

# Phorbol Esters Rapidly Stimulate Amiloride-sensitive $\text{Na}^+/\text{H}^+$ Exchange in a Human Leukemic Cell Line

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**ABSTRACT** The human, leukemic cell line, HL-60, undergoes differentiation in response to tumor-promoting phorbol esters. Recent studies have implicated stimulation of a  $\text{Na}^+/\text{H}^+$  antiporter as an initial event in cellular differentiation and/or proliferation. The effects of phorbol esters on  $\text{Na}^+$ -dependent  $\text{H}^+$  efflux from HL-60 cells were studied by pH-stat titration. Tumor-promoting phorbol diesters, but not the inactive parent alcohol, stimulated  $\text{Na}^+$ -dependent  $\text{H}^+$  efflux in a rapid (within 1 min at  $37^\circ\text{C}$ ) 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  $\text{Na}^+$ -dependent  $\text{H}^+$  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  $\text{H}^+$  efflux indicate that stimulation of a  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+$ -dependent  $\text{H}^+$  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 promoters corresponded with their potency as stimulators of  $\text{Na}^+$ -dependent  $\text{H}^+$  efflux. Stimulation was inhibited completely by amiloride, and overall characterization indicated phorbol diester activation of a  $\text{Na}^+/\text{H}^+$  antiporter. These results suggest that activation of  $\text{Na}^+/\text{H}^+$  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  $\mu\text{g}/\text{ml}$  streptomycin, pH 7.3. Cells were maintained at between  $0.2\text{--}1.0 \times 10^6$  cells/ml (log phase growth) at  $37^\circ\text{C}$  in air.

**$\text{H}^+$  Efflux Assay:** For  $\text{H}^+$  efflux determinations,  $20\text{--}30 \times 10^6$  HL-60 cells were washed in a standard, unbuffered assay solution (5.4 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgCl}_2$ , 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  $37^\circ\text{C}$ . Acid release was measured using a combined pH electrode, coupled to a pH meter and a pen recorder. The rate of  $\text{H}^+$  efflux was calculated from the amount of  $\text{OH}^-$  (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  $\text{CO}_2$  was made for all experiments. As well, after a series of additions to a cell sample, the  $\text{Na}^+/\text{H}^+$  ionophore, monensin, was added (1.44  $\mu\text{M}$ ) as a positive control. Any response short of a burst in the rate of  $\text{H}^+$  extrusion was taken as an indication of decreased cell viability (lack of an intact, transmural  $\text{Na}^+$  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  $\text{H}^+$  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  $\text{Na}^+$ -dependent  $\text{H}^+$  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 described (17).

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

TABLE I  
Stimulation of H<sup>+</sup> Efflux by Phorbol Esters

Assay condition		Stimulation of H <sup>+</sup> efflux rate in excess of basal rate (nmol H <sup>+</sup> /min · 10 <sup>6</sup> cells)
Addition	Presence of Na <sup>+</sup>	
0.2% DMSO (solvent)	+	0.22 ± 0.08
4 $\alpha$ -Phorbol (1 $\mu$ M)	+	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 $\mu$ M)	+	0.23 ± 0.05
Amiloride (200 $\mu$ M) + PDBU (100 nM)	+	0.25 ± 0.10

H<sup>+</sup> efflux was assayed as described in Materials and Methods. Each value represents the mean  $\pm$  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); 4 $\alpha$ -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<sup>+</sup>-free medium (choline substituted for Na<sup>+</sup>) extrude acid at a rate of 1.3 nmol H<sup>+</sup>/min · 10<sup>6</sup> cells at 37°C, as assayed by pH-stat titration. The effect of Na<sup>+</sup> addition on the steady-state rate of H<sup>+</sup> efflux is illustrated in Fig. 1. Half-maximal stimulation of H<sup>+</sup> efflux was obtained at ~15 mM NaCl. Maximum stimulation to nearly twice the Na<sup>+</sup>-free level occurred at ~80 mM Na<sup>+</sup>. The Na<sup>+</sup>-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<sup>+</sup> extrusion in HL-60 cells appears dependent, in part, on an inwardly directed Na<sup>+</sup> gradient. This Na<sup>+</sup>-dependent component accounts for approximately one third to one half of the steady-state rate of basal H<sup>+</sup> efflux in this cell type. Furthermore, as might be expected for H<sup>+</sup> extrusion, the rate of Na<sup>+</sup>-dependent H<sup>+</sup> 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<sup>+</sup> (or Li<sup>+</sup>)-stimulated H<sup>+</sup> efflux. Amiloride (200  $\mu$ M) inhibited basal H<sup>+</sup> efflux (assayed in the presence of 110 mM Na<sup>+</sup>) by 32  $\pm$  4%, ( $\bar{x}$   $\pm$  SEM, *n* = 6); this represented almost 90% inhibition of the Na<sup>+</sup>-dependent basal component. The basal rate of H<sup>+</sup> efflux occurring in the absence of Na<sup>+</sup> was not inhibited by amiloride. The diuretic, furosemide (1 mM), known to inhibit Na<sup>+</sup>, K<sup>+</sup>/Cl<sup>-</sup> co-transport and ouabain-insensitive K<sup>+</sup> fluxes (26), did not affect Na<sup>+</sup>-stimulated H<sup>+</sup> efflux. Likewise, neither did diisothiocyanostilbene disulfonic acid (100  $\mu$ M) an anion-exchange blocker (5) and inhibitor of endosomal and lysosomal H<sup>+</sup>-ATPases (36), or diethylstilbenediol (10  $\mu$ M), a known inhibitor of plasmalemmal H<sup>+</sup> ATPases in plants (3).

Table I illustrates that the tumor-promoting phorbol esters,

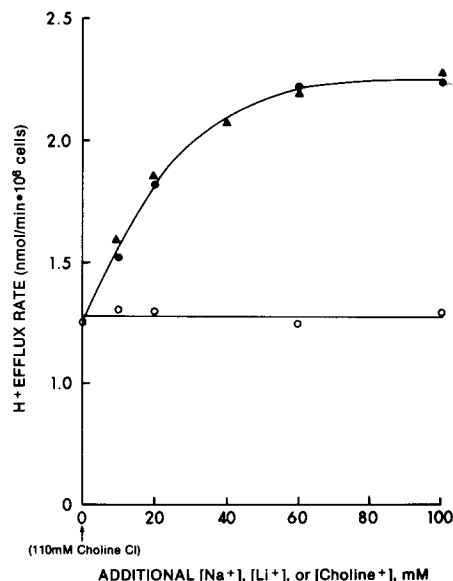


FIGURE 1 Effect of extracellular Na<sup>+</sup>, Li<sup>+</sup>, or choline on the rate of H<sup>+</sup> efflux. H<sup>+</sup> efflux was determined as described in Materials and Methods. Cell suspensions were assayed in standard medium containing 110 mM choline chloride and either additional NaCl (●),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

phorbol 12,13-dibutyrate (PDBU),<sup>1</sup> and 12-O-tetradecanoylphorbol-13-acetate (TPA), markedly stimulated H<sup>+</sup> 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<sup>+</sup>. Amiloride completely inhibited stimulation of Na<sup>+</sup>-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 4 $\alpha$ -phorbol, the parent alcohol of the phorbol ester series and a nonpromotor, did not stimulate H<sup>+</sup> efflux in excess of that attributable to the DMSO solvent alone, even at micromolar concentrations. Dose-response curves (Fig. 2) indicated that the *K<sub>m</sub>* 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<sup>+</sup>-dependent H<sup>+</sup> efflux, the rate of phorbol ester stimulated Na<sup>+</sup>-dependent H<sup>+</sup> 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<sup>+</sup>-dependent H<sup>+</sup> efflux was reversible, we incubated cells in Na<sup>+</sup>-complete medium and a basal rate recorded (Fig. 3). After PDBU addition and establishment of the new increased steady-state rate of H<sup>+</sup> efflux, the cells were pelleted, washed twice, and resuspended in PDBU-free medium. Results clearly indicate that upon reassaying for H<sup>+</sup> 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<sup>+</sup>-dependent H<sup>+</sup> efflux, when expressed as a Lineweaver-Burk plot, revealed that PDBU reduced the *K<sub>m</sub>* for Na<sup>+</sup> from ~15 to ~7 mM,

<sup>1</sup> Abbreviations used in this paper: PDBU, phorbol 12,13-dibutyrate; TPA, 12-O-tetradecanoylphorbol-13-acetate.

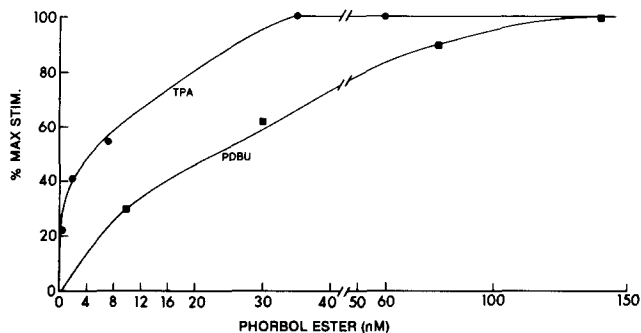


FIGURE 2 Effect of phorbol ester concentration on the rate of  $\text{Na}^+$ -dependent  $\text{H}^+$  efflux.  $\text{H}^+$  efflux was assayed as described in Materials and Methods. All suspensions were assayed in standard medium containing 150 mM NaCl. Maximal stimulation (100%) was similar for both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

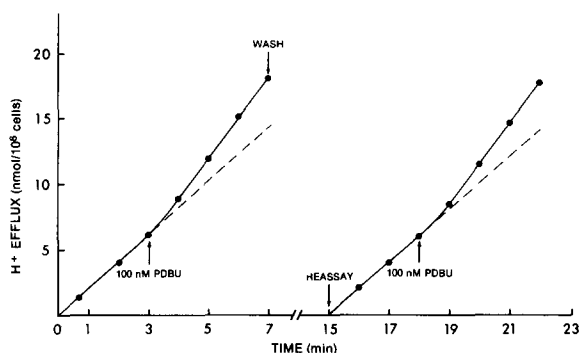


FIGURE 3 Reversible stimulation of  $\text{Na}^+$ -dependent  $\text{H}^+$  efflux by phorbol ester.  $\text{H}^+$  efflux was assayed as described in Materials and Methods. The experiment was performed as described in the text. The dashed line represents extrapolation of the basal rate of  $\text{H}^+$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

and increased the  $V_{\text{max}}$  from 1.3 nmol/ $\text{min} \cdot 10^6$  cells to 2.0 nmol/ $\text{min} \cdot 10^6$  cells (Fig. 4). Although the alterations in the  $K_m$  and  $V_{\text{max}}$  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  $\text{Na}^+$ -dependent  $\text{H}^+$  efflux in HL-60 cells show great similarity to the  $\text{Na}^+/\text{H}^+$  antiporters described in other cell types (20, 27, 33). One important difference appears to exist, that is, basal  $\text{H}^+$  efflux in HL-60 cells contains a  $\text{Na}^+$ -dependent, amiloride inhibitable component. This indicates that the  $\text{Na}^+/\text{H}^+$  antiporter functions constitutively in HL-60 cells, unlike cell types heretofore described in which basal  $\text{H}^+$  efflux shows very little if any inhibition by amiloride (2, 13, 22, 29).

$\text{Na}^+$  influx (via electroneutral  $\text{Na}^+/\text{H}^+$  exchange) increases rapidly in cells stimulated by serum or defined growth factors (2, 13, 21, 22, 28, 29, 33). Increased  $\text{Na}^+$  entry, in turn, stimulates the  $\text{Na}^+/\text{K}^+$  pump, resulting in an increased rate of  $\text{K}^+$  (or  $\text{Rb}^+$ ) 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  $^{86}\text{Rb}^+$  uptake was an early response to TPA in Swiss 3T3 cells. Dicker and Rozengurt (10) confirmed this finding and hy-

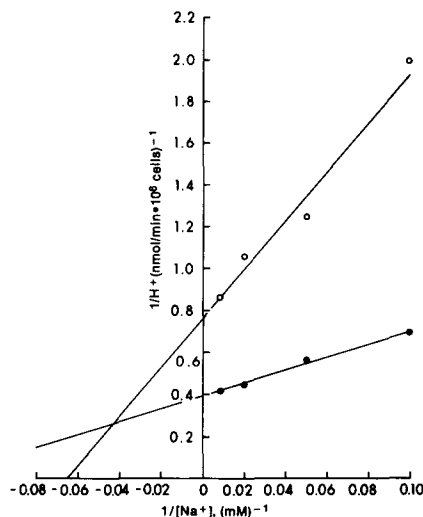


FIGURE 4 Effect of the phorbol ester, PDBU, on the rate of  $\text{Na}^+$ -dependent  $\text{H}^+$  efflux.  $\text{H}^+$  efflux was determined as described in Materials and Methods. Cell suspensions were assayed in standard medium containing various combinations of NaCl and choline chloride, the sum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

pothesized that stimulation of the  $\text{Na}^+/\text{K}^+$  pump resulted from a TPA-induced increase in  $\text{Na}^+$  influx. Our results provide evidence in support of that hypothesis.

Stimulation of  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+$ -dependent  $\text{H}^+$  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  $\text{Na}^+/\text{H}^+$  exchange may be mediated via phosphorylation of the  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+/\text{H}^+$  exchange.

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