Vol. 134, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 14, 1986 Pages 8-13

OSMOTIC ACTIVATION OF THE Na^+/H^+ ANTIPORT IN PROTEIN KINASE C-DEPLETED LYMPHOCYTES

Sergio Grinstein[*q], Esther Mack[*] and Gordon B. Mills[S]

[*]Department of Cell Biology, The Hospital for Sick Children, 555 University Ave.,
[q]Department of Biochemistry, University of Toronto and
[S]Division of Oncology, Toronto General Hospital
Toronto, Canada, M5G 1X8

Received November 8, 1985

SUMMARY. The Na^+/H^+ antiport of rat thymic lymphocytes is activated when protein kinase C is stimulated by phorbol esters. A similar activation of the antiport is obtained when the cells are treated with hypertonic solutions. We tested the possibility that protein kinase C also mediates the osmotic activation of Na^+/H^+ exchange. Protein kinase C was depleted by preincubation of thymocytes for 24 hr in the presence of high concentrations of phorbol ester. Disappearance of the enzyme was assessed by direct measurement of phosphotransferase activity, and by the loss of biological responses to phorbol esters. The Na^+/H^+ antiport in protein kinase C-depleted cells was not stimulated by addition of phorbol ester, but responded normally to hypertonic treatment. The results indicate that the osmotic activation of countertransport does not require stimulation of protein kinase C.

An amiloride-sensitive, electroneutral Na^+/H^+ exchanger has been detected in the plasma membranes of most mammalian cell types (see 1 and 2 for review). This antiport is thought to be essential for pH_i[a] regulation (3), and transepithelial ion transport (4) and may play a role in the initiation of mitogenesis (5).

In resting lymphocytes the antiport is nearly quiescent at physiological pH_i, but becomes activated by addition of tumor-promoting phorbol esters (6). The primary target for phorbol esters, which are structural analogs of diacylglycerol, is the Ca^{2+}- and phospholipid-dependent protein kinase C (7,8). Several lines of evidence suggest that in lymphocytes the activation of Na^+/H^+ exchange by phorbol esters is mediated by stimulation of protein kinase C: a) the concentrations of TPA required for activation of countertransport are similar to those reported to activate the kinase; b) only those phorbol derivatives that accelerate kinase activity have an effect on Na^+/H^+ exchange (6) and c) the antiport is also stimulated by diacylglycerol (9), the putative physiological activator of protein kinase C.

In erythrocytes (10,11) and epithelial cells (12), as well as in lymphocytes (9,13), the Na^+/H^+ antiport can also be stimulated at normal pH_i by osmotic cell shrinking. In this instance,

[a]ABBREVIATIONS: pH_i: cytoplasmic pH; TPA: 12-O-tetradecanoylphorbol 13,acetate; BCECF: 2',7' bis(carboxyethyl)-5,6 carboxyfluorescein.

0006-291X/86 $1.50

Na^+/H^+ exchange appears to play a role in volume regulation by inducing reswelling of shrunken cells (11). The osmotic activation of the antiport bears a remarkable resemblance to that produced by phorbol esters: both responses are attributable to an alkaline shift in the pH_i dependence of transport (9,13). Moreover, both are blocked by depletion of ATP or by addition of trifluoperazine or N-ethylmaleimide (13). These similarities led to the suggestion that the osmotic activation of the antiport might also be due to activation of protein phosphorylation, perhaps mediated by protein kinase C (13).

It was recently reported that prolonged incubation of fibroblasts with phorbol esters causes a decrease in the number of phorbol ester-binding sites (14) and in the activity of protein kinase C (15). Further, the cells become unresponsive to the biological effects normally elicited by phorbol esters in untreated cells (16). These protein kinase C-depleted cells provide a useful model to test the involvement of this enzyme in the osmotic activation of Na^+/H^+ exchange. Disappearance of the osmotic response along with the kinase would be indicative of a role of this enzyme in cell volume regulation. We here report that down-regulation of protein kinase C by phorbol esters can also be induced in rat thymic lymphocytes, and that the antiport in kinase-depleted cells fails to respond to activation by TPA. In contrast, the osmotic activation persists in these cells.

MATERIALS AND METHODS

Materials and solutions. Histone type III-S, TPA and phosphatidylserine were obtained from Sigma Chemical Co. N-methyl-D-glucamine was from Aldrich. Medium RPMI 1640 (HCO_3^--free), and 100X concentrated antibiotic-antimycotic mixture were from GIBCO. [gamma-^{32}P]-ATP was from ICN. BCECF acetoxymethylester was purchased from Molecular Probes. Amiloride was the kind gift of Merck Frosst Canada. Na^+-solution contained (in mM): 140 NaCl, 1 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 20 Hepes (pH 7.3). Na^+-free solution contained 140 mM N-methyl-D-glucamine chloride instead of NaCl, but was otherwise identical.

Methods. Thymocytes were isolated from male Wistar rats (150-200 g), counted and maintained in Hepes-buffered RPMI 1640 as previously described (9,13). For overnight incubations, the medium was supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone.

For pH_i measurements the cells were sedimented, resuspended at 40×10^6/ml and loaded with BCECF by incubation with the parent acetoxymethylester (2-3 µg/ml) for 30 min at 37°C. pH_i was determined fluorimetrically at 37°C using a Perkin-Elmer 650-40 spectrofluorimeter and calibrated with nigericin KCl as described (9,13).

For protein kinase C activity determinations, the cells were sedimented, washed and lysed by resuspension in a medium containing 0.1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonylfluoride, 10 mM mercaptoethanol and 20 mM Tris.HCl, pH7.5. The suspension was homogenized by 20 strokes in a Dounce-type homogenizer, and then centrifuged at 4°C for 30 min at 48,000 x g. The supernatant was fractionated with $(NH_4)_2SO_4$ as described by Niedel et al (17). The fraction precipitated between 33% and 70% saturation, which contains all the protein kinase C in the extract (15,17), was resuspended at 1-3 mg protein/ml in a medium containing 0.1 mM EDTA, 50 mM mercaptoethanol, 1 mM EGTA, 25% glycerol and 20 mM Tris.HCl, pH 7.5. The fractionation step serves to concentrate the kinase and to eliminate low molecular weight effectors present in the crude extract (15,17). For comparison, in some experiments the unfractionated extract was used directly for kinase activity measurements.

Protein kinase C activity was determined by measuring the incorporation of ^{32}Pi from [gamma-^{32}P]-ATP into histone type III-S. The assays were performed for 3 min at 30°C using

5-20 µg protein in a total volume of 250 µl of medium containing 0.5-1.0 µCi [gamma-^{32}P]-ATP, 100 µM ATP, 20 mM magnesium acetate, 1 mM $CaCl_2$, 50 µg histone and 20 mM Tris.HCl, pH 7.5. The determinations were performed in triplicate in the presence and absence of TPA (10^{-7}M, final) and phosphatidylserine (24 µg per assay), to define the protein kinase C-mediated phosphorylation. The reaction was terminated by addition of 1 ml of ice-cold 25% trichloroacetic acid. The precipitated histone was then separated by filtration on 0.45 µm HA Millipore filters, followed by four washes, each with 3 ml of 5% trichloroacetic acid. The filters were counted by liquid scintillation.

RESULTS AND DISCUSSION

Table 1 summarizes measurements of protein (histone) kinase activity in extracts obtained from cells incubated for 24 hr in the presence and absence of 2×10^{-7} M TPA. The cells were extracted with Triton X-100 and fractionated with $(NH_4)_2 SO_4$ as described by Niedel et al (17) and Rodriguez-Peña and Rozengurt (15), to remove phospholipids and soluble effectors. The assays were performed in the presence and absence of phosphatidylserine and TPA, to define the fraction of the kinase activity attributable to protein kinase C. In control cells, protein kinase C accounts for approximately 40% of the total phosphotransferase activity. In contrast, only a very small, statistically insignificant fraction of the activity is due to protein kinase C in TPA-treated cells. This reduction is not due to carryover of TPA and/or phospholipid by the TPA-treated cells, since the basal activity (-PL in Table 1) is not increased. Moreover, the total phosphotransferase activity (+PL in Table 1) is decreased in the treated cells, consistent with disappearance of protein kinase C activity. Qualitatively similar results were obtained when the unfractionated Triton X-100 extract was used for the assays, indicating that the TPA-induced decrease in activity is not artifactually generated by the fractionation procedure. These data resemble those reported for 3T3 cells (15) and are consistent with functional down-regulation of protein kinase C upon prolonged incubation with phorbol esters.

Table 1

Determination of protein kinase C activity in control and TPA-treated cells

	-PL	+PL	▲PL
	(pmol ^{32}P/min/mg protein)		
Control	880 ± 124	1403 ± 120	523
TPA-treated	657 ± 118	714 ± 265	57

Thymocytes (20×10^6/ml) were incubated for 24 hr at 37°C in Hepes-buffered medium RPMI 1640 with or without 2×10^{-7} M TPA. After washing, the cells were lysed in Triton X-100 and a protein kinase C-rich fraction was isolated by $(NH_4)_2 SO_4$ fractionation as described (15; see Methods). Protein kinase was measured for 3 min at 30°C in the presence (+PL) or absence (-PL) of phosphatidylserine and TPA as described under Methods. ▲PL refers to the phosphatidylserine and TPA-induced kinase activity, i.e. protein kinase C. The data are means ± SE of six determinations.

Further evidence for the disappearance of functional protein kinase C was obtained by measuring the effects of TPA on Na^+/H^+ countertransport. The activity of the antiport was assessed by measuring pH_i, since activation has been reported to result in cytoplasmic alkalinization (6,9). As shown in Fig. 1, cells incubated in the absence of TPA for 24 hr respond to the addition of the phorbol ester with alkalinization (top trace, Fig 1A), as has been reported for freshly isolated cells (6). That this alkalinization is due to stimulation of the Na^+/H^+ antiport is evidenced by its requirement for external Na^+ (the response is absent in N-methyl-D-glucamine$^+$ solution; bottom trace, Fig 1A) and by its sensitivity to amiloride, an inhibitor of the antiport (middle trace, Fig 1A).

The pH_i of TPA-treated cells was typically ≈ 0.1 units lower than that of untreated controls (in three preparations pH_i averaged 7.11± 0.03 [SEM] in control cells and 6.98 ± 0.05 in phorbol ester-treated cells). This indicates that the TPA-induced alkalinization did not persist after 24 hr. This could be attributed to washout of the phorbol ester during the BCECF-loading and resuspension procedures, or alternatively to disappearance of functional protein kinase C. That the latter explanation is likely correct is indicated by the experiment shown in Fig. 1C. Addition to the chronically phorbol ester-treated cells of 10^{-7} M TPA, a concentration that produces maximal stimulation in control cells (6), failed to elicit a cytoplasmic alkalinization. An alkalinization

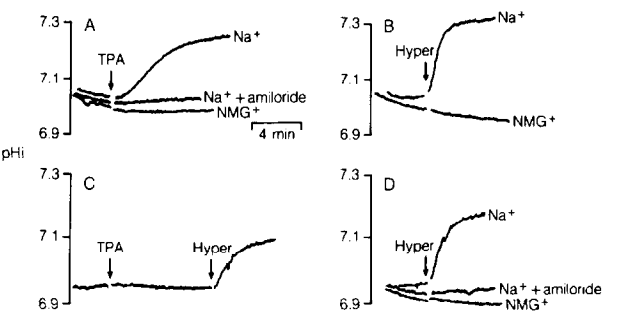

Fig. 1. Effects of TPA and hypertonicity on pH_i in control and protein kinase C-depleted rat thymic lymphocytes. Thymocytes were preincubated for 24 hr at 37°C in the presence (C-D) or absence (A-B) of 2 x 10^{-7}M TPA. The cells were then washed, resuspended and loaded with BCECF by incubation with the parent acetoxymethylester. After one additional wash and resuspension, the cells were used for pH_i determination as described under Methods. A) Control cells were suspended in either Na^+-medium, with or without 200 µM amiloride, or in N-methyl-D-glucamine$^+$ medium (NMG^+). Where indicated, 10^{-7}M TPA (final) was added to the cuvette; B) Control cells were suspended in Na^+ or N-methyl-D-glucamine$^+$ media (isotonic). Where indicated, the media were made hypertonic (450 ± 5 mosM) by addition of a small volume of concentrated NaCl or N-methyl-D-glucamine chloride, respectively. Similar results were obtained when N-methyl-D-glucamine chloride was used to make the Na^+-medium hypertonic (not shown; see ref. 13); C) Cells preincubated for 24 hr with TPA prior to BCECF loading and washing were suspended in Na^+-medium. Where indicated, 10^{-7} M TPA was added. Finally, the solution was made hypertonic by addition of concentrated NaCl; D) Cells preincubated in TPA for 24 hr were suspended in Na^+-medium with or without 200 µM amiloride or in N-methyl-D-glucamine$^+$ medium. Where indicated, the solutions were made hypertonic with the appropriate solute as above. The traces are representative of three separate experiments. The time scale applies to A-D. Temperature: 37°C.

could be induced in these cells by osmotic shrinking (Fig 1C; see discussion below), indicating that the antiport remains functional and responsive to stimulation following addition of the phorbol ester. Thus, this biological effect of the phorbol ester receptor(s) disappears concomitantly with the decrease in protein kinase C activity.

As shown in Fig. 1B, increasing the osmolarity of the medium from 290 ± 5 mosM to 450 ± 5 mosM by addition of NaCl resulted in activation of the antiport in untreated cells. As reported for fresh cells (13), the resulting alkalinization is eliminated when extracellular Na^+ is replaced by N-methyl-D-glucamine$^+$ (Fig. 1B) or by addition of amiloride (not illustrated), indicating stimulation of Na^+/H^+ countertransport. A distinct alkalinization was also observed when cells chronically treated with TPA were challenged osmotically (Fig. 1D). The alkalinization was similarly Na^+-dependent and amiloride-sensitive (Fig. 1D, middle and bottom traces), indicating activation of the Na^+/H^+ antiport. The magnitude of the osmotic response was similar in untreated (ΔpH_i= 0.26 ±0.02) and in TPA-treated cells (ΔpH_i = 0.20 ± 0.03).

The present results indicate that the osmotic activation of the Na^+/H^+ exchanger can be accomplished in cells largely devoid of functional protein kinase C, ruling out a role of this enzyme in the stimulation of the antiport during volume regulation. This conclusion is consistent with recent observations that migration of cytoplasmic kinase C to the membrane, which is indicative of activation of this enzyme (18), was obtained when the cells were treated with TPA but not with hypertonic solutions (Grinstein et al, submitted for publication). Moreover, osmotic shrinking did not decrease the cellular content of phosphatidylinositol 4,5 bisphosphate or increase the levels of inositol trisphosphate, indicating that phospholipase C does not liberate diacylglycerol from phosphoinositides under these conditions. It must be concluded that at least two distinct mechanisms can activate the Na^+/H^+ antiport in lymphocytes: phosphorylation of the exchanger or an ancillary protein by protein kinase C, and an unidentified process triggered by osmotic cell shrinking, which neither stimulates phospholipase C activity nor requires the presence of functional protein kinase C. The two pathways must share the final step, inasmuch as the phorbol ester- and osmotically-induced responses are not additive (9). This conclusion resembles that obtained for the growth factor-induced stimulation of the antiport, which also appears to be unrelated to protein kinase C (16).

ACKNOWLEDGMENTS

Supported by the National Cancer Institute (Canada) and the Medical Research Council (Canada). S. Grinstein is the recipient of a Medical Research Council Scientist award.

REFERENCES

1. Aronson, P. S. (1985) Annu. Rev. Physiol. 47, 545-560.
2. Mahnensmith, R. L., and Aronson, P. S. (1985) Circ. Res. 56, 773-788.
3. Boron, W. F. (1983) J. Membrane Biol. 72, 1-1.
4. Aronson, P. S. (1983) Am. J. Physiol. 245, F647-F659.
5. L'Allemain, G., Franchi, A., Cragoe, E., and Pouyssegur, J. (1984) J. Biol. Chem. 259, 4313-4319.

6. Grinstein, S., Cohen, S., Goetz, J. D., Rothstein, A., and Gelfand, E. W. (1985) Proc. Nat. Acad. Sci. (USA) 82, 1429-1433.
7. Nishizuka, Y. (1984) Science 225, 1365-1370.
8. Berridge, M. J. (1984) Biochem. J. 220, 345-360.
9. Grinstein, S., Cohen, S., Goetz, J. D., and Rothstein, A. (1985) J. Cell Biol. 101, 269-276.
10. Parker, J. C., and Castranova, V. (1984) J. Gen. Physiol. 84, 379-401.
11. Cala, P. M. (1985) Fed. Proc. 44, 2500-2507.
12. Spring, K. R., and Ericson, A. C. (1982) J. Membrane Biol. 69, 167-176.
13. Grinstein, S., Cohen, S., and Rothstein, A. (1985) J. Gen. Physiol. 85,765-787.
14. Collins, M. K. L., and Rozengurt, E. (1984) J. Cell. Physiol. 118, 133-142.
15. Rodriguez-Pena, A., and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. 120, 1053-1059.
16. Vara, F., and Rozengurt, E. (1985) Biochem. Biophys. Res. Commun. 130, 646-653.
17. Niedel, J. E., Kuhn, L. J., and Vandenbark, G. R. (1983) Proc. Nat. Acad. Sci. USA 80, 36-40.
18. Kraft, A. S., and Anderson, W. B. (1983) Nature 301, 621-624.