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)

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ABSTRACT Phorbol 12-myristate 13-acetate and other phorbol esters induce an intracellular alkalization 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 22Na⁺, suggesting activation of Na⁺/H⁺ 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 cytoplasmic pH (pHe) 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 Ca²⁺- 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 of 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 Ca²⁺ dependence of the stimulation of the antiport were also investigated.

EXPERIMENTAL PROCEDURES

Materials. Phorbol and phorbol esters were purchased from Sigma. 22Na⁺ was from Amersham. Bis(1,3-diethyl-thiobarbituric)trimethine oxonol (bis-oxonol) was from Molecular Probes (Junction City, OR). Amiloride was the gift of Merck Sharp & Dohme. Quin-2-acetoxyethyl 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 tetracetoxyethyl ester of bis(carboxyethyl)carboxyfluorescein (BCECF) was synthesized by M. Ramjeesingh (Hospital for Sick Children, Toronto). RPMI-1640 medium (with or without HCO₃⁻) was purchased from Gibco. Na⁺ solution was 140 mM NaCl/1 mM KCl/1 mM CaCl₂/1 mM MgCl₂/10 mM glucose/20 mM Tris/2-N-morpholinoethanesulfonylic acid, pH 7.2. Where indicated, CaCl₂ was omitted. K⁺ solution and N-methylglycine ñ solution were prepared by isomotic replacement of Na⁺ by K⁺ or N-methylglycine ñ but were otherwise identical.

Methods. Rat thymocytes were isolated as described (2) and maintained in nominally HCO₃⁻-free RPMI-1640 medium buffered with 20 mM Hepes. Cell sizing and counting were done in a Coulter Counter with attached Channelizer as described (1). pH of was determined fluorimetrically using BCECF as described (2). Where indicated, pH was manipulated by means of nigerin in N-methylglycine ñ 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 10⁶ cells per ml. Free cytosolic Ca²⁺ concentration was estimated using quin-2 as described by Tsien et al. (14), calibrating with ionomycin and Mn²⁺ (15). The rate of acidification of lightly buffered medium was measured as described (2). Uptake of 22Na 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. The resting pH of thymocytes in Na⁺ solution (Na⁺ = 140 mM) at 37°C was 7.23 ± 0.017 (n = 11). As shown in Fig. 1A, addition of phorbol 12-myristate 13-acetate (PMA) to these cells induces a sizable alkalization, which becomes apparent ~45 sec after addition of the phorbol ester. In 11 experiments using 10 nM PMA, the final pH (measured 6–8 min after addition of the phorbol ester) averaged 7.42 ± 0.03. As shown in Fig. 1B, this cytoplasmic alkalization is accompanied by the appearance of proton equivalents in the external medium, mea-

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2-3 maximal proton calculated medium, volume of dent ApH reported age mer mmol/liter-l to thymocytes (2). As hypothesis. completely alkalinization produced significant (Fig. 1A). In thymocytes (17) was used to test this hypothesis. As shown in Fig. 1A, 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. The acidification of the external medium was similarly inhibited (Fig. 1B).

Effect of Phorbol Esters on Na⁺ Uptake. Additional evidence for the involvement of Na⁺/H⁺ exchange was obtained by measuring the effects of PMA on isotopic Na⁺ uptake. As reported elsewhere (16), the fluxes of 22Na⁺ 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 22Na⁺-containing (Na⁺ = 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 22Na⁺ 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⁺/H⁺ 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, 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. In contrast to the 4β diesters, un-

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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\(_I\) ≈ 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\(_I\) ≥ 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 cytosol, 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\(_2\)CO\(_3\), 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 swel-

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**Table 2.** Effect of phorbol derivatives on pH\(_I\) and correlation with tumor-promoting and protein kinase C-stimulating activity

<table>
<thead>
<tr>
<th>Analog</th>
<th>Concentration, M</th>
<th>Maximal rate of alkalization, ΔpH/min(^{-1})</th>
<th>Tumor-producing activity, relative units*</th>
<th>Protein kinase C activation, %†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>10(^{-6})</td>
<td>0.058 ± 0.003 (7)</td>
<td>++ +</td>
<td>100</td>
</tr>
<tr>
<td>4β-Phorbol 12,13-didecanoate</td>
<td>5 × 10(^{-8})</td>
<td>0.051 ± 0.005 (3)</td>
<td>++</td>
<td>81</td>
</tr>
<tr>
<td>4β-Phorbol 12,13-dibutyrate</td>
<td>10(^{-7})</td>
<td>0.052 ± 0.003 (3)</td>
<td>++</td>
<td>88</td>
</tr>
<tr>
<td>4β-Phorbol 12,13-dibenzoate</td>
<td>10(^{-6})</td>
<td>0.015 ± 0.009 (3)</td>
<td>[90%]</td>
<td>26%</td>
</tr>
<tr>
<td>4a-Phorbol 12,13-didecanoate</td>
<td>10^{-6}</td>
<td>0.052 ± 0.004 (3)</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td>4β-Phorbol</td>
<td>2.5 × 10(^{-6})</td>
<td>0</td>
<td>Nonpromoter</td>
<td>0</td>
</tr>
<tr>
<td>4α-Phorbol</td>
<td>2.5 × 10(^{-6})</td>
<td>0</td>
<td>Nonpromoter</td>
<td>0</td>
</tr>
<tr>
<td>4α-Phorbol 12,13-didecanoate</td>
<td>10^{-6}</td>
<td>0</td>
<td>Nonpromoter</td>
<td>0</td>
</tr>
</tbody>
</table>

pH\(_I\) was measured in BCECF-loaded cells suspended in Na\(^+\) solution at 37°C. Maximal rate of alkalization 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.
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\(^+\)/H\(^+\) 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\(^+\)/H\(^+\) 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\(^+\)], inasmuch as it is ouabain sensitive and K\(_S\) 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\(^+\).**

**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\(^{2+}\)] indicator quin-2 to determine whether a similar mechanism underlies the effect of PMA. As illustrated in Fig. 6A, 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\(^{2+}\) 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. 6A). In fact, a slight decrease in Ca\(^{2+}\) 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 alkalization also in Ca\(^{2+}\)-free medium (Fig. 6B). The magnitude of the ΔpH\(_i\) was in fact slightly larger in the nominal absence than in the presence of Ca\(^{2+}\). The results indicate that the

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**Fig. 3.** Cytoplasmic pH (pHi) 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 into a fluorescence cuvette containing Na\(^+\) solution with (triangles) or without (squares) 100 μM amiloride. H\(^+\) efflux 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.

**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 Channelizer 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. ΔF/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.
activation of Na⁺/H⁺ exchange is not mediated by an increased [Ca²⁺]. Instead, it is conceivable that the increased uptake of Ca²⁺ is secondary to the change in pHᵢ, in [Na⁺]ᵢ, or in membrane potential. This idea is consistent with the somewhat slower kinetics of [Ca²⁺]ᵢ increase (Fig. 6A) compared to the course of alkalization (Figs. 1 and 6B). However, the [Ca²⁺]ᵢ response may have been spuriously slowed by the Ca²⁺-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 Ca²⁺- 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 alkalization (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 pHᵢ dependence of the antiport. The pHᵢ 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 pHᵢ [~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 pHᵢ, in spite of the prevalent chemical Na⁺ gradient, which is energetically capable of driving pHᵢ 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 pHᵢ 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 pHᵢ 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 alkalization 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 702/3 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.

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