Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 111-117

CYCLOSPORIN A INHIBITS KINASE C-INDEPENDENT ACTIVATION OF THE Na^+/H^+ EXCHANGER BY PDGF AND VANADATE

Thomas O. Daniel[1,2] and Harlan E. Ives[1,3]

Division of Nephrology, [1]Cardiovascular Research Institute and [2]Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA 94143

Received April 10, 1987

SUMMARY: Mitogen-induced activation of Na^+/H^+ exchange was studied in Swiss 3T3 fibroblasts. Phorbol myristic acetate (PMA) caused amiloride inhibitable cell alkalinization. PDGF and vanadate, but not bombesin or thrombin, caused additional alkalinization when given 10 min after a maximal dose of PMA. Down-regulation of kinase C by 24 hr PMA exposure prevented the alkalinization response to bombesin and thrombin, but not to PDGF or vanadate. Cyclosporin A specifically blocked the additional alkalinization after PDGF or vanadate in cells acutely exposed to PMA and in kinase C down-regulated cells. Thus, there are at least two independent pathways which activate Na^+/H^+ exchange. PMA, bombesin, and thrombin act via kinase C. PDGF and vanadate cause additional stimulation of the Na^+/H^+ exchanger by a kinase C-independent pathway, inhibitable by cyclosporin A.

Intracellular alkalinization resulting from the action of an amiloride inhibitable Na^+/H^+ exchanger is a common response to agents which have mitogenic activity (for reviews, see 1,2). The biochemical pathways mediating this alkalinization response are still not well defined. Because activation of Na^+/H^+ exchange accompanies commitment to cell division, understanding the mechanisms that regulate Na^+/H^+ exchange may provide further insight

[2] Present Address: Division of Nephrology, Vanderbilt Univ., Nashville, TN 37232.

[3] To whom correspondence should be addressed: Box 0532, 1065 HSE, Univ. of California, San Francisco, SF, CA 94143.

Abbreviations: PMA, phorbol-12-myristate-13-acetate; PDGF, platelet derived growth factor; BCECF, (2',7')-bis(carboxyethyl)-(5,6)-carboxyfluorescein.

0006-291X/87 $1.50

to the biochemical events coupling growth factor binding with cell division.

Since the Na^+/H^+ exchanger is activated both by agents which generate diacylglycerol and by biologically active phorbol esters, it has been proposed that kinase C is involved in the activation of Na^+/H^+ exchange (1-4). However, activation of the Na^+/H^+ exchanger by epidermal growth factor has been observed under conditions where kinase C is already maximally activated by phorbol esters (5) or down-regulated by phorbol esters (6). This suggests a kinase C-independent mechanism for activation of the Na^+/H^+ exchanger.

We provide additional evidence that some mitogens exhibit a kinase C-independent mechanism for activation of the Na^+/H^+ exchanger and show that cyclosporin A blocks specifically this pathway.

MATERIALS AND METHODS

Materials. Unless otherwise specified, all materials were reagent grade and were obtained from Sigma. BCECF-AM was obtained from Molecular Probes (Junction City, OR), ionomycin was obtained from Calbiochem (LaJolla, CA), BSA was fraction V, fatty acid poor Pentex albumin (Miles Scientific, Naperville, IL), bombesin was from Peninsula Labs (Belmont, CA) and PDGF was a gift from L.T. Williams. Cyclosporin A was a gift from Sandoz, Inc. (East Hanover, NJ)

Cell Culture. Swiss 3T3 fibroblasts (65 - 72 passage, American type culture collection) were maintained in Dulbecco modified Eagle's medium with 10% fetal calf serum. Cells for experimentation were grown to confluence in Leighton tubes (Costar) without medium change for the 4 days prior to experimentation.

Intracellular pH Measurement. The 1 cm wide plastic strip containing the confluent monolayer of cells was removed from the Leighton tubes and cut to 3 cm length. These plastic strips were mounted at a 60° angle in 1 x 1 cm acrylic fluorescence cuvettes (Sarstedt) with a magnetic stirrer below the cell strip. Cells were washed 3 times and maintained at 37° C in medium consisting of 140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 25 mM glucose, 25 mM Hepes/NaOH (pH 7.20), and 0.5 mg/ml BSA. Cells were loaded with 5 μM BCECF for 20 min and washed twice with the incubation medium. Fluorescence (excitation 440 or 505 nm, emission 530 nm) was measured with an SLM (Urbana, IL) 4800 spectrofluorometer in steady state mode. Background light scattering and autofluorescence was < 10% of the fluorescence signal and was subtracted during data analysis. Data was recorded and stored in an IBM PC XT computer. At the end of each experiment the dye response was calibrated by changing medium to a similar incubation solution, but with 140 mM KCl, 5 mM NaCl, and 10 ug/ml nigericin. pH of this medium was changed to 7.2, 7.0, then 6.8 and fluorescence at 440 and 505 nm excitation was measured at each pH. Under these conditions, intracellular pH is equal to extracellular pH (7).

RESULTS

Intracellular pH was measured in Swiss 3T3 fibroblasts after stimulation by 100 ng/ml PMA (Fig. 1). Subsequent addition of 100 ng/ml PMA had no additional effect on cell pH. When given after this dose of PMA, bombesin and thrombin caused cell acidification and recovery to the higher baseline pH, but caused no additional net alkalinization. In contrast, PDGF and vanadate both induced an additional net increase in cell pH after maximal stimulation by PMA. This second alkalinization was blocked by 60 μM dimethylamiloride (data not shown).

In the next series of experiments, kinase C activity was down-regulated by 24 hr. exposure to 100 ng/nl PMA (8). Under these conditions, PMA did not cause cell alkalinization (Fig. 2).

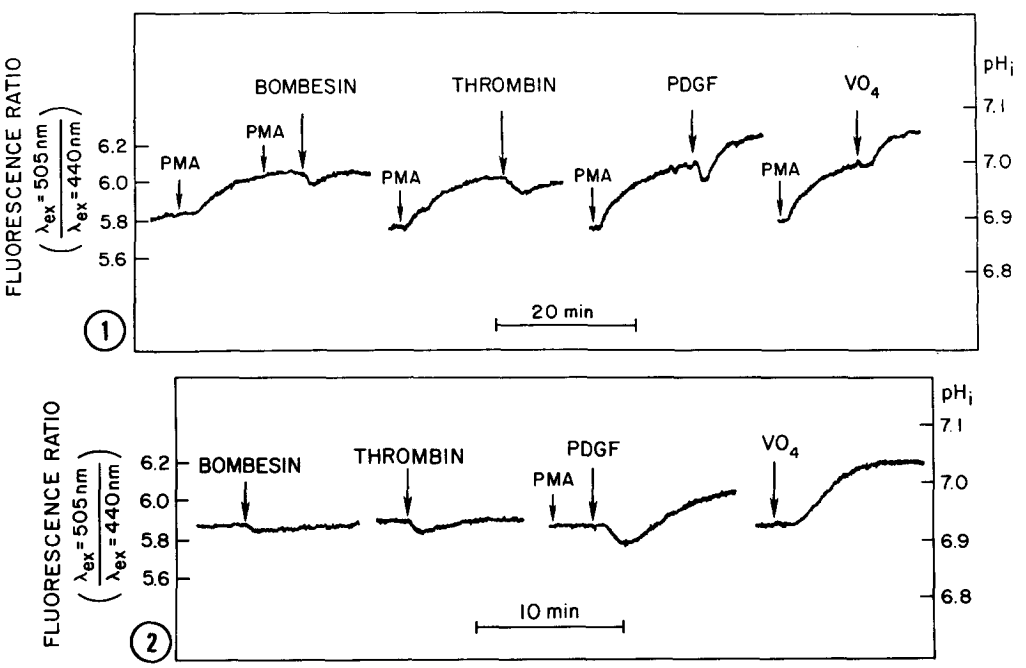

Fig. 1. Intracellular pH changes after exposure to mitogens. BCECF-loaded cells were exposed to 100 ng/ml PMA. Subsequent addition of 100 ng/ml PMA had no additional effect. After stabilization of pH_i, 1.5 x 10^{-9} M bombesin, 0.4 U/ml thrombin, 7.5 nM PDGF, or 100 uM sodium orthovanadate was added at the indicated times. PDGF was electrophoretically pure; in other experiments, partially purified PDGF gave identical results. Fluorescence ratio was measured and pH_i was calculated as described in METHODS. Results are typical of 3 similar experiments.

Fig. 2. Effect of kinase C down-regulation on pH_i response to mitogens. Cells were exposed to 100 ng/ml PMA for 24 hr. prior to experimentation. Concentrations of agonists were the same as in Fig. 1. To show that cells were deficient in kinase C (8), 100 ng/ml PMA is shown to have no effect on cell pH. Result is typical of 5 similar experiments.

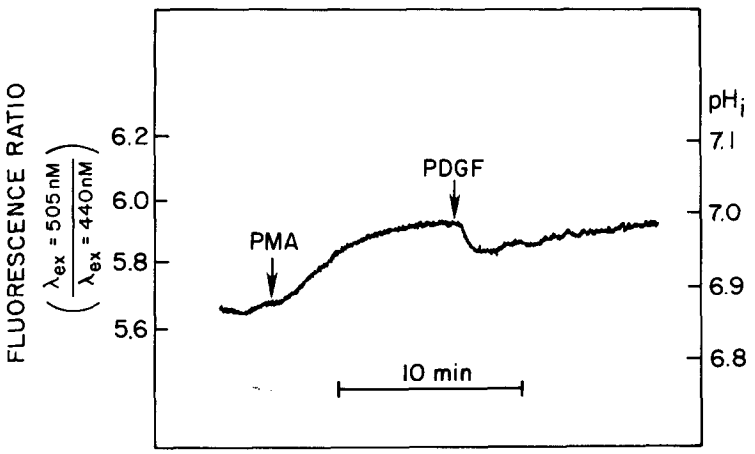

Fig. 3. Effect of cyclosporin A on pH_i response to PMA and PDGF. Cells were exposed to 2 uM cyclosporin A. 5 min. later, 100 ng/ml PMA was added. After stabilizaiton of pH_i, 7.5 nM PDGF was added. Result is typical of 3 similar experiments (see Table 1).

Similar to what was found after maximal PMA exposure, bombesin and thrombin caused slight acidification and recovery of cell pH, but no net alkalinization in kinase C down-regulated cells. In contrast to this, PDGF and vanadate both caused significant increases in cell pH. The experiments shown in Figs. 1 and 2, when taken together, provide evidence for a pathway for activation of the Na^+/H^+ exchanger which is independent of kinase C.

The immunosuppressive cyclic peptide, cyclosporin A, has been reported to prevent activation of the Na^+/H^+ exchanger by anti T3 receptor antibodies in T cells (9). In Swiss 3T3 cells, cyclosporin A (1-5 μM) had no apparent effect on activation of the Na^+/H^+ exchanger by PMA. However, cyclosporin A prevented the additional alkalinization observed with PDGF or vanadate when given after PMA (Fig. 3, Table 1). In kinase C down-regulated cells, 5 μM cyclosporin A prevented the alkalinization response to both PDGF and vanadate (Fig. 4).

Cyclosporin A, at the concentrations used above, had no direct effect on the Na^+/H^+ exchanger, as assessed by the rate of recovery from an intracellular acid load (.022 $\pm$ .007 pH unit/min in controls, .020 $\pm$ .004 pH units/min after wash out of 2mM NH_4Cl). It also did not affect cell alkalinization in response to 5 uM monensin, an ionophore with Na^+/H^+ exchange activity (0.46 $\pm$ 0.03 pH units in control, 0.48 $\pm$ 0.05 pH units after 5 μM cyclosporin A). Thus, cyclosporin A does not alter the transmembrane gradients driving Na^+/H^+ exchange.

Table 1. Effect of cyclosporin A on pH_i response to PMA and PDGF

Cyclosporin A (uM)	delta pH_i (PMA)	delta pH_i (PDGF)
0	.11 ± .01	.05 ± .01
1	.09 ± .01	.01 ± .005 *
2	.12 ± .01	.01 ± .004 *
5	.11 ± .01	0 ± .007 *

* Significantly different from control ($p<.05$, N=3 in all cases)

BCECF-loaded cells were incubated with the indicated concentrations of cyclosporin A for 5 min. 100 ng/ml PMA was added, the pH_i was allowed to stabilize and delta pH_i was determined as the difference in pH_i before and after addition of PMA. Then, 7.5 nM PDGF was added and additional delta pH_i was determined.

DISCUSSION

These experiments provide evidence for two independent pathways for activation of the Na^+/H^+ exchanger. The first pathway most likely involves kinase C, since it can be activated by PMA and obliterated by kinase C down-regulation. Bombesin and thrombin, both of which cause increased kinase C activity (10,11), both fail to stimulate the Na^+/H^+ exchanger after kinase

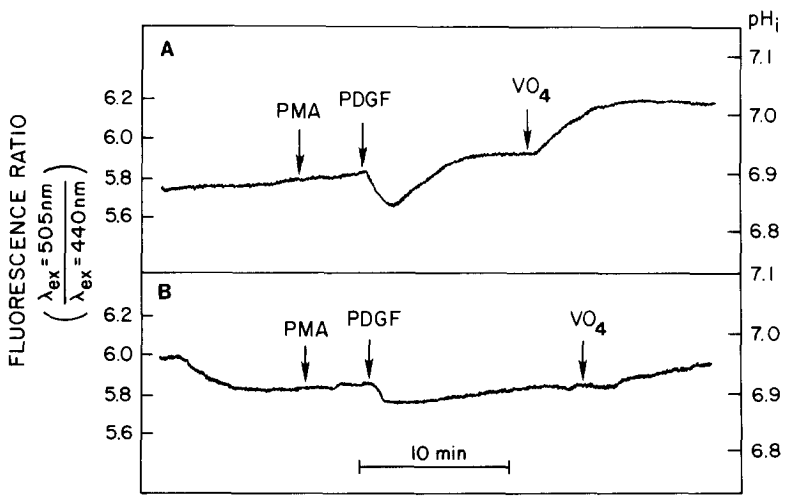

Fig. 4. Effect of cyclosporin A on pH_i response in kinase C down-regulated cells. Cells were exposed to 100 ng/ml PMA for 24 hr. In A, cells were given 100 ng/ml PMA, 7.5 nM PDGF, and 100 uM sodium orthovanadate at the indicated times. In B, cells were treated with 2 uM cyclosporin A 5 min before addition of PMA. Lack of response to PMA shows that cells are deficient in kinase C. Result is typical of 3 similar experiments.

C is either maximally stimulated or down-regulated. Thus, these two agents appear to exert their effects on the Na^+/H^+ exchanger exclusively through kinase C. In contrast, PDGF (which also activates kinase C) and vanadate (which does not activate kinase C (12)) both cause additional amiloride inhibitable increases in cell pH after kinase C is maximally stimulated or down-regulated. This argues for the existence of a kinase C independent pathway for activation of the Na^+/H^+ exchanger, stimulated only by certain agents like PDGF and vanadate.

Kinase C-independent activation of the Na^+/H^+ exchanger has been suggested by others (5,6,9,12). The data presented here demonstrate an additional distinction between the two mechanisms for activating the Na^+/H^+ exchanger - inhibition of the kinase C-independent mechanism by cyclosporin A. In T cells, cyclosporin A blocks cell alkalinization induced by T3 receptor antibodies but not by PMA (9). In both systems, the intact response to PMA demonstrates that the Na^+/H^+ exchanger remins functional and responsive to activation. In addition, we demonstrate that the ion gradients for Na^+/H^+ exchange remain intact after cyclosporin A. Thus, both kinase C-dependent and cyclosporin A inhibitable, kinase C-independent activation of the Na/H exchanger has been found in two distinct systems.

Activation of the Na^+/H^+ exchanger is associated with stimuli which cause cell division in many systems. Therefore, cyclosporin A may be a useful tool for investigation of cellular events that are a part of growth factor signal transduction , but separate from kinase C.

ACKNOWLEDGMENTS

We would like to thank Floyd C. Rector, Jr. and Lewis T. Williams for their support and criticisms of this work. This research was supported by N.I.H. grants AM-34127, AM-27045, HL-32898, A.H.A. grant-in-aid 83-1107, and by the Howard Hughes Medical Institute. Parts of this work were presented in preliminary form at the December 7, 1986 meeting of the American Society for Nephrology in Washington, D.C.

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