counter-Channelyzer system.

pH_i was measured fluorimetrically using BCECF essentially as described earlier (10, 12), using 650-40 or LS-5 spectrofluorometers (Perkin-Elmer Corp., Norwalk, CT) with thermostatted and magnetically stirred cell compartments, attached to R-100 recorders (Perkin-Elmer Corp.). Briefly, the cells (5 × 10^7/ml) were loaded with BCECF by incubation with 3 μg/ml of the parent acetoxymethyl ester for 30 min at 37°C. The cells were then sedimented, washed once, and resuspended in HEPES-buffered RPMI 1640 at 10^8 cells/ml. Aliquots of this suspension (3–5 × 10^6 cells) were used for each measurement. Calibration was performed using the nigericin/K^+ method of Thomas et al. (28). In some experiments pH_i was manipulated using nigericin in N-methyl-D-glucamine$^+$ solution as previously described (12).

Free cytoplasmic Ca^{2+} concentration ([Ca^{2+}]$_i$) was determined using quin-2 essentially as described by Tsien et al. (29). Thymocyte suspensions (5 × 10^7 cells/ml) in HEPES-buffered RPMI 1640 were loaded for 30 min at 37°C with 10 μM quin-2 acetoxymethylester. The cells were then washed and resuspended in the required medium at 5 × 10^6 cells/ml. Fluorescence was measured with excitation at 339 nm and emission at 492 nm using 2- and 15-nm slits, respectively. Calibration was made with ionomycin and Mn^{2+} as described by Rink et al. (24).

Intracellular Na^+ and K^+ contents were determined by flame photometry as described earlier (12). ATP was determined by using the aforementioned luciferin–luciferase ATP determination kit. Protein was determined according to the method of Bradford (6). Unless otherwise indicated, all the experiments were performed at 37°C. The results are presented either as representative traces or as the mean ± SEM of at least three experiments. Statistical comparisons were made using Student's t test for unpaired samples.

RESULTS

Effects of Diacylglycerol on Na^+/H^+ Exchange

Phorbol diesters are structural analogs of diacylglycerols, which are thought to be the physiological activators of protein kinase C (4, 21). Further evidence that this enzyme is responsible for the activation of the Na^+/H^+ antiport in rat thymocytes was obtained by testing the effects of exogenously added diacylglycerol. We determined the effects of OAG, a relatively permeant diacylglyceride, on the activity of the antiport detected as a change in pH_i in Na^+ solution. The measurements were performed fluorimetrically using BCECF in nominally HCO_3^--free solutions in order to maximize the pH_i changes (and thereby minimize cell volume changes). In agreement with earlier determinations, the resting pH_i of thymocytes in Na^+ solution at 37°C was 7.23 ± 0.02 ($n = 11$). As illustrated in Fig. 1, addition of the diacylglycerol (25 μg/ml, final concentration) produced a cytoplasmic alkalinization, resembling the reported effect of TPA on these and other cells (5, 10, 20, 25). In six experiments, the alkalinization averaged 0.094 ± 0.011 pH units, with a maximal rate of 0.036 ± 0.006 pH/min. Similar effects of OAG on Na^+/H^+ exchange in fibroblasts were recently reported by Moolenaar et al. (20). The diacylglycerol-induced change in pH_i in thymocytes was strictly dependent on the presence of extracellular Na^+. No effect on pH_i was observed when the diacylglycerol was added to cells suspended in either N-methyl-D-glucamine$^+$ solution or in K^+ solution (not illustrated). This Na^+-dependence of the efflux of H^+ equivalents suggests that exchange of extracellular Na^+ (Na^+_o) for internal H^+ (H^+_i) is involved. Amiloride, an inhibitor of Na^+_o/H^+_i exchange in a variety of cell types (3) including thymocytes, was used to test this hypothesis. As shown in Fig. 1, a concentration of the diuretic known to inhibit the antiport in these cells abolished the effect of the diacylglycerol on pH_i. Together with the results obtained with phorbol diesters (reference 10 and see below), the effects of diacylglycerol strongly suggest that protein kinase C is responsible for the activation of the Na^+/H^+ exchanger. This suggested the possibility that an analogous mechanism might underlie the osmotic activation of the antiport.

Comparison of the Phorbol Ester– and Volume-induced Activation of the Antiport

ONSET AND REVERSIBILITY: Several features of the stimulation of the antiport by hypertonic solutions and by phorbol esters were compared in order to establish possible similarities between their mechanisms of activation. Fig. 2 compares the magnitude and time course of the onset of the response, as well as the course and extent of the reversal upon removal of the stimuli. As observed earlier at room temperature (13), increasing the medium osmolarity from the original isotonic level of 290 mosM to 550 mosM with N-methyl-D-glucamine chloride while keeping $[Na^+]_o$ constant at 140 mM results in a pronounced cytoplasmic alkalinization. The change in pH_i becomes apparent after a lag of 10–15 s and
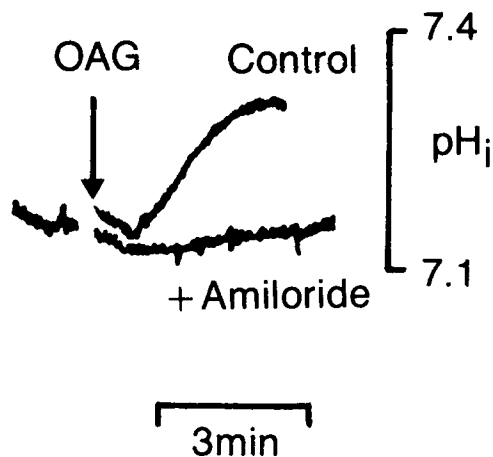

FIGURE 1 Effect of OAG on pH_i. BCECF-loaded thymocytes (3 × 10^6 cells/ml) were suspended in Na^+ solution with or without 100 μM amiloride. Where indicated, 25 μg/ml (final concentration) of OAG was added to both samples and recording resumed. The pH_i was measured fluorimetrically and calibration was made with ni-gericin-K^+ according to the method of Thomas et al. (28). The figure is a composite of representative traces from three similar experiments. Temperature: 37°C.
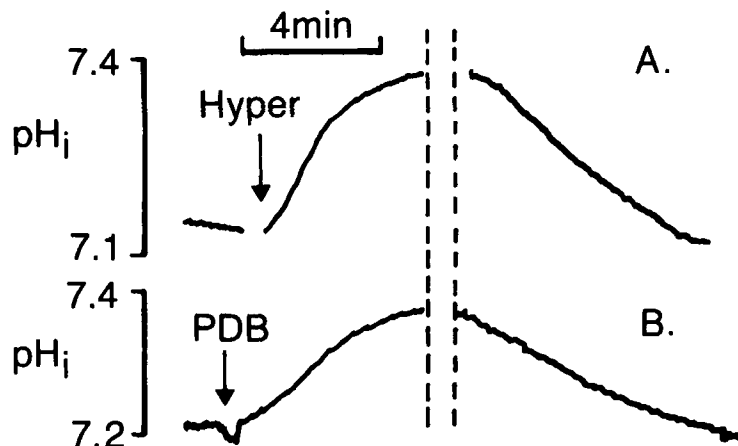
within 5 min reaches a maximum value of 0.22 ± 0.01 (mean ± SEM of 16 determinations). The cells were then sedimented (indicated in the figure by the dotted lines) and resuspended in isotonic Na^+ solution. Restoration of the normal osmolarity led to a gradual decline of pH_i towards the original level. The half-time of the recovery was 3–4 min.

Fig. 2B illustrates the effects of 4β-phorbol-12,13-dibutyrate, a short chain diester, on pH_i. This phorbol ester was chosen for comparison because, unlike TPA, it dissociates readily from hydrophobic binding sites, allowing the determination of the rate of reversal. In five experiments, 4β-phorbol-12,13-dibutyrate produced a maximal alkalinization of 0.15 ± 0.01 units; the effect was detectable after ~20 s and reached a maximum after 4–6 min. After the addition of albumin (5 mg/ml, final concentration) to scavenge the phorbol ester, sedimentation, and resuspension in fresh medium, pH_i returned to the initial level with a half-time of ~4 min.

ADDITIVITY: The similarities between the two modes of activation (see above and Discussion) are suggestive of a common underlying mechanism. If this conclusion is correct, then maximal stimulation by either procedure should preclude further stimulation by the other, i.e., the responses would not be additive. Conversely, additivity would be expected if independent processes are involved. To distinguish between these alternatives, cells were osmotically shrunk under conditions known to produce maximal activation of the antiport (13), while pH_i was monitored fluorimetrically with BCECF. TPA was then added and recording continued (Fig. 3A). In three similar experiments, no further alkalinization was observed even at concentrations of the phorbol ester known to induce a maximal response (10). In contrast, addition of NH_4^+ (which is in equilibrium with NH_3, a permeable weak base) or of monensin (an exogenous Na^+/H^+-exchanging ionophore) after maximal osmotic activation, produced the expected additional cytoplasmic alkalinization (Fig. 3, B and C). Experiments in which TPA was added first (Fig. 3D) confirmed that the responses are not additive. The subsequent osmotic challenge produced only a small further alkalinization. As before, a marked additional alkalinization could be

FIGURE 2 Comparison of the effects of hypertonic medium and of 4β-phorbol-12,13-dibutyrate (PDB) on pH_i. (A) BCECF-loaded cells were suspended in isotonic (290 mosM) Na^+ solution and their pH_i monitored fluorimetrically as described in Materials and Methods. Where indicated by the arrow, the medium was made hypertonic (550 mosM) by addition of concentrated N-methyl-D-glucamine chloride. After several min, the sample was sedimented (dotted line), resuspended in isotonic Na^+ solution, and recording was resumed. Representative of six similar experiments. (B) BCECF-loaded cells were suspended in Na^+ solution and their pH_i monitored as above. PDB (2 × 10^{-8} M, final concentration) was added where indicated by the arrow. The phorbol ester was then removed by addition of bovine serum albumin (5 mg/ml, final concentration) followed by centrifugation of the cells (dotted line). The thymocytes were then resuspended in fresh Na^+ solution and recording resumed. Representative of five similar experiments. Temperature: 37°C.
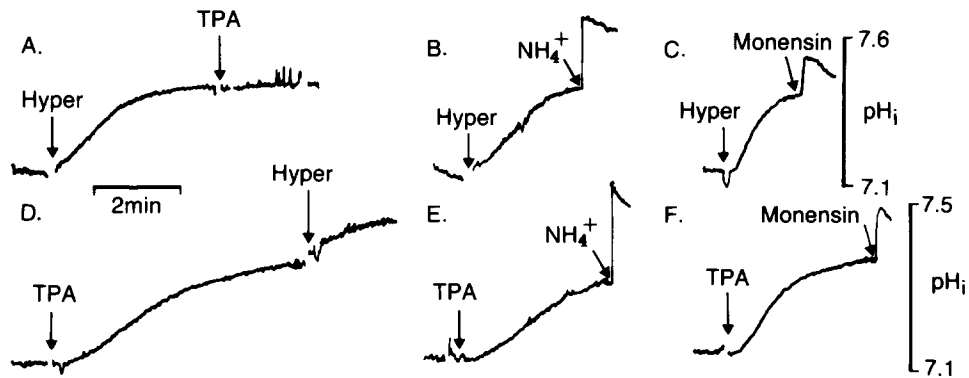
Figure 3   Additivity of the osmotically and phorbol ester–induced cytoplasmic alkalinization. Thymocytes were loaded with BCECF and their pH_i was monitored by fluorescence measurement as described in Materials and Methods. (A) The cells were initially suspended in isotonic Na^+ solution. Where indicated, the medium was made hypertonic (550 mosM) by addition of concentrated N-methyl-D-glucamine chloride to the cuvette. Finally, TPA (2×10^{-8} M, final concentration) was added and recording was continued. (B) Cells in Na^+ solution were first treated hypertonically as in A and then with 30 mM NH_4Cl. (C) Cells in Na^+ solution were treated hypertonically as in A, followed by addition of 5 μM monensin where indicated. (D) Cells in Na^+ solution were initially treated with TPA (2×10^{-8} M). The medium was then made hypertonic as in A. (E) Cells were treated with TPA, followed by NH_4Cl (30 mM). (F) Cells were treated with TPA first, then with monensin (5 μM). The traces are representative of at least three similar experiments. The time scale is 2 min for A, B, D, and E and 4 min for C and F. Temperature: 37°C.

obtained after TPA treatment by addition of either NH_4^+ (Fig. 3E) or monensin (Fig. 3F). These findings with monensin and NH_4^+ indicate that the existing Na^+ gradient can still drive protons outward and that the lack of additivity of the volume- and TPA-induced responses was not due to failure of the fluorescent dye to detect more alkaline levels of pH_i.

CYTOPLASMIC CA^{2+}:   We have reported earlier that one of the features in common to both the hypertonic and phorbol ester treatments is an increase in $[Ca^{2+}]_i$ concentration, which is detectable using quin-2 (10, 13). This increase is delayed with respect to the cytoplasmic alkalinization, and appears to be a consequence, rather than the cause, of the activation of the antiport. This is supported by the finding that neither the hypertonically induced (13) nor TPA-induced increases in $[Ca^{2+}]_i$ are present in cells suspended in Na^+-free media. Moreover, the increased $[Ca^{2+}]_i$ can be blocked by concentrations of 5,N-substituted amiloride analogs that inhibit the antiport.[3] If correct, this hypothesis predicts that, since the activation of Na^+/H^+ exchange is not additive, neither should be the changes in $[Ca^{2+}]_i$. An experimental test is illustrated in Fig. 4. Osmotic shrinking produced the reported increase in $[Ca^{2+}]_i$ but the subsequent addition of a maximal dose of TPA produced no further change (Fig. 4A). In contrast, addition of the nonfluorescent Ca^{2+} ionophore ionomycin increased quin-2 fluorescence further, indicating that saturation of the probe had not occurred. If TPA was added first, the subsequent hypertonic stress produced only a small further increase in $[Ca^{2+}]_i$ (Fig. 4B), closely resembling the effects on pH_i (Fig. 3).

PHARMACOLOGICAL   STUDIES: Further similarities between the hypertonically induced and phorbol ester–induced responses were revealed by pharmacological studies. We recently reported that the transport function of the antiport, measured in acid-loaded cells, can be inhibited by prolonged exposure to comparatively high concentrations of N-ethylmaleimide (e.g., 1 mM for 30 min) (14). Fig. 5 shows evidence that a much milder treatment with the alkylating agent (0.25 mM for 5 min at 22°C) completely eliminates the

[3] Amiloride itself could not be used for these determinations since, at the concentrations required for inhibition, the intrinsic fluorescence of the diuretic precludes measurements with quin-2.
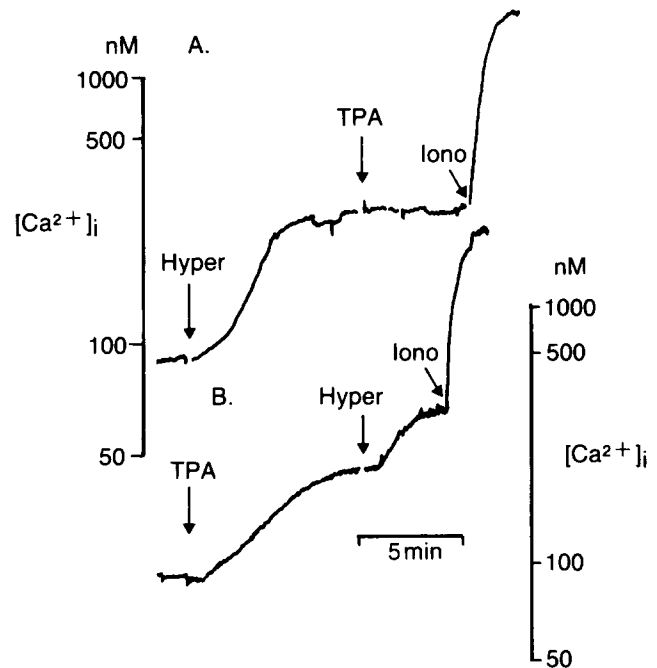
Figure 4   Effect of osmotic shrinking and of phorbol ester on the level of $[Ca^{2+}]_i$. Thymocytes were loaded with quin-2 by incubation with the parent tetra-acetoxymethylester and $[Ca^{2+}]_i$ was determined in Na^+ solution by fluorescence measurements as described in Materials and Methods. (A) Cells were initially suspended in isotonic Na^+ solution. Where indicated, the medium was made hypertonic (550 mosM) with concentrated NaCl. Then, 2×10^{-7} M TPA was added and, where indicated, 1 μM ionomycin (Iono) was also added. (B) Cells suspended in isotonic Na^+ solution were first exposed to TPA, then treated hypertonically as in A. Finally, ionomycin was added where indicated. Calibration was made according to the ionomycin and Mn^{2+} method of Rink et al. (24). The traces are representative of three similar experiments. Temperature: 37°C.

activation by both hypertonicity (Fig. 5A) and TPA (Fig. 5B), while producing only partial inhibition of the acid-induced response (Fig. 5C). The latter was measured following addition of Na^+ to cells that were acid loaded by suspension in
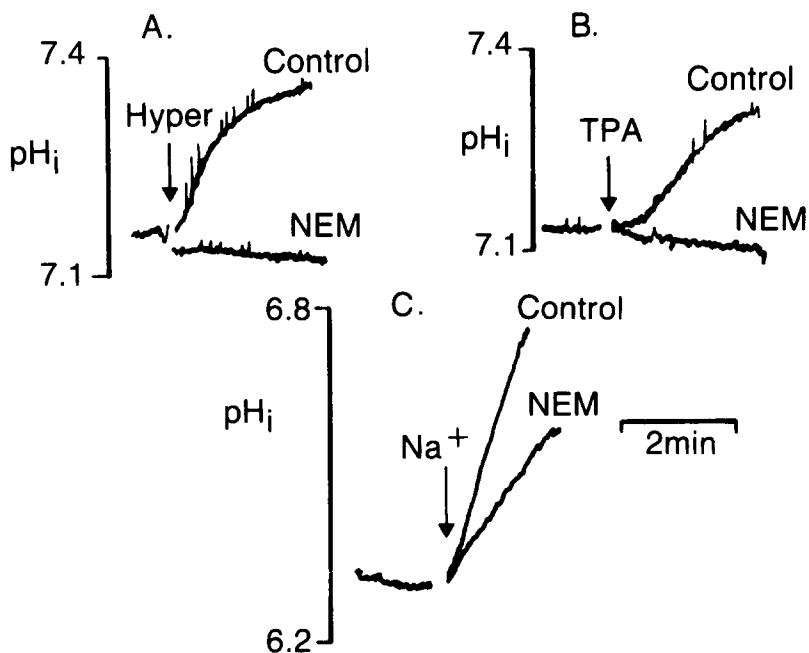
FIGURE 5 Effects of N-ethylmaleimide (NEM) on Na^+/H^+ exchange induced by hypertonicity (A), phorbol ester (B), or acid loading (C). The thymocytes were initially loaded with BCECF and an aliquot was then treated with 0.25 mM N-ethylmaleimide in Na^+ solution for 5 min at room temperature (20–22°C). (A) Untreated and N-ethylmaleimide–treated cells were suspended in isotonic Na^+ solution. Where indicated, the medium was made hypertonic (550 mosM) by addition of concentrated N-methyl-D-glucamine chloride. (B) Control and N-ethylmaleimide–treated cells were suspended in Na^+ solution. Where indicated by the arrow, TPA (2×10^{-8} M) was added to both suspensions. (C) Control and N-ethylmaleimide–treated cells were suspended in N-methyl-D-glucamine$^+$ solution and then acid loaded by addition of the K^+/H^+ exchange ionophore nigericin (0.2 μg/ml, final concentration; not shown). The recording starts following addition of albumin (5 mg/ml, final concentration) to scavenge the ionophore and thereby terminate acid loading. Where indicated by an interruption of the trace, the cells were rapidly sedimented, resuspended in a small volume (50 μl) of N-methyl-D-glucamine$^+$ solution and, where noted by the arrow, they were injected to a cuvette containing 1 ml of Na^+ solution. The traces are composites, representative of a minimum of three experiments. Temperature during the pH$_i$ recording period was 37°C.

Na^+-free, N-methyl-D-glucamine$^+$ medium with nigericin (see reference 12 for details). These results allow a distinction between basal (acid-activated) and stimulated forms of transport, and indicate that the latter is more susceptible to inhibition by the maleimide. Indeed, using an even lower concentration of N-ethylmaleimide (0.1 mM for 5 min), the hypertonically induced and TPA-induced responses were almost completely eliminated, with no significant effect on the response to acid loading (not shown).

We also tested the effects of TFP, a substance reported to block the activity of protein kinase C in vitro (21), as a potential inhibitor of the activation of the antiport. As above, the activity of the exchanger was detected as an amiloride-sensitive intracellular alkalinization. Typical results are illustrated in Fig. 6. Whereas increasing the tonicity of the medium produced the expected pH$_i$ increase in control cells, the response was largely eliminated by 50 μM TFP (Fig. 6, top). Similarly, the response induced by TPA was abolished by a comparable concentration of the phenothiazine (Fig. 6, bottom). Independent experiments (not illustrated) showed that this concentration of TFP has no direct effect on the antiport itself (measured in acid-loaded cells as above), but only blocks the activation mechanism.

A more detailed analysis of the effects of TFP is given in Fig. 7, which shows the concentration-dependence of the inhibitory effects of the phenothiazine on both the osmotically induced and the TPA-induced responses. In both instances, half-maximal inhibition was attained at ~15 μM TFP and

nearly complete inhibition at 50 μM. Higher concentrations of the drug could not be tested since they produced a spontaneous apparent acidification of the cytoplasm. The similarity in the susceptibility to TFP is suggestive of a common step in the osmotically induced and phorbol ester–induced activations of the antiport. Because half-maximal inhibition was attained at a concentration similar to the K_i for inhibition of protein kinase C, it is conceivable that this enzyme is activated by both processes.

ATP-DEPLETION EXPERIMENTS: Because the studies with inhibitors suggested involvement of a protein kinase, it was reasoned that depletion of ATP, the putative substrate of the kinase, might prevent activation of the antiport by phorbol esters and perhaps also by osmotic shrinking. Preliminary experiments were performed to determine whether the basal (acid-stimulated) rate of transport was affected by the depletion process. Cells were incubated at 22°C in glucose-free medium in the presence of antimycin A, a blocker of mitochondrial respiration. The incubation was carried out in K^+ solution that contained 20 mM Na^+ to prevent changes in the internal cation composition of the cells, which was routinely monitored by flame photometry. The levels of cellular ATP were assayed at increasing intervals by the luciferin–luciferase assay, and equivalent samples were used for the determination of Na^+/H^+ countertransport. The latter was determined as the rate of amiloride-sensitive cellular swelling (measured electronically) of cells suspended in Na-propionate medium (pH 6.7) (see footnote 2). As shown in Fig. 8, ATP is virtually
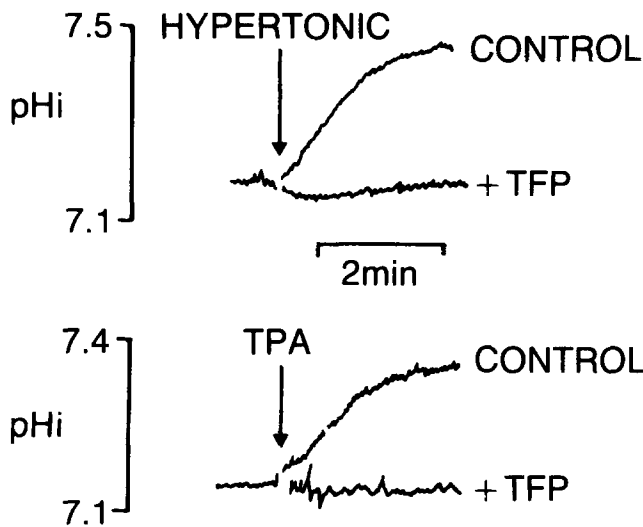
FIGURE 6 Inhibition of the cytoplasmic alkalinization by TFP. BCECF-loaded cells were suspended in Na$^+$ solution with or without 50 μM TFP. (Top) Effect on osmotically induced alkalinization. After a control period, the medium was made hypertonic (550 mosM) by addition of concentrated N-methyl-D-glucamine chloride. (Bottom) Effect on phorbol ester-induced alkalinization. Where indicated by the arrow, 2 × 10^{-8} M TPA was added to the samples. The traces are composites, representative of three similar experiments.
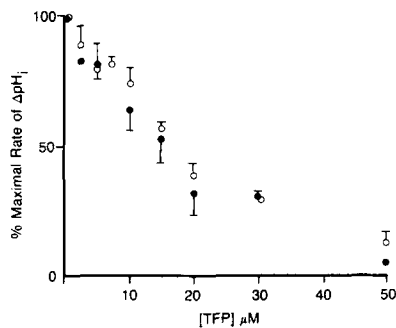

FIGURE 7 Concentration dependence of the inhibitory effect of TFP on the osmotically and phorbol ester-induced alkalinization. BCECF-loaded cells in Na$^+$ solution were activated either osmotically (open circles) or with 2 × 10^{-8} M TPA (solid circles) as described for Fig. 6, in the presence of increasing concentrations of TFP. The maximal rate of amiloride-sensitive alkalinization (ΔpH$_i$/min) was measured and is expressed in the ordinate as percent of the maximal (uninhibited) response. The data are means ± SEM of three determinations. Where not indicated, the SEM was smaller than the symbol.

completely depleted after 45 min under these conditions, with half-maximal depletion occurring within the first 10 min. The rate of acid-induced Na$^+$/H$^+$ exchange was found to decrease after ATP depletion. Inhibition was not due to a decrease in Na$^+$ driving force, since [Na$^+$]$_i$ remained approximately constant. The inhibition of transport was not complete even after two hours, and proceeded more slowly than the depletion of the nucleotide (Fig. 8). After 20 min, the ATP content had been reduced by ~90%, whereas the rate of countertransport was inhibited only 25–30%. This enabled us to assess the effect of ATP depletion on the activation process induced by either phorbol ester or hypertonic shrinking (Fig. 9). Using the fluorimetric pH$_i$ assay, the response of cells suspended in Na$^+$ solution made hypertonic with N-methyl-D-glucamine
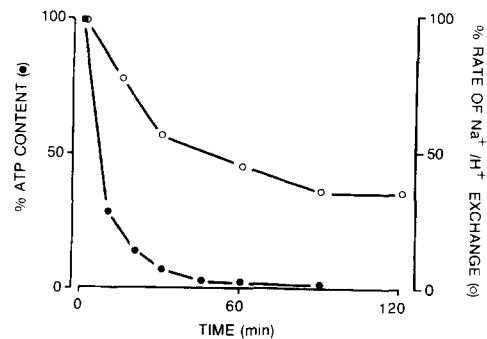

FIGURE 8 Na$^+$/H$^+$ exchange and ATP depletion in thymocytes. Thymocytes were depleted of ATP by incubation in glucose-free medium containing antimycin A (1 μg/ml) at 20–22°C for the length of time indicated in the abscissa. The cellular ATP content (left ordinate, solid circles) was determined by the luciferin–luciferase technique. The rate of Na$^+$/H$^+$ exchange (right ordinate, open circles) was estimated in parallel samples by measuring the rate of amiloride-sensitive swelling of cells suspended in Na-propionate medium at 20–22°C (15). To facilitate comparison, the data have been expressed as percent of the initial levels. The points are means of three experiments with duplicate determinations.

chloride was found to be inhibited by 81 ± 5% (mean ± SEM of three experiments) after a 20-min depletion. Under comparable conditions, the TPA-induced alkalinization was virtually eliminated. In contrast, the rate of alkalinization of acid-loaded cells (Fig. 9C) was reduced to a much lesser degree (41 ± 10% inhibition), in reasonable agreement with the swelling detection method of Fig. 8. Thus, the basal and activated forms of the transporter can also be distinguished on the basis of their ATP-dependence. Moreover, though not identical, the ATP requirement for activation by either shrinking or TPA appears to be similar, reinforcing the analogy between the two stimuli.

DISCUSSION

Under certain conditions, an amiloride-sensitive, nonconductive Na$^+$/H$^+$ exchange activity can be induced in thymic lymphocytes, measurable by isotopic Na$^+$ flux or as pH changes in the medium or cytoplasm (10, 12, 16). This antiport is virtually quiescent at normal pH$_i$ (7.1–7.2), but can be markedly activated by an intracellular acidification. Quiescence is not a consequence of thermodynamic equilibrium, inasmuch as the Na$^+$ gradient is capable of driving pH$_i$ well above the resting level (see results with monensin in Fig. 3, and references 11 and 16 for detailed discussion). Instead, the behavior of the antiport, which is suggestive of a role in pH$_i$ homeostasis, appears to be dictated kinetically by a "modifier" site. The existence of such a pH$_i$-controlling mechanism was originally proposed by Aronson et al. (2), who suggested that protons may bind to an allosteric "modifier" site on the cytoplasmic face of the antiport to activate transport.

Transport through the antiport can also be activated, without cytoplasmic acidification, by addition of a variety of phorbol diesters, including TPA. This activation is not due to changes in the affinity for external Na$^+$ or H$^+$ (a competitive inhibitor of Na^+_o uptake), but appears to be associated with a shift in the pH$_i$ sensitivity of the transport rate of the antiport (10). The shift in the H^+_i-dependence of the antiport presumably reflects an altered behavior of the "modifier" site, in that this site largely determines the pH$_i$-sensitivity of the exchanger. According to this model, the set point of the "modifier,"
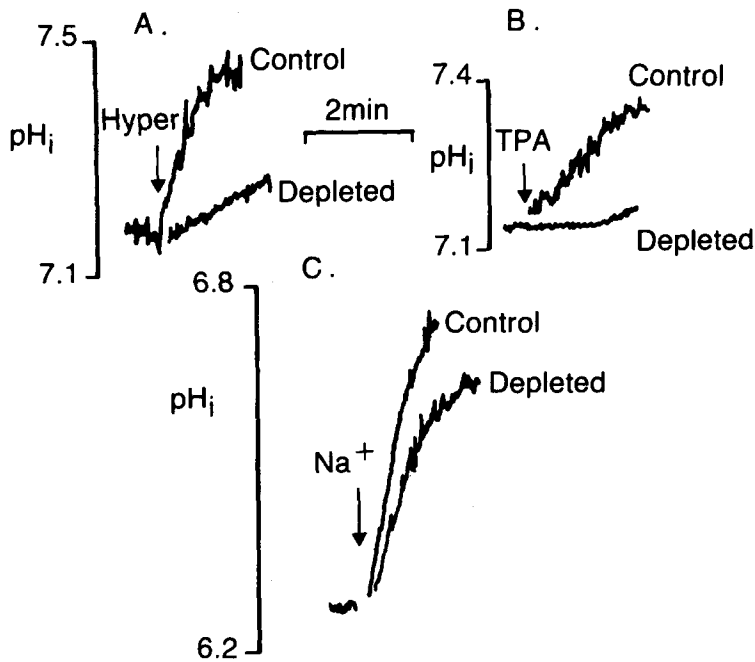
FIGURE 9 Effect of ATP depletion on Na^+/H^+ exchange induced in thymocytes by hypertonicity (A), phorbol esters (B), and by cytoplasmic acidification (C). Cells were loaded with BCECF and an aliquot was then depleted of ATP for 20 min at 20–22°C as described for Fig. 8. (A) Control and ATP-depleted cells were suspended in Na^+ solution at 37°C. Where indicated, the medium was made hypertonic (550 mosM) with N-methyl-D-glucamine chloride. (B) Control and depleted cells were resuspended in Na^+ solution to which TPA (2×10^{-8} M) was added at the arrow. (C) Control and ATP-depleted cells were acid loaded in N-methyl-D-glucamine$^+$ solution as described for Fig. 5C. The samples were then sedimented (indicated by the discontinuity in the trace), resuspended in a small volume of N-methyl-D-glucamine$^+$ solution, and injected to a fluorescence cuvette containing 1 ml Na^+ solution. The traces are composites representative of at least three similar experiments.

which normally prevents transport at $pH_i \geq 7.1$, is adjusted upward. As a result, the nearly quiescent exchanger is activated, but the activation persists only until pH_i attains a value of ≈ 7.35, the new set point.

Several lines of evidence indicate that the alkaline shift in the set point of the "modifier" site of the antiport produced by phorbol esters is mediated by protein kinase C. (a) The concentrations of TPA required for activation of counter-transport are similar to those reported to activate the kinase (10). (b) Only those phorbol diesters that are known to be tumor promoters and to accelerate the kinase had a stimulatory effect on Na^+/H^+ exchange (10). (c) OAG, which stimulates the kinase (4, 21), produced an Na^+-dependent amiloride-sensitive alkalinization (Fig. 1), consistent with activation of the antiport. (d) TFP (Figs. 6 and 7) and 8-(diethylamino)octyl-3,4,5-trimethoxybenzoate HCl (not shown) inhibited the activation of the antiport by TPA, at concentrations reported to block the activity of protein kinase C (27). (e) Finally treatment of thymocytes with TPA under conditions that activate transport was shown to induce protein phosphorylation (unpublished observations).

Exposure to hypertonic solutions can also activate the antiport without prior acidification, resulting in an increase in pH_i above the normal resting level. As in the case of TPA, the affinity for extracellular Na^+ and H^+ is not affected by shrinking. In fact, the osmotic stimulation bears a remarkable resemblance to that of phorbol esters, being attributable to a shift in the pH_i-dependence of the "modifier" site (11, 13). That the osmotically induced and TPA-induced responses share a common pathway is suggested not only by the analogous mechanism of activation, but also by the following kinetic and pharmacological similarities. (a) The extent and time course of onset and reversal are similar in both cases (e.g., Fig. 2; the slightly longer lag of the phorbol ester response can be accounted for by the time required for its diffusion into the cell). (b) The responses are not additive either in terms of changes of pH_i (Fig. 3) or of $[Ca^{3+}]_i$ (Fig. 4). (c) Both are inhibited by similar concentrations of TFP (Figs. 6 and 7). (d) Pretreatment with N-ethylmaleimide eliminates osmotically induced and TPA-induced activation under condi-

tions where basal (acid-activated) transport is minimally affected (Fig. 5). (e) Finally, depletion of cellular ATP can reduce both responses with minimal effect on the basal response (Fig. 9).

In view of the similarities between the osmotically induced and phorbol ester–induced activation, it is hypothesized that the latter is also associated with stimulation of a protein kinase. This prediction was borne out by analysis of protein phosphorylation, which showed an increased labeling of 50–55 and 60-kD polypeptides of a membrane-rich fraction (unpublished observations). It is therefore conceivable that phosphorylation of the exchanger itself or of an ancillary protein mediates the shift in the set point of the "modifier" site, a phenomenon that is common to the phorbol ester and osmotic activations. On this basis, the sequence of events leading to regulatory volume increase could be described as follows: (a) cellular shrinking activates a protein kinase; (b) the kinase phosphorylates the Na^+/H^+ exchanger or a neighboring protein which affects the operation of the antiport; (c) phosphorylation produces a shift in the pH_i-dependence of the exchanger, resulting from a readjustment of the set point of the "modifier" site to a more alkaline pH_i; (d) Na^+/H^+ exchange is activated, with simultaneous uptake of osmotically active Na^+ and cytoplasmic alkalinization (the H^+ ejected are largely replaced by the intracellular buffers); (e) in the presence of external HCO_3^- (which is in equilibrium with the permeant CO_2), the internal HCO_3^- concentration increases; (f) extracellular Cl^- exchanges for internal HCO_3^-; and (g) the intracellular accumulation of Na^+, Cl^- and HCO_3^- drives osmotically obliged water into the cells, which swell toward their normal (isotonic) volume.

In view of the similarities between the osmotically induced and TPA-induced activations of Na^+/H^+ exchange, it is conceivable that cell shrinking liberates diacylglycerol which stimulates protein kinase C. Though attractive, this model is only tentative and fails to explain a few observations. First, while similar, the ATP-dependence (Fig. 9) and the protein phosphorylation patterns (unpublished observations) are not identical for TPA-treated and shrunken cells. Second, migration of soluble kinase C to the membrane fraction, which has been

reported to occur in phorbol ester–treated cells (17) is also observed in TPA-treated, but not in shrunken thymocytes (Grinstein, S., and E. Mack, unpublished observations). Some of these observations can be rationalized if it is assumed that, whereas TPA diffuses throughout the cell, the more labile diacylglycerol generated upon shrinking acts only locally at or near the plasma membrane. Alternatively, it is possible that shrinking directly activates a protein kinase different from C kinase. In this regard it is important to point out that in A431 cells, the osmotic activation of the antiport is considerably greater than that obtained with TPA (9). Instead, the magnitude of the osmotic response is comparable to the one obtained with epidermal growth factor (26, 31), which is thought to activate a tyrosine kinase.

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