Okadaic Acid, a Phosphatase Inhibitor, Induces Activation and Phosphorylation of the Na\textsuperscript{+}/H\textsuperscript{+} Antiport* (Received for publication, February 20, 1991)

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We determined the effect of okadaic acid (OA), a potent phosphoprotein phosphatase inhibitor, on the intracellular pH (pHi) of rat thymic lymphocytes and human bladder carcinoma cells. OA induced a rapid and sustained cytosolic alkalization. This pH\textsubscript{i} increase was Na\textsuperscript{+}-dependent and was inhibited by 5,N-disubstituted analogs of amiloride, indicating mediation by the Na\textsuperscript{+}/H\textsuperscript{+} antiport. As described for other stimuli, such as mitogens and hypertonic challenge, activation of the antiport by OA is attributable to an upward shift in its pHi dependence. Accordingly, the alkalization produced by the phosphatase inhibitor was not additive with that induced osmotically. Activation of the antiport by OA was accompanied by a marked increase in phosphoprotein accumulation, revealing the presence of active protein kinases in otherwise unstimulated cells. We considered the possibility that phosphorylation of the antiport itself or of an ancillary protein is responsible for activation of Na\textsuperscript{+}/H\textsuperscript{+} exchange. Consistent with this notion, the alkalization induced by OA was absent in ATP depleted cells. More importantly, immunoprecipitation experiments demonstrated increased phosphorylation of the antiport following treatment with OA. We conclude that, upon inhibition of phosphoprotein phosphatase activity, constitutively active kinases induce activation of the Na\textsuperscript{+}/H\textsuperscript{+} exchange, possibly by direct phosphorylation of the antiport.

Maintenance of the cytosolic pH (pHi)\textsuperscript{\dagger} in the physiological range is essential for cell function and survival. It is now well established that mammalian cells have pHi levels near or slightly above neutrality (1). Because most mammalian cells have transmembrane potentials in excess of 50 mV (negative inside), the measured pHi, levels are considerably higher than expected if H\textsuperscript{+} (OH\textsuperscript{–}) and HCO\textsubscript{3}\textsuperscript{−} were passively distributed across the plasma membrane. These findings imply that, in order to maintain pHi, within the physiological range, active processes have evolved to extrude excess H\textsuperscript{+} (equivalents) which are driven electrophoretically into the cell, or are generated metabolically therein.

One of the primary mechanisms involved in the extrusion of H\textsuperscript{+} from mammalian cells is the Na\textsuperscript{+}/H\textsuperscript{+} antiport. This ubiquitous molecule, recently identified as a glycoprotein of molecular mass \(\approx 110\) kDa (2), catalyzes the electroneutral exchange of one Na\textsuperscript{+} for one H\textsuperscript{+}, a process that is susceptible to inhibition by amiloride and, more potently, by its 5,N-disubstituted analogs (3–5). The activity of the antiport is exquisitely dependent on the intracellular pHi; it is virtually quiescent at physiological pHi, but is markedly activated when the cytoplasm becomes acidic (5). This behavior is primarily dictated by the allosteric effect of an internal "modifier" site, which curtails the activity of the antiport as pHi approaches the physiological set point (5, 6). The antiport can also be stimulated without prior intracellular acidification by a variety of stimuli, including several hormones and growth factors (7, 8). In lymphoid cells, stimulation can be attained by addition of mitogenic lectins or phorbol esters, and by osmotically induced shrinking (9, 10). While the mechanism underlying the osmotic activation remains unresolved, stimulation of the antiport by mitogens and phorbol esters is thought to be associated with activation of protein kinase activity (7, 11). Indeed, recent immunoprecipitation experiments have demonstrated that the phosphorylation of the antiport increases under these conditions (2). It has therefore been speculated that phosphorylation of the antiport leads to a shift in the pHi sensitivity of the modifier site, accounting for activation of exchange activity and the reported cytosolic alkalization.

Phosphoprotein accumulation can result not only from activation of kinase activity, but also from inhibition of protein phosphatases. It is therefore conceivable that phosphatase activity also influences the rate of Na\textsuperscript{+}/H\textsuperscript{+} exchange. This hypothesis was tested in the present work using okadaic acid (OA), a potent inhibitor of protein phosphatases 2A and 1 (13, 14). OA induced a rapid and sustained cytosolic alkalinization. This pHi increase was Na\textsuperscript{+}-dependent and was inhibited by 5,N-disubstituted analogs of amiloride, indicating mediation by the Na\textsuperscript{+}/H\textsuperscript{+} antiport. As described for other stimuli, such as mitogens and hypertonic challenge, activation of the antiport by OA is attributable to an upward shift in its pHi dependence. Accordingly, the alkalization produced by the phosphatase inhibitor was not additive with that induced osmotically. Activation of the antiport by OA was accompanied by a marked increase in phosphoprotein accumulation, revealing the presence of active protein kinases in otherwise unstimulated cells. We considered the possibility that phosphorylation of the antiport itself or of an ancillary protein is responsible for activation of Na\textsuperscript{+}/H\textsuperscript{+} exchange. Consistent with this notion, the alkalization induced by OA was absent in ATP depleted cells. More importantly, immunoprecipitation experiments demonstrated increased phosphorylation of the antiport following treatment with OA. We conclude that, upon inhibition of phosphoprotein phosphatase activity, constitutively active kinases induce activation of the Na\textsuperscript{+}/H\textsuperscript{+} exchange, possibly by direct phosphorylation of the antiport.

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\textsuperscript{\dagger} The abbreviations used are: pHi, intracellular pH; OA, okadaic acid; W7, N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide; H7, 1-(5-isouquinolinylsulfonfonyl)-2-methyl piperazine; BCECF, 2',7'-bis(carboxyethyl)-5-(6)-carboxyfluorescein; H8, N-(2-(methylamino)ethyl)-5-isouquinoline sulfonamide; EPA, 5-(N-ethyl-N-propyl)-amiloride; HEpes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; EGTA, ethyleneglycol-bis(oxyethylenenitrilo)tetrasacetic acid; [Ca\textsuperscript{2+}], cytoplasmic free calcium concentration; pHi, extracellular pH; [Na\textsuperscript{+}], extracellular Na\textsuperscript{+} concentration; SDS, sodium dodecyl sulfate.

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ACTIVATION OF Na\textsuperscript{+}/H\textsuperscript{+} ANTIPORT BY OKADAIC ACID

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kineses, and inositol 1,4,5-triphosphatase (13, 14). In the experiments described below, we tested the effects of OA on cytosolic pH and protein phosphorylation in rat thymic lymphocytes and human bladder carcinoma cells. OA proved to be a potent stimulus of phosphoprotein accumulation and of Na\textsuperscript{+}/H\textsuperscript{+} exchange in these cells.

EXPERIMENTAL PROCEDURES

Materials

Solution RPMI (bicarbonate-free), Coomassie Blue, molecular weight standards, tetrasodium pyrophosphate, O-phosphantholine, iodoacetamide, phenylmethylsulfonyl fluoride, calmodulin antagonist W1, and protein kinase inhibitor H8 were from Sigma, St. Louis, Missouri; ionomycin, staurosporine, and the luciferin-luciferase ATP determination kit were from Calbiochem-Behring. N-methyl-D-glucamine and sodium orthovanadate were obtained from Aldrich. Bovine pancreas DNase and RNase were from Boehringer Mannheim. The nonionic detergent Nikkol was purchased from Nikko, Japan. Protein A-Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. The acetoxymethyl esters of B-Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. The acetoxymethyl esters of 2',7'-biscarboxyethyl)-5(6)-carboxyfluorescein (BCECF) and indo-1 were purchased from Molecular Probes. The cyclic nucleotide-dependent protein kinase inhibitor H8 was from Seikagaku America. [1,2,3,4,5,6,7,8,9,10]ATP and [32P]orthophosphate were from ICN. [N-ethyl-N-propyl-nitrosonornicotine (EPAn) was either the kind gift of Merck. OA was purified as described (15). Polyclonal antibodies against the Na\textsuperscript{+}/H\textsuperscript{+} antiport were raised by injecting rabbits with the Na\textsuperscript{+}/H\textsuperscript{+} antiport, as described (2).

The acetoxymethyl esters of OA-Sepharose CL-4B was from Pharmacia LKB Biotechnology Inc. The acetoxymethyl esters of fluoroein (BCECF) and indo-1 were purchased from Molecular Probes. The cyclic nucleotide-dependent protein kinase inhibitor H8 was from Seikagaku America. [1,2,3,4,5,6,7,8,9,10]ATP and [32P]orthophosphate were from ICN. [N-ethyl-N-propyl-nitrosonornicotine (EPAn) was either the kind gift of Merck. OA was purified as described (15). Polyclonal antibodies against the Na\textsuperscript{+}/H\textsuperscript{+} antiport were raised by injecting rabbits with the Na\textsuperscript{+}/H\textsuperscript{+} antiport, as described (2).

Methods

Cell Isolation and Culture—Thymocytes were isolated from male Wistar rats (150–200 g) as previously described (17). The cells were counted using a Coulter counter and maintained in Hepes-buffered RPMI 1640 at room temperature until use. Cell viability was >95% and was not affected by any of the procedures or reagents used.

For some experiments, we utilized monolayer cultures of MGH-U1 cells, a human bladder carcinoma line. A clone expressing high Na\textsuperscript{+}/H\textsuperscript{+} exchange activity, originally isolated by Rotin et al. (18) and called HSPP-spheroid, was utilized. The cells were maintained in complete α-minimal essential medium supplemented with antibiotics and 10% fetal bovine serum and buffered to pH 7.4 with 25 mM bicarbonate. Cultures, free of Mycoplasma, were reestablished from frozen stock approximately every 3 months.

Cell Permeabilization—For electroporation, thymocytes were sedimented, solubilized, and treated with or without the indicated concentrations of OA in Na\textsuperscript{+} solution for 10 min at 37°C. The thymocytes were next sedimented, the pellet resuspended in a small volume (50 μl) of 150 mM Tris-HCl, pH 7.5, diluted in 200 μl of boiling 2% SDS sample buffer containing mercaptoethanol and immediately boiled. Samples were loaded on 15% polyacrylamide gels. Molecular weight standards and of molecular weight standards was performed in the presence of SDS on 10% gels by the method of Laemmli (21) using the Bio-Rad Mini Protean II system. The gels were then stained using Coomassie Blue, dried overnight at room temperature using Bio-Gel Wrap (Bio Design Inc.), and used for autoradiography with intensifying screens at −70°C.

To analyze lysosome permeabilized cells, 10⁷ thymocytes were electroporated as described above and then suspended in 1 ml of a modified permeabilization medium containing only 50 μM ATP plus 25–50 μg of [γ-32P]ATP. The cells were then treated with or without the indicated concentrations of OA for 6 min at 37°C. Cells were then washed and incubated with or without the indicated concentrations of OA in Na+ solution for 10 min at 37°C. The thymocytes were next sedimented, the pellet resuspended in a small volume (50 μl) of 150 mM Tris-HCl, pH 7.5, diluted in 200 μl of boiling 2% SDS sample buffer containing mercaptoethanol and immediately boiled. Samples were loaded on 15% polyacrylamide gels. Molecular weight standards was performed in the presence of SDS on 10% gels by the method of Laemmli (21) using the Bio-Rad Mini Protean II system. The gels were then stained using Coomassie Blue, dried overnight at room temperature using Bio-Gel Wrap (Bio Design Inc.), and used for autoradiography with intensifying screens at −70°C.
were scanned using a Pharmacia LKB 2222–020 Ultrascan XL Laser densitometer, coupled to a Packard Bell PB500 computer.

Other Methods—Cellular ATP content was determined using the aforementioned luciferin–luciferase kit. Unless otherwise specified, all experiments were performed at 37 °C. Data are presented as representative traces or radiograms or as the mean ± one standard error of the indicated number of experiments.

RESULTS

Effect of OA on pH.—In agreement with earlier determinations (17, 22), the resting pH of rat thymocytes in nominally bicarbonate-free Na+ solution ([Na+]i = 140 mM) at 37 °C was found to average 7.11 ± 0.04 (n = 18). As shown in Fig. 1, treatment of these cells with 1 μM OA resulted in a substantial cytoplasmic alkalinization which became apparent 1 min after addition of the phosphatase inhibitor and attained its full magnitude within ≈9 min. In 18 preparations, the final pHi averaged 7.27 ± 0.04. The maximal rate of alkalinization induced by OA, determined in 12 experiments, was 0.028 ± 0.003 pH units/min.

The concentration dependence of the effect of OA on pHi was analyzed in experiments like those in Fig. 1, and the accumulated data are summarized in Table I. Within the period of observation (15 min), only a marginal effect was obtained with 1 × 10⁻⁷ M OA. Maximal effects were obtained using 1 × 10⁻⁶ M OA; higher concentrations of the phosphatase inhibitor failed to further alkalinize pH; or to increase the rate of alkalinization. The lag period required to attain the maximal response was found to be somewhat shorter as the concentration of OA was increased (Table I).

Characterization of the Transport System Involved in OA-induced Alkalinization.—The process responsible for the OA-induced alkalinization was investigated next. Three distinct transport systems are the primary determinants of pHi in mammalian cells: the Na+/H+ antiport, cation-independent HCO₃⁻/Cl⁻ exchange, and Na⁺-dependent HCO₃⁻/Cl⁻ exchange. Because the experiments in Fig. 1 were performed in the nominal absence of HCO₃⁻, stimulation of Na⁺-dependent HCO₃⁻/Cl⁻ exchange is unlikely to account for the observed pH changes. Similarly, inhibition of cation-independent HCO₃⁻/Cl⁻ exchange, which is poised to drive the cell to a pHi near or slightly below the resting level, is not likely responsible for the alkalinosis. We therefore turned our attention to the cation antiport. The presence of a robust Na+/H⁺ antiport in the plasma membrane of thymocytes has been demonstrated by a variety of complementary techniques (17). Moreover, stimulation of this antiport by mitogens and by osmotic stress results in pHi changes comparable to that elicited by OA. To test the involvement of Na+/H⁺ exchange in the alkalinization produced by OA, cells were treated with the phosphatase inhibitor in media devoid of Na⁺. As shown in Fig. 2B, the effect of OA (1 μM) on pHi was completely abolished (∆pHi = 0.0 in four experiments) in cells suspended in Na⁺-free (K⁺-rich) solution. Experiments where Na⁺ was substituted by N-methyl-D-glucammonium⁺ confirmed that the OA-induced change in pHi is strictly dependent on the presence of extracellular Na⁺ (∆pHi = 0.06 ± 0.02 (n = 4), not illustrated).

These observations are consistent with the notion that Na⁺/H⁺ exchange is responsible for the effects of OA on pHi. EPA, an amiloride analog which is a potent and relatively specific inhibitor of the antiport, was used to confirm this hypothesis. In rodent lymphocytes, EPA was reported to block the antiport with an apparent Kᵢ = 25 mM (determined at [Na⁺]i = 70 mM; Ref. 23). Cells were incubated in Na⁺ solution in the presence of 5 μM EPA, a concentration sufficient to fully inhibit the antiport even at [Na⁺]i = 140 mM. As shown in Fig. 2A, the amiloride analog completely abolished the effect of OA on pHi. These results strongly suggest that the effect of OA on thymocyte pHi is mediated by activation of the Na⁺/H⁺ antiport.

As mentioned above, it has been demonstrated in lymphocytes that the Na⁺/H⁺ antiport, which is nearly quiescent in unstimulated cells suspended in isotonic solutions, becomes rapidly activated when the osmolarity of the medium is increased, resulting in cytosolic alkalinization (9). We reasoned that, if a similar mechanism underlies the effect of OA, the hypertonic response and that of OA should not be additive. Conversely, if independent pathways mediate the two responses, additivity would be anticipated. This hypothesis was tested in the experiments illustrated in Fig. 3. Addition of an extra 70 mM NaCl to the isotonic Na⁺ solution resulted in a pronounced cytoplasmic alkalinization, the change in pHi...
becoming apparent after a lag of only 10–15 s and reaching a maximal value of 0.18 ± 0.02 pH units (n = 17) within 3 min (Fig. 3A). Subsequent addition of the maximally stimulatory dose of OA (1 μM) to such hypertonically stimulated cells produced no further effect on pHi (ΔpH = 0.011 ± 0.005, n = 8). When the cells were first treated with OA (1 μM), subsequent osmotic challenge produced only a small further alkalinization: ΔpH = 0.08 ± 0.01 (n = 8), consistent with the greater effect of the osmotic treatment (Fig. 3A). Together with the Na+ dependence and EPA sensitivity, the nonadditivity of the OA and osmotic responses indicates that the phosphatase inhibitor elevates pHi through stimulation of the Na+/H+ antiport.

Mechanism of Activation of Na+/H+ Exchange by OA—As discussed in the Introduction, hypertonic stress, as well as mitogenic agents, activate the antiport by shifting the pH dependence of the modifier site, adjusting the set point upward to promote Na+/H+ exchange at physiological values of pH. Because the hypertonic and OA-induced responses are not additive, it is likely that a similar mechanism underlies the effect of the phosphatase inhibitor. To test this possibility, the intracellular pH dependence of the response to OA was determined. Cells were pre-equilibrated in Na+ solution at pHi = 7.7 in order to increase their intracellular pH. In seven experiments, pH averaged 7.27 ± 0.01 when pHi was 7.7, compared to 7.16 ± 0.03 (n = 4) for control cells (suspended in solution at pHi = 7.3). In the cells that were artificially alkalinized, the effect of the phosphatase inhibitor on pHi was shown to be greatly reduced. Addition of 1 μM OA induced a cytoplasmic alkalinization of only 0.07 ± 0.02 (n = 7) pH units, compared to 0.14 ± 0.02 (n = 4) for control cells. It is noteworthy that the final pH reached in both cases is similar, suggesting that attainment of the set point deactivated the antiport at the same level in both instances, regardless of the initial pH. These observations are consistent with an OA-induced alkaline shift in the pH dependence of the antiport.

By analogy with the effects of mitogens and tumor promoters, it is likely that the effect of OA on the antiport is mediated by phosphoprotein accumulation. This would result from kinase activation in the case of the former stimuli and from inhibition of phosphoprotein phosphatases in the case of OA. In this event, stimulation of Na+/H+ exchange by OA would be predicted to depend on the availability of cellular ATP, as has been demonstrated for the growth promoters (9). To test this prediction, cells were preincubated at 37°C in glucose-free medium in the presence of antimycin A, a blocker of mitochondrial respiration. The incubation was carried out in a K+-rich solution that contained 20 mM Na+ (see “Experimental Procedures”), to prevent changes in the internal cation composition of the cells subsequent to inhibition of the Na+/K+ ATPase. After 20 min of incubation in this medium, the ATP content, determined by the luciferin-luciferase assay, had been reduced by ≈97% (Fig. 4D, stippled bars). The pHi of these ATP-depleted cells was found to be more acidic than that of control cells (cf. top with middle traces in Fig. 4, A and B). This is consistent with the reported partial inhibition of Na+/H+ exchange activity upon ATP depletion (9), attributed to an acidic shift in the pH dependence of the modifier site (24). To confirm the effectiveness of the ATP depletion procedure, the cells were subjected to hypertonic shock (Fig. 4B); in agreement with earlier findings (9), the osmotically

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**Fig. 3. Additivity of the osmotically induced and OA-induced cytoplasmic alkalinization.** Thymocytes were loaded with BCECF and their pHi was monitored fluorimetrically. A, cells in isotonic Na+ solution were initially treated with OA (1 μM). Where indicated, the medium was made hypertonic by addition of 70 mM NaCl from a 4 M stock and recording was continued. B, cells in isotonic Na+ solution were first treated hypertonerically as in A, followed by addition of 1 μM OA where indicated. The traces are representative of at least eight similar experiments. Temperature, 37°C.

**Fig. 4. Inhibitory effect of ATP-depletion on the activation of Na+/H+ exchange induced by OA and hypertonicity.** Where indicated, thymocytes were depleted of ATP by incubation in glucose-free medium containing antimycin A (1.5 μg/ml) at 37°C for 20 min. The cells were loaded with BCECF during the depletion process. Control cells were loaded with BCECF in standard Na+ solution. A, control (top trace) and ATP-depleted cells (lower trace) were suspended in Na+ solution, and OA (1 μM) was added to both samples where indicated. B, control (top trace) and ATP-depleted cells (lower trace) were suspended in Na+ solution; where indicated, the medium was made hypertonic by addition of 70 mM NaCl. C, ATP-depleted cells in Na+ solution were initially treated with OA (1 μM); where indicated, the medium was made hypertonically as in B. The traces are representative of at least four experiments of each type. Temperature, 37°C. D, correlation between depletion of intracellular ATP and inhibition of osmotically induced and OA-induced alkalinization. The data are taken from the experiments described in A and B. Stippled bars, OA-induced pH change; Hatched bars, osmotically induced pH change (right ordinate). Values are means ± S.E. of at least four determinations.
induced alkalization was greatly (though not completely) inhibited in ATP-depleted cells: \( \Delta p_{H} = 0.04 \pm 0.01 \) (n = 4) versus 0.18 ± 0.02 pH units (n = 17) in control cells. Under comparable conditions, the response to OA was completely obliterated by the ATP depletion protocol \( \Delta p_{H} = 0.015 \pm 0.005 \) (n = 10), despite the acidic basal pH. (lower trace in Fig. 4A). These findings are consistent with the notion that the effect of OA is mediated by phosphoprotein accumulation.

We also determined the effect of sequential application of OA and hypertonic stress in ATP-depleted cells (Fig. 4C). Unexpectedly, when depleted cells were first treated with OA (1 \( \mu \)M) and then challenged osmotically, the response to hypertonicity was restored. The kinetics of the response was slower than in ATP replete cells, but the final magnitude of the alkalization was comparable \( \Delta p_{H} = 0.18 \pm 0.02 \) (n = 10). In contrast, when ATP-depleted cells were subjected first to hypertonic shock and then treated with OA, the response to OA remained greatly inhibited \( \Delta p_{H} = 0.06 \pm 0.01 \) (n = 3); not illustrated. The significance of these results is addressed under “Discussion.”

The ATP dependence of the stimulation of \( Na^+/H^+ \) exchange by OA suggests that the effect is mediated by phosphoprotein accumulation, resulting from inhibition of phosphatases 1 and/or 2A. Indeed, OA has been reported to stimulate protein phosphorylation in adipocytes, hepatocytes, and chromaffin cells (14, 25). To determine whether thymocyte phosphorylation was also affected by OA, cells were preloaded with \( [\text{32P}] \)orthophosphate to allow incorporation of the isotope into the nucleotide pool. This was followed by incubation of the cells at 37°C in the presence or absence of OA (5 \( \mu \)M) for the length of time required for full activation of the antiport (10 min, e.g. Fig. 1). The cells were then sedimented and analyzed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate, followed by autoradiography.

A typical autoradiogram is shown in Fig. 5A. While OA induced a marked accumulation of phosphoproteins throughout the range of molecular mass resolved by 10% polyacrylamide gels, the increase was particularly noticeable in the 45–205-kDa range. Similar results were obtained in five experiments. The enhancement induced by OA was even more noticeable when the background phosphorylation that accompanies metabolic labeling was eliminated. This was accomplished by exposing the cells to radiolabel only during the period of incubation with or without OA. For this purpose, \( [\gamma^{32P}] \)ATP was added immediately before the incubation period to electropermeabilized cells, which allow the passage of solutes of molecular weight \( \leq 700 \) while retaining macromolecules (26). As shown in Fig. 5B, this maneuver significantly reduced the labeling of control cells, magnifying the effect of 1 \( \mu \)M OA. Similar results were obtained in four experiments.

The finding that OA, a phosphatase inhibitor, increases phosphoprotein accumulation implies that protein kinases are constitutively active in otherwise untreated cells. One or more of these kinases must be responsible for the stimulation of the antiport. The aim of the experiments described below was to identify the kinase(s) involved in the activation of \( Na^+/H^+ \) exchange by OA. For this purpose, we tested the sensitivity of the pH response to a variety of inhibitors of specific protein kinases.

In lymphocytes, activation of \( Na^+/H^+ \) exchange can be obtained by stimulation of protein kinase C with phorbol esters (11). Because substrates of this kinase are primarily dephosphorylated by phosphatases 1 and 2A, the effects of OA could reflect the existence of constitutively active protein kinase C. This possibility was analyzed using H7, a potent antagonist of protein kinase C (27), which has been shown to inhibit the cytoplasmic alkalization produced by 12-O-tetradecanoylphorbol 13-acetate in thymocytes (10). At 100 \( \mu \)M, a concentration that greatly inhibits the response to phorbol esters (10), H7 failed to inhibit the alkalization induced by OA. In four experiments, the alkalization induced by the phosphatase inhibitor averaged 0.10 ± 0.005 pH units compared to 0.11 ± 0.01 in its absence. Two other protein kinase C inhibitors, trifluoperazine (10 \( \mu \)M) and staurosporine (20 nM), also failed to inhibit the OA-induced stimulation of the antiport, providing further evidence against the involvement of protein kinase C in the activation of the antiport by OA.

In some systems, stimulation of \( Na^+/H^+ \) exchange has been attributed to \( Ca^{2+} \)-calmodulin-dependent kinases, activated in response to an elevation in \( [Ca^{2+}] \) (28). The effects of OA may be mediated by a similar process. This could result from inhibition of dephosphorylation coupled to a constitutively active \( Ca^{2+} \)-dependent kinase or from changes in \( [Ca^{2+}] \), induced by OA. The latter could conceivably occur by phosphorylation of proteins involved in \( [Ca^{2+}] \), homeostasis, with concomitant changes in their transport activity. To analyze the second possibility, the effect of OA on \( [Ca^{2+}] \), was measured using the fluorescent indicator indo-1. As illustrated in Fig. 6B, addition of 1 \( \mu \)M OA to cells in standard Na+ medium did not increase \( [Ca^{2+}] \), during the time required for the appearance of the pH response. Further evidence against a role of \( Ca^{2+} \) was obtained by manipulation of the intracellular \( [Ca^{2+}] \). The alkalization produced by OA was unaffected when the cells were acutely suspended in nominally \( Ca^{2+} \)-free solution (not illustrated), or when they were \( Ca^{2+} \)-depleted by preincubation for 20 min at 37°C in \( Ca^{2+} \)-free medium containing 1 mM EGTA (Fig. 6A). Taken together, these results

![Fig. 5. A. effect of OA on protein phosphorylation in isolated intact thymocytes. Cells were preloaded with \( [\gamma^{32P}] \)orthophosphate for 2.5 h at 37°C. The cells were then resuspended in Na+ medium and incubated at 37°C for 10 min with or without 5 \( \mu \)M OA, as indicated. Samples were analyzed by polyacrylamide gel electrophoresis through the method of Laemmli (21). A typical autoradiogram, representative of five similar experiments, is illustrated. The arrow indicates the location of the \( Na^+/H^+ \) antipport (\( \approx 110 \) kDa), determined by immunoprecipitation of \( [32P] \)-labeled cells. B. effect of OA on protein phosphorylation in isolated electropermeabilized thymocytes. Cells were electropermeabilized in medium devoid of ATP and then incubated in permeabilization medium containing 50 \( \mu \)M ATP, 25 \( \mu \)Ci/ml \( [\gamma^{32P}] \)ATP with or without 1 \( \mu \)M OA, as indicated. After a 6-min incubation at 37°C, the reaction was stopped and the samples were analyzed by polyacrylamide gel electrophoresis as in A. A typical autoradiogram, representative of four similar experiments is illustrated. The arrow indicates the location of the \( Na^+/H^+ \) antipport.](image-url)
Activation of Na+/H+ Antiport by Okadaic Acid

The Introduction, stimulation of the antiport by mitogens and pretreatment with forskolin greatly inhibited the alkalinizing activity. Indeed, using antibodies raised against the carboxylic pend in this medium for pH, recording. OA (1 μM) was added to both samples where indicated. Representative of six similar experiments. 

The latter conclusion was confirmed by experiments testing the effect of the calmodulin antagonist W7 on the OA-induced alkalinization. The pH, response to OA was found to be insensitive to W7 (100 μM), a concentration reported to inhibit calmodulin-dependent processes in a variety of systems (29, 30). Thus, activation of the Na+/H+ antiport by OA is most likely not mediated by a Ca2+/calmodulin-sensitive protein kinase.

In some cell types cAMP can act as a modulator of the Na+/H+ antiport. In fish erythrocytes, activation of the antiport by catecholamines is mediated by cAMP (31). Similarly, elevation of intracellular cAMP levels has been shown to stimulate Na+/H+ exchange activity in murine macrophages (32). It was therefore of interest to test whether cAMP was involved in the OA-induced alkalinization. For this purpose we used H8, a potent inhibitor of cyclic nucleotide-dependent kinases (33). As shown in Fig. 7A, the activation of the antiport by OA was unaffected by the presence of 10 μM H8, suggesting that protein kinase A was not essential to the response. Further evidence was obtained using forskolin, which has been shown to produce a massive increase in intracellular cAMP in thymocytes (10). As illustrated in Fig. 7B, incubation of the cells with the adenylate cyclase activator (100 μM) had no direct effect on pH. More importantly, pretreatment with forskolin greatly inhibited the alkalinizing response to OA:ΔpH = 0.03 ± 0.01 (n = 7) in the presence of forskolin versus 0.12 ± 0.01 pH units (n = 6) in control cells. Together, these findings indicate that cAMP-dependent kinase is unlikely to mediate the effects of OA. Instead, this kinase appears to antagonize the OA response, as was reported for the activation of the antiport by the mitogenic lectin concanavalin-A (10).

Phosphorylation of the Na+/H+ antiport—As discussed in the Introduction, stimulation of the antiport by mitogens and phorbol esters is attributed to activation of protein kinase activity. Indeed, using antibodies raised against the carboxylic domain of the human Na+/H+ antiport, Sardet et al. (2) have recently shown that mitogenic activation of fibroblasts and A431 human epidermoid cells stimulates phosphorylation of the antiport. In order to determine whether activation of Na+/H+ exchange by OA is similarly mediated by direct phosphorylation of the antiport, we performed immunoprecipitation experiments using the above mentioned affinity-purified antibody. Immunoprecipitates obtained from [32P]orthophosphate-labeled thymocytes yielded very weak and inconsistent signals, sufficient to assign a molecular weight to the rat lymphoid antiporter (Fig. 5), but too unreliable for the quantification of the effects of OA on phosphate incorporation. The low yield of the immunoprecipitates could be attributed to scarcity of the antigen in thymocyte membranes and/or, more likely, to poor immunoreactivity of the rodent antigen with the antibody, which was raised against the human antiporter. Differences in the immunoreactivity of antiporters from different species were also reported by Sardet et al. (2).

To circumvent these difficulties, we performed immunoprecipitation experiments in control and OA-treated human cells. For these experiments we utilized a clone of the urinary bladder carcinoma line MGH-U1, which expresses high Na+/H+ exchange activity. To ensure that OA also activates the antiport in these cells, pH, measurements using BCECF were performed first. As shown in Fig. 8A, treatment of MGH-U1 cells with OA (1-2 μM) led to a substantial rise in pH,ΔpH = 0.20 ± 0.05 (n = 6), comparable to that observed in thymocytes. The alkalinization became apparent shortly after addition of the phosphatase inhibitor, and the maximal response was achieved within ~12 min. The OA-induced alkalinization was inhibited by 5-N,N-dimethylamino amiloride (not illustrated). We next analyzed the effect of OA on the phosphorylation of the antiport. Cells were labeled with [32P]orthophosphate, incubated in the presence or absence of OA under conditions leading to activation of the antiport, and then subjected to immunoprecipitation. The immunoprecipitate was analyzed by SDS-polyacrylamide gel electrophoresis and a typical autoradiogram is shown in Fig. 8B. Untreated
Activation of Na\(^+\)/H\(^+\) Antiport by Okadaic Acid

Fig. 8. A, effect of OA on cytoplasmic pH of MGH-U1 cells. B, CECF-loaded MGH-U1 cells grown on a glass coverslip were placed in Na\(^+\) solution, and pH, was measured fluorimetrically as described under "Experimental Procedures." Where indicated, 3 \(\mu\)M OA was added and recording was resumed. Representative of six similar experiments. Temperature, 37 °C. B, effect of OA on phosphorylation of the antiporter in MGH-U1 cells. Cells grown to confluency were preloaded with \(^{32}\)Porthophosphate for 3 h at 37 °C. The cells were then washed with ice-cold phosphate-buffered saline and treated with or without OA (1 \(\mu\)M) for 15 min in Na\(^+\) solution. The reaction was stopped by washing the cells twice with ice-cold saline and freezing in liquid N\(_2\). Proteins were then solubilized and immunoprecipitated as described under "Experimental Procedures." N, nonimmune serum; C, immune serum, control samples; O, immune serum, okadaic acid-treated samples. The immunoprecipitates were analyzed by polyacrylamide (9%) gel electrophoresis and autoradiography. Representative of two similar experiments.

samples (lane C) showed a modest amount of phosphorylation in a diffuse band corresponding to \(\approx\)110 kDa, which was not detectable when using non-immune serum (N). This band resembles the phosphorylated polypeptides detected by Sardet et al. (2), and its molecular weight is close to that predicted for the glycosylated Na\(^+\)/H\(^+\) antiporter (34). OA was found to greatly stimulate the phosphorylation of the immunoprecipitated band (lane O), indicating that inhibition of phosphatase activity leads to enhanced phosphorylation of the antiport, possibly accounting for its increased activity. Integration of scans of autoradiograms from two experiments showed that OA treatment led to a 3-fold increase in the intensity of the 110-kDa band. It must be pointed out that OA increased the phosphorylation of contaminating bands that coprecipitated nonspecifically with the antiport during immunoprecipitation. The background contributed by such nonspecific radioactivity flanking the 110-kDa band, however, was subtracted when integrating the autoradiograms.

DISCUSSION

The objective of this study was to assess the effect of OA, a potent and specific inhibitor of protein phosphatases 1 and 2A (35), on cytosolic pH and protein phosphorylation in rat thymic lymphocytes. At micromolar OA, OA was found to produce a substantial and persistent intracellular alkalization (Fig. 1). The Na\(^+\) dependence of this response and its sensitivity to amiloride analogs (Fig. 2, A and B) strongly support the notion that the effect of the phosphatase inhibitor is mediated by activation of the Na\(^+\)/H\(^+\) antiport. OA appears to activate the antipore by the same mechanism as other stimuli such as mitogenic agents or osmotically induced shrinking, i.e., by shifting its pH\(_{\text{dependence}}\) to more alkaline levels.

Several lines of evidence support the view that the activation of Na\(^+\)/H\(^+\) exchange by OA in lymphocytes is mediated by inhibition of protein phosphatase activity. First, maximal effects were obtained at 1 \(\mu\)M OA, which is similar to the concentration required for maximal inhibition of protein phosphatase 1 (IC\(_{50}\) = 10–20 nM) as well as the more sensitive protein phosphatase 2A (IC\(_{50}\) = 0.1–0.2 nM) (13, 14). Second, stimulation of the Na\(^+\)/H\(^+\) antipore by OA was stringently dependent on the availability of cellular ATP (Fig. 4). Third, as reported for other cell types (14, 25), treatment with OA was found to produce a marked accumulation of phosphoproteins in thymocytes (Fig. 5, A and B). Finally, immunoprecipitation experiments revealed that OA treatment greatly stimulates the phosphorylation of the antipore itself (Fig. 8). In a recent report, Sardet et al. (2) demonstrated that activation of hamster fibroblasts and A431 human epidermoid cells with growth factors or phorbol esters similarly leads to an increased phosphorylation of the antipore. Taken together, these observations suggest that the shift in the pH\(_{\text{dependence}}\) of the modifier site leading to activation of the antipore is mediated by phosphorylation of the transporter. However, given the complexity of the effects of okadaic acid, as evident from the phosphorylation patterns (Fig. 5), this conclusion should be regarded as tentative and other possible mechanisms should not be discounted at present.

The finding that the antipore is activated by addition of OA alone, a purportedly specific phosphatase inhibitor, implies that one or more kinases are constitutively active in otherwise unstimulated cells. Experiments using inhibitors suggested that protein kinase C and Ca\(^{2+}\)-calmodulin-dependent kinases are not responsible for the effects of OA. Similarly, an inhibitor of cyclic nucleotide-dependent kinases failed to prevent the effect of OA. On the contrary, treatment of the cells with forskolin at concentrations that increase intracellular cAMP levels was found to greatly inhibit the OA-induced activation of Na\(^+\)/H\(^+\) exchange. An inhibitory effect of forskolin was earlier reported on the activation of the antipore by concanavalin A (10), suggesting that a common pathway is utilized by mitogens and by the phosphatase inhibitor. The following explanations may account for the inhibitory effect of cAMP on the OA-induced stimulation of the antipore. First, cAMP-dependent kinase may activate an OA-insensitive phosphatase. Protein phosphatases can indeed be regulated by cAMP, but the effects of the cyclic nucleotide reported so far are inhibitory (36). Alternatively, the constitutively active kinase unmasked by OA could be inhibited by cAMP. Finally, in addition to the activating phosphorylation site, the antipore may possess a protein kinase A substrate site with inhibitory effects on transport. These alternatives should be resolved in the future by detailed quantitation of phosphorylation and phosphopeptide mapping of the antipore isolated from cells treated with OA in the presence and absence of forskolin.

In view of the results summarized above, the identity of the kinase(s) responsible for the stimulatory effects of OA remains to be elucidated. Inasmuch as the OA-sensitive phosphatases 1 and 2A are generally regarded as typical P-Ser/P-Thr phosphatases (37), the relevant kinase would be anticipated to be a Ser/Thr kinase. Indeed, Ser is the sole residue of the antipore phosphorylated by epidermal growth factor and thrombin in cultured cells (2). However, caution must be exercised in predicting the phosphorylated residues, since recent studies showed that OA treatment of fat cells can also lead to an increase in tyrosine phosphorylation (38).

The Na\(^+\)/H\(^+\) antipore appears to be the target of multiple signaling pathways, including those activated by tyrosine kinase receptors and by G-protein-coupled phosphoinositide metabolism. The similarity of the responses elicited by such diverse pathways would suggest convergence of the pathways at a common site. This putative site could be the antipore itself or, alternatively, a preceding step. Mitogen-activated protein (MAP) kinase and the proto-oncogene product Raf-1 are seemingly ubiquitous Ser-Thr protein kinases that can be activated by a variety of mitogens (39–41). These kinases can be activated by Tyr and/or Ser and Thr phosphorylation (41–43), suggesting a role in the integration of signals from distinct pathways.
transduction pathways. Interestingly, a kinase of 42 kDa, with properties consistent with mitogen-activated protein kinase 2, was recently found to be activated in cells treated with OA (38, 44, 45). It is therefore tempting to suggest that mitogen-activated protein kinase 2 or a closely related kinase is responsible for the activation of the antiport by the phosphatase inhibitor.

The experiments using OA also shed some light on the mechanism underlying the osmotic activation of the antiport. The hypertonic and OA-induced responses are not additive (Fig. 3) and both seem to require ATP (Fig. 4, A and B), suggesting that a common pathway may mediate the two effects. Interestingly, whereas ATP depletion was found to greatly (but not completely) inhibit the response to hypertonic stress, pretreatment of ATP-depleted cells with OA was shown to restore the response to hypertonicity (Fig. 4C). This observation could be explained by assuming that hypertonicity activates the phosphorylation of substrates that are normally dephosphorylated by phosphatases 1 and/or 2A. In the absence of okadaic acid, phosphorylation in ATP-depleted cells would be insufficient to activate the antiport. When the phosphatases are inhibited by OA, however, the residual ATP (about 3% of the normal content) would suffice to phosphorylate and thereby stimulate the Na+/H+ antiport.

It is noteworthy that, unlike the OA-induced stimulation of the antiport, activation by hypertonic stress was not impaired by pretreatment with forskolin (results not shown). Thus, while the osmotic effects and those of the phosphatase inhibitor are similar, the underlying mechanisms are not identical. If a common pathway does exist, the hypertonic effect must be exerted downstream of the site inhibited by cAMP. Experiments designed to evaluate the effect of osmotic shrinking on the phosphorylation of the antiport are currently underway.

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