Phorbol Esters Rapidly Stimulate Amiloride-sensitive Na^+/H^+ Exchange in a Human Leukemic Cell Line

JEFFREY M. BESTERMAN and PEDRO CUATRECASAS
Department of Molecular Biology, The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709

ABSTRACT The human, leukemic cell line, HL-60, undergoes differentiation in response to tumor-promoting phorbol esters. Recent studies have implicated stimulation of a Na^+/H^+ antiporter as an initial event in cellular differentiation and/or proliferation. The effects of phorbol esters on Na^+-dependent H^+ efflux from HL-60 cells were studied by pH-stat titration. Tumor-promoting phorbol diesters, but not the inactive parent alcohol, stimulated Na^+-dependent H^+ efflux in a rapid (within 1 min at 37°C) and reversible manner. Stimulation was dependent on the concentration of extracellular sodium; lithium could substitute for sodium, but choline could not. Stimulation was dependent on the activity of extracellular protons and was inhibited completely by amiloride. The concentrations of phorbol diesters at which we observed half-maximal stimulation of Na^+-dependent H^+ efflux are very similar to the K_d reported in the literature for binding of these phorbol diesters to the phorbol ester receptor and the K_m for phorbol diester activation of protein kinase C. Overall characterization of basal and phorbol ester-stimulated H^+ efflux indicate that stimulation of a Na^+/H^+ antiporter constitutes a primary event in phorbol ester interaction with HL-60 cells.

The tumor-promoting phorbol esters initiate, in vitro, a multitude of diverse cellular changes (for reviews see references 1, 9), paramount of which is the ability to affect cellular differentiation and proliferation. The molecular mechanism(s) underlying the actions of phorbol esters has yet to be identified. Recent studies on growth factors suggest that stimulation of a Na^+/H^+ antiporter in the plasmalemma may be a critical, initiating event for cellular differentiation and/or proliferation (2, 21, 22, 28, 29, 33). We now report that phorbol esters rapidly stimulate Na^+-dependent H^+ efflux in HL-60 cells, a human leukemic, promyelocytic cell line (7) that undergoes differentiation into macrophage-like cells in response to phorbol esters (31, 32). The potency of the phorbol esters as tumor promotors corresponded with their potency as stimulators of Na^+-dependent H^+ efflux. Stimulation was inhibited completely by amiloride, and overall characterization indicated phorbol diester activation of a Na^+/H^+ antiporter. These results suggest that activation of Na^+/H^+ exchange may comprise an early event in the mechanism of phorbol ester–induced cellular differentiation.

MATERIALS AND METHODS

Cells: The human, leukemic, promyelocytic cell line, HL-60, was grown in spinner culture in RPMI 1640, containing 10% fetal calf serum, 20 mM HEPES, 75 U/ml penicillin, 75 μg/ml streptomycin, pH 7.3. Cells were maintained at between 0.2–1.0 × 10^6 cells/ml (log phase growth) at 37°C in air.

H^+ Efflux Assay: For H^+ efflux determinations, 20–30 × 10^6 HL-60 cells were washed in a standard, unbuffered assay solution (5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, containing various combinations of NaCl and its substitute, choline chloride, the sum of the two always equaling 300 mosmol), and suspended in 12.5 ml of the same. The cell suspension was continuously stirred and its temperature was maintained at 37°C. Acid release was measured using a combined pH electrode, coupled to a pH meter and a pen recorder. The rate of H^+ efflux was calculated from the amount of OH^- (5 mM NaOH in 110 mM NaCl) that had to be added per unit time to maintain the pH of the cell suspension at a constant 7.3 (pH stat). All data represent steady-state values, usually determined over the first 5 min after an addition to the cell suspension. A correction for the background acidification rate due to the constant stirring in of CO_2 was made for all experiments. As well, after a series of additions to a cell sample, the Na^+/H^+ ionophore, monensin, was added (1.44 μM) as a positive control. Any response short of a burst in the rate of H^+ extrusion was taken as an indication of decreased cell viability (lack of an intact, transmural Na^+ gradient), and the data from that cell sample discounted.

All three phorbol derivatives were used as concentrated stocks dissolved in dimethylsulfoxide (DMSO). Experiments reported in Table I used 500-fold concentrated stocks, resulting in a final DMSO concentration of 0.2% (26 mM). As shown in Table I, this concentration of DMSO slightly stimulated H^+ efflux. A 10-fold lower concentration of DMSO (0.02%, 2.6 mM) showed little if any stimulation (data not shown). Thus, most other experiments were carried out using 5,000-fold concentrated stocks of phorbol diesters. Where necessary, results obtained with phorbol esters have been corrected for stimulation due to the DMSO solvent alone. It must be noted that on occasion the rate of Na^+-dependent H^+ efflux in HL-60 cells was unresponsive to phorbol esters. Increasing the final concentration of phorbol ester failed to induce stimulation. The cause of this apparent insensitivity to phorbol esters is not known. However, phorbol ester–insensitive variants of HL-60 cells have been descirbed (17).

Materials: The following materials were obtained from the indicated sources: RPMI-1640 (Gibco Laboratories, Grand Island, NY); fetal bovine

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TABLE I

Stimulation of H^+ Efflux by Phorbol Esters

Assay condition		Stimulation of H^+ efflux rate in excess of basal rate
Addition	Presence of Na^+	
		(nmol H^+/min · 10^6 cells)
0.2% DMSO (solvent)	+	0.22 ± 0.08
4α-Phorbol (1 μM)	+	0.20 ± 0.07
PDBU (100 nM)	−	0.10 ± 0.03
	+	0.90 ± 0.09
TPA (35 nM)	−	ND
	+	1.00 ± 0.10
PDBU (100 nM) + amiloride (200 μM)	+	0.23 ± 0.05
Amiloride (200 μM) + PDBU (100 nM)	+	0.25 ± 0.10

H^+ efflux was assayed as described in Materials and Methods. Each value represents the mean ± SEM of determinations on two to nine cell suspensions. All three phorbol derivatives were used as concentrated stocks dissolved in DMSO, resulting in a final DMSO concentration of 0.2%. *ND*, not determined.

serum (Hyclone Laboratories, Logan, UT); 4α-phorbol, phorbol dibutyrate, tetradecanoylphorbol acetate, and diisothiocyanostilbene (Sigma Chemical Co., St. Louis, MO); furosemide (Hoechst, Somerville, NJ); monensin (Calbiochem-Behring Corp., San Diego, CA); diethylstilbenediol (Pfaltz and Bauer, Stamford, CT). Amiloride HCl was a gift from Merck, Sharp and Dohme, (Rahway, NJ). All other chemicals were reagent grade.

RESULTS AND DISCUSSION

HL-60 cells suspended in Na^+-free medium (choline substituted for Na^+) extrude acid at a rate of 1.3 nmol H^+/min · 10^6 cells at 37°C, as assayed by pH-stat titration. The effect of Na^+ addition on the steady-state rate of H^+ efflux is illustrated in Fig. 1. Half-maximal stimulation of H^+ efflux was obtained at ~15 mM NaCl. Maximum stimulation to nearly twice the Na^+-free level occurred at ~80 mM Na^+. The Na^+-induced stimulation occurred immediately (within seconds of addition of NaCl) and remained elevated thereafter. Lithium chloride could substitute for sodium chloride with equal effectiveness, but choline chloride could not (Fig. 1). Thus, the rate of steady-state basal H^+ extrusion in HL-60 cells appears dependent, in part, on an inwardly directed Na^+ gradient. This Na^+-dependent component accounts for approximately one third to one half of the steady-state rate of basal H^+ efflux in this cell type. Furthermore, as might be expected for H^+ extrusion, the rate of Na^+-dependent H^+ efflux was inversely proportional to the activity of extracellular protons over the pH range tested, 6.5–8.0 (data not shown).

The diuretic, amiloride, inhibited the rate of Na^+ (or Li^+)-stimulated H^+ efflux. Amiloride (200 μM) inhibited basal H^+ efflux (assayed in the presence of 110 mM Na^+) by 32 ± 4%, ($\bar{x}$ ± SEM, n = 6); this represented almost 90% inhibition of the Na^+-dependent basal component. The basal rate of H^+ efflux occurring in the absence of Na^+ was not inhibited by amiloride. The diuretic, furosemide (1 mM), known to inhibit Na^+, K^+/Cl^- co-transport and ouabain-insensitive K^+ fluxes (26), did not affect Na^+-stimulated H^+ efflux. Likewise, neither did diisothiocyanostilbene disulfonic acid (100 μM) an anion-exchange blocker (5) and inhibitor of endosomal and lysosomal H^+-ATPases (36), or diethylstilbenediol (10 μM), a known inhibitor of plasmalemmal H^+ ATPases in plants (3).

Table I illustrates that the tumor-promoting phorbol esters,

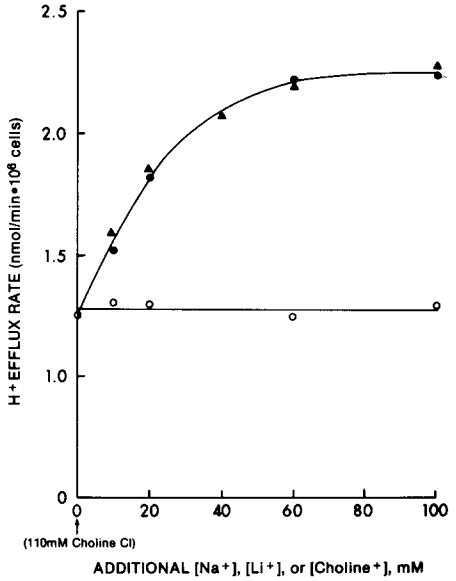

FIGURE 1 Effect of extracellular Na^+, Li^+, or choline on the rate of H^+ efflux. H^+ efflux was determined as described in Materials and Methods. Cell suspensions were assayed in standard medium containing 110 mM choline chloride and either additional NaCl (●), LiCl (▲), or choline chloride (○).

phorbol 12,13-dibutyrate (PDBU),[1] and 12-O-tetradecanoylphorbol-13-acetate (TPA), markedly stimulated H^+ efflux. The onset of this stimulation was extremely rapid, certainly noticeable within 1 min (see Fig. 3). This stimulation required the presence of extracellular Na^+. Amiloride completely inhibited stimulation of Na^+-dependent proton efflux. This inhibition occurred by either treating the cells first with amiloride and then with the phorbol ester or equally as well if the amiloride was added subsequent to the phorbol ester. Addition of 4α-phorbol, the parent alcohol of the phorbol ester series and a nonpromotor, did not stimulate H^+ efflux in excess of that attributable to the DMSO solvent alone, even at micromolar concentrations. Dose-response curves (Fig. 2) indicated that the K_m for stimulation by the active phorbol esters are in accord with their known tumor promoting potency: ~5 nM for TPA and ~20 nM for PDBU (35). As with basal Na^+-dependent H^+ efflux, the rate of phorbol ester stimulated Na^+-dependent H^+ extrusion was related inversely to the activity of extracellular protons over the pH range 6.5–8.0 (data not shown).

Stimulation of cellular events by PDBU has been shown to be reversible (8). To test whether PDBU stimulation of Na^+-dependent H^+ efflux was reversible, we incubated cells in Na^+-complete medium and a basal rate recorded (Fig. 3). After PDBU addition and establishment of the new increased steady-state rate of H^+ efflux, the cells were pelleted, washed twice, and resuspended in PDBU-free medium. Results clearly indicate that upon reassaying for H^+ efflux, rapid and complete reversal to the basal state had occurred. Moreover, when PDBU was readded, stimulation again was immediate and as strong as previously.

The kinetics of the PDBU-stimulated Na^+-dependent H^+ efflux, when expressed as a Lineweaver-Burk plot, revealed that PDBU reduced the K_m for Na^+ from ~15 to ~7 mM,

[1] *Abbreviations used in this paper:* PDBU, phorbol 12,13-dibutyrate; TPA, 12-O-tetradecanoylphorbol-13-acetate.

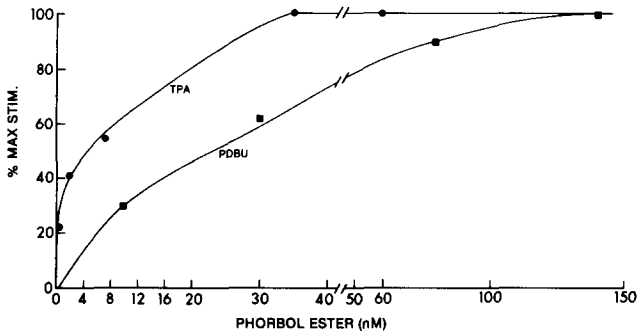

FIGURE 2 Effect of phorbol ester concentration on the rate of Na^+-dependent H^+ efflux. H^+ efflux was assayed as described in Materials and Methods. All suspensions were assayed in standard medium containing 150 mM NaCl. Maximal stimulation (100%) was similar for both PDBU and TPA, representing ~1 nmol H^+ extruded/min·10^6 cells in excess of the basal rate (see Table I).

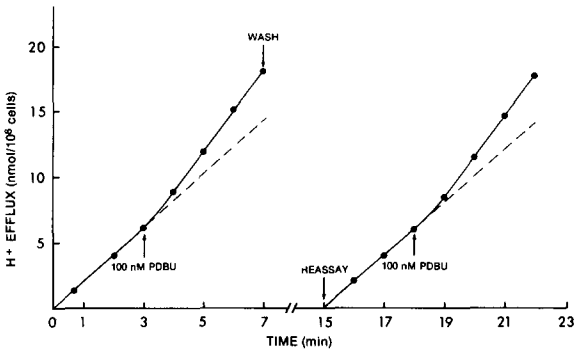

FIGURE 3 Reversible stimulation of Na^+-dependent H^+ efflux by phorbol ester. H^+ efflux was assayed as described in Materials and Methods. The experiment was performed as described in the text. The dashed line represents extrapolation of the basal rate of H^+ efflux and is provided for comparison with the PDBU-stimulated rate. The washing procedure was performed at 37°C and required 5–10 min.

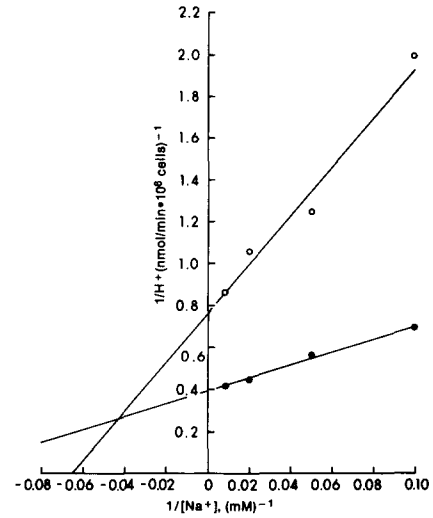

FIGURE 4 Effect of the phorbol ester, PDBU, on the rate of Na^+-dependent H^+ efflux. H^+ efflux was determined as described in Materials and Methods. Cell suspensions were assayed in standard medium containing various combinations of NaCl and choline chloride, the sum always equaling 300 mosmol, in the absence (○) or presence (●) of 100 nM PDBU. Both the basal and PDBU-stimulated steady-state rates of H^+ efflux have been corrected for the Na^+-independent component, and the data presented as a Lineweaver-Burk plot.

and increased the V_{max} from 1.3 nmol/min·10^6 cells to 2.0 nmol/min·10^6 cells (Fig. 4). Although the alterations in the K_m and V_{max} were only about twofold, they were observed repeatedly. The mechanisms responsible for the effects of PDBU on the antiporter are currently being investigated.

The characteristics of the Na^+-dependent H^+ efflux in HL-60 cells show great similarity to the Na^+/H^+ antiporters described in other cell types (20, 27, 33). One important difference appears to exist, that is, basal H^+ efflux in HL-60 cells contains a Na^+-dependent, amiloride inhibitable component. This indicates that the Na^+/H^+ antiporter functions constitutively in HL-60 cells, unlike cell types heretofore described in which basal H^+ efflux shows very little if any inhibition by amiloride (2, 13, 22, 29).

Na^+ influx (via electroneutral Na^+/H^+ exchange) increases rapidly in cells stimulated by serum or defined growth factors (2, 13, 21, 22, 28, 29, 33). Increased Na^+ entry, in turn, stimulates the Na^+/K^+ pump, resulting in an increased rate of K^+ (or Rb^+) influx (2, 10, 21, 22, 24, 33). We suggest that phorbol esters stimulate a similar series of events. Moroney et al. (24) reported that stimulation of ouabain-sensitive $^{86}Rb^+$ uptake was an early response to TPA in Swiss 3T3 cells. Dicker and Rozengurt (10) confirmed this finding and hypothesized that stimulation of the Na^+/K^+ pump resulted from a TPA-induced increase in Na^+ influx. Our results provide evidence in support of that hypothesis.

Stimulation of Na^+/H^+ exchange by serum or by defined growth factors has been reported to result in intracellular alkalosis (23, 30, 33). Consistent with our finding that phorbol diesters stimulate Na^+/H^+ exchange, Burns and Rozengurt (4) have reported just recently that phorbol esters cause an increase in intracellular pH.

High affinity, saturable, stereospecific binding of phorbol esters to cells in culture has been demonstrated (11, 12, 15, 16, 34, 35), along with the purification of a phorbol ester activated, calcium and phospholipid dependent protein kinase (protein kinase C) (6, 25). The concentrations of TPA and PDBU at which we observe half-maximal stimulation of Na^+-dependent H^+ efflux are very similar to the K_d reported for binding of phorbol esters to the phorbol ester receptor (8, 11, 12, 34, 35) and the K_m for phorbol ester activation of protein kinase C (6, 25). As well, phorbol esters have been reported to rapidly stimulate the phosphorylation of specific cell surface receptors (18, 19) and of specific cytosolic proteins (14) in HL-60 and other cell types. In this regard, it has been hypothesized that growth factor stimulation of Na^+/H^+ exchange may be mediated via phosphorylation of the Na^+/H^+ antiporter (23). In light of the known ability of phorbol esters to stimulate protein kinase C (6, 25), an analogous hypothesis is an appealing, albeit unproven, mechanism for the phorbol ester stimulation of Na^+/H^+ exchange.

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REFERENCES

1. Abrahm, J., and G. Rovera. 1980. The effect of tumor-promoting phorbol diesters on terminal differentiation of cells in culture. *Mol. Cell. Biochem.* 31:165–175.
2. Boonstra, J., W. H. Moolenaar, P. H. Harrison, P. Moed, P. T. van der Saag, and S. W. de Laat. 1983. Ionic responses and growth stimulation induced by nerve growth factor and epidermal growth factor in rat pheochromocytoma (PC12) cells. *J. Cell Biol.* 97:92–98.
3. Bowman, B. J., S. E. Mainzer, K. E. Allen, and C. W. Slayman. 1978. Effects of inhibitors on the plasma membrane and mitochondrial adenosine triphosphatases of *Neurospora crassa*. *Biochim. Biophys. Acta.* 512:13–28.
4. Burns, C. P., and E. Rozengurt. 1983. Serum, platelet-derived growth factor, vasopressin and phorbol esters increase pH in Swiss 3T3 cells. *Biochem. Biophys. Res. Commun.* 116:931–938.
5. Cabantchik, Z. I., P. A. Knauf, and A. Rothstein. 1978. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of probes. *Biochim. Biophys. Acta.* 515:239–302.
6. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847–7851.
7. Collins, S. J., R. C. Gallo, and R. E. Gallagher. 1977. Continuous growth and differentiation of human myeloid leukaemic cells in suspension growth. *Nature (Lond.).* 270:347–349.
8. Cooper, R. A., A. D. Braunwald, and A. L. Kuo. 1982. Phorbol ester induction of leukemic cell differentiation is a membrane-mediated process. *Proc. Natl. Acad. Sci. USA.* 79:2865–2869.
9. Diamond, L., T. G. O'Brien, and G. Rovera. 1978. Tumor promoters: effects on proliferation and differentiation of cells in culture. *Life Sci.* 23:1979–1988.
10. Dicker, P., and E. Rozengurt. 1981. Phorbol ester stimulation of Na influx and Na-K pump activity in Swiss 3T3 cells. *Biochem. Biophys. Res. Commun.* 100:433–441.
11. Driedger, P. E., and P. M. Blumberg. 1980. Specific binding of phorbol ester tumor promoters. *Proc. Natl. Acad. Sci. USA.* 77:567–571.
12. Dunphy, W. G., R. J. Kochenburger, M. Castagna, and P. M. Blumberg. 1981. Kinetics and subcellular localization of specific [^{3}H]phorbol 12,13-dibutyrate binding by mouse brain. *Cancer Res.* 41:2640–2647.
13. Frelin, C., P. Vigne, and M. Lazdunski. 1983. The amiloride-sensitive Na^+/H^+ antiport in 3T3 fibroblasts. Characterization and stimulation by serum. *J. Biol. Chem.* 258:6272–6276.
14. Feuerstein, N., and H. L. Cooper. 1983. Rapid protein phosphorylation induced by phorbol ester in HL-60 cells. *J. Biol. Chem.* 258:10786–10793.
15. Goodwin, B. J., and J. B. Weinberg. 1982. Receptor-mediated modulation of human monocyte, neutrophil, lymphocyte, and platelet function by phorbol diesters. *J. Clin. Invest.* 70:699–706.
16. Horowitz, A. D., E. Greenebaum, and I. B. Weinstein. 1981. Identification of receptors for phorbol ester tumor promoters in intact mammalian cells and of an inhibitor of receptor binding in biological fluids. *Proc. Natl. Acad. Sci. USA.* 78:2315–2319.
17. Huberman, E., C. Weeks, A. Herrmann, M. Callaham, and T. Slaga. 1981. Alterations in polyamine levels induced by phorbol diesters and other agents that promote differentiation in human promyelocytic leukemia cells. *Proc. Natl. Acad. Sci. USA.* 78:1062–1066.
18. Jacobs, S., N. E. Sahyoun, A. R. Saltiel, and P. Cuatrecasas. 1983. Phorbol esters stimulate the phosphorylation of receptors for insulin and somatomedin C. *Proc. Natl. Acad. Sci. USA.* 80:6211–6213.
19. May, W. S., S. Jacobs, and P. Cuatrecasas. 1984. Association of phorbol ester induced hyperphosphorylation and reversible regulation of transferrin membrane receptors in HL-60 cells. *Proc. Natl. Acad. Sci. USA.* 81:2016–2020.
20. Moolenaar, W. H., J. Boonstra, P. T. van de Saag, and S. W. de Laat. 1981. Sodium/proton exchange in mouse neuroblastoma cells. *J. Biol. Chem.* 256:12883–12887.
21. Moolenaar, W. H., C. L. Mummery, P. T. van der Saag, and S. W. de Laat. 1981. Rapid ionic events and the initiation of growth in serum-stimulated neuroblastoma cells. *Cell.* 23:789–798.
22. Moolenaar, W. H., Y. Yarden, S. W. de Laat, and J. Schlessinger. 1982. Epidermal growth factor induces electrically silent Na^+ influx in human fibroblasts. *J. Biol. Chem.* 257:8502–8506.
23. Moolenaar, W. H., R. Y. Tsien, P. T. van der Saag, and S. W. de Laat. 1983. Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature (Lond.).* 304:645–648.
24. Moroney, J., A. Smith, L. D. Tomei, and C. E. Wenner. 1978. Stimulation of $^{86}Rb^+$ and $^{32}P_i$ movements in 3T3 cells by prostaglandins and phorbol esters. *J. Cell. Physiol.* 95:287–294.
25. Niedel, J. E., L. J. Kuhn, and G. R. Vandenbark. 1983. Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA.* 80:36–40.
26. O'Brien, T. G., and K. Krzeminski. 1983. Phorbol ester inhibits furosemide-sensitive potassium transport in Balb/c3T3 preadipose cells. *Proc. Natl. Acad. Sci. USA.* 80:4334–4338.
27. Paris, S., and J. Pouyssegur. 1983. Biochemical characterization of the amiloride-sensitive Na^+/H^+ antiport in Chinese hamster lung fibroblasts. *J. Biol. Chem.* 258:3503–3508.
28. Pouyssegur, J., J. C. Chambard, A. Franchi, S. Paris, and E. Van Obberghen-Schilling. 1982. Growth factor activation of an amiloride-sensitive Na^+/H^+ exchange system in quiescent fibroblasts: coupling to ribosomal protein S6 phosphorylation. *Proc. Natl. Acad. Sci. USA.* 79:3935–3939.
29. Rothenberg, P., L. Glaser, P. Schlesinger, and D. Cassel. 1983. Epidermal growth factor stimulates amiloride-sensitive $^{22}Na^+$ uptake in A431 cells. *J. Biol. Chem.* 258:4883–4889.
30. Rothenberg, P., L. Glaser, P. Schlesinger, and D. Cassel. 1983. Activation of Na^+/H^+ exchange by epidermal growth factor elevates intracellular pH in A431 cells. *J. Biol.Chem.* 258:12644–12653.
31. Rovera, G., T. G. O'Brien, and L. Diamond. 1979. Induction of differentiation in human promyelocytic leukemia cells by tumor promoters. *Science (Wash. DC).* 204:868–870.
32. Rovera, G., D. Santoli, and C. Damsky. 1979. Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. *Proc. Natl. Acad. Sci. USA.* 76:2779–2783.
33. Schuldiner, S., and E. Rozengurt. 1982. Na^+/H^+ antiport in Swiss 3T3 cells: mitogenic stimulation leads to cytoplasmic alkalinization. *Proc. Natl. Acad. Sci. USA.* 79:7778–7782.
34. Shoyab, M., and G. J. Todaro. 1980. Specific high affinity cell membrane receptors for biologically active phorbol and ingenol esters. *Nature (Lond.).* 288:451–455.
35. Solanki, V., and T. J. Slaga. 1981. Specific binding of phorbol ester tumor promoters to intact primary epidermal cells from Sencar mice. *Proc. Natl. Acad. Sci. USA.* 78:2549–2553.
36. Yamashiro, D. J., S. R. Fluss, and F. R. Maxfield. 1983. Acidification of endocytic vesicles by an ATP-dependent proton pump. *J. Cell Biol.* 97:929–934.