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 HCO3− 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+-sparring diuretic amiloride, is believed to play a major role in the regulation of cytoplasmic pH (pHi) (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]), 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 Ca2+ 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) required for activation of mRNA antiport 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 (which in turn drive the accumulation of HCO3- 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 HCO3-) was purchased from Gibco Laboratories, Grand Island, NY; 42-phorbol 12,13-dibutyrate, TPA, N-ethylmaleimide, antimycin A, 2-deoxyglucose, HEPES, 24-N-morpholinoethanesulfonic 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-octeyl-2-acetylglycerol (OAG) was obtained from Molecular Probes (Junction City, OR); Bis(carboxyethyl)carboxyfluorescein BCFE) acetyoxymethylester 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; aminolide 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 acetyoxymethylesters of quin-2 and BCFE were made in dimethylsulfoxide. Aminolide and TFP were kept as 10 mM aqueous solutions. RPMI 1640 (HCO3- free) solution was buffered with 20 mM Hepes-Na to pH 7.3. Na+ solution contained 140 mM NaCl, 1 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 20 mM Tris- MES, pH 7.2. N-Methyl-D-glucamine solution and K+ solution were prepared by isotonic dilution of Na+ and K+ solutions. Free cytoplasmic Na+ concentration ([Na+]c) and pHi were manipulated using nigericin and K+ in N-methyl-D-glucamine solution as previously described (12).

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. As shown in Fig. 1, addition of the diacylglycerol (25 μM/ml, final concentration) produced a cytoplasmic alkalinization, resembling the reported effect of TPA on Na+/H+ exchange in fibroblasts (20). The diacylglycerol-induced change in pH in thymocytes was strictly dependent on the presence of extracellular Na+. No effect on pH 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 effect of H+ equivalents suggests that exchange of extracellular Na+ (Na+o) for internal H+ (H+) is involved. Aminolide, an inhibitor of Na+/H+ 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. 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 pHi becomes apparent after a lag of 10-15 s and 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 pHi 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 pHi. 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, pHi 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 pHi 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 NH4+ (which is in equilibrium with NH3, 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

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**Figure 1** Effect of OAG on pHi. BCECF-loaded thymocytes (3 x 10⁶ 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 pHi was measured fluorimetrically and calibration was made with nigericin-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.

**Figure 2** Comparison of the effects of hypertonic medium and of 4β-phorbol-12,13-dibutyrate (PDB) on pHi. (A) BCECF-loaded cells were suspended in isotonic (290 mosM) Na+ solution and their pHi 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 pHi monitored as above. PDB (2 x 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 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 \(\times\) 10\(^{-4}\) 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\(_4\)Cl. (C) Cells in Na\(^+\) solution were treated hypertonically as in A, followed by addition of 5 \(\mu\)M monensin where indicated. (D) Cells in Na\(^+\) solution were initially treated with TPA (2 \(\times\) 10\(^{-4}\) M). The medium was then made hypertonic as in A. (E) Cells were treated with TPA, followed by NH\(_4\)Cl (30 mM). (F) Cells were treated with TPA first, then with monensin (5 \(\mu\)M). 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 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

\(^3\) 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.
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 hypertonicity-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, increase in control cells, the response was largely eliminated by 50 \(\mu M\) 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 \(-15 \mu M\) TFP and nearly complete inhibition at 50 \(\mu M\). 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
After a control period, the medium was made hypertonic (550 mosM) by addition of concentrated N-methyl-D-glucamine chloride. According to this model, the set point of the "modifier," this site largely determines the pHi-sensitivity of the exchange.

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 antipporter is virtually quiescent at normal pH, 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 antipporter, which is suggestive of a role in pH homeostasis, appears to be dictated kinetically by a "modifier" site. The existence of such a pH-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 antipporter to activate transport.

Transport through the antipporter 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⁺ uptake), but appears to be associated with a shift in the pH sensitivity of the transport rate of the antipporter (10). The shift in the pH-dependence of the antipporter presumably reflects an altered behavior of the "modifier" site, in that this site largely determines the pH-sensitivity of the exchange.

According to this model, the set point of the "modifier,"
which normally prevents transport at pH_i ≥ 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 exchanger, resulting from a readjustment of the set point (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^{2+}] (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 conditions 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- to 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-dependent behavior 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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