Phorbol Esters Induce Differentiation in a Pre-B-lymphocyte Cell Line by Enhancing Na+/H+ Exchange*

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The murine pre-B-lymphocyte cell line 70Z/3 may be induced to differentiate to a surface immunoglobulin-positive phenotype by the polyclonal B-cell mitogen, lipopolysaccharide. This is accomplished via activation of an amiloride-sensitive Na⁺-uptake system (Rosoff, P. M., and Cantley, L. C. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 7547–7550). Here we show that the active tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) also induces surface IgM expression in 70Z/3 cells. TPA also appears to work by activating a plasma membrane Na⁺/H⁺ exchange system. A significant increase in cellular Na⁺ was detected within 10 min after TPA addition and by 2 h an 80% increase was observed. Amiloride blocked both the induction of surface IgM and the increase in cellular Na⁺. Further evidence that both TPA and lipopolysaccharide activate Na⁺/H⁺ exchange was provided by measurements of intracellular pH with carboxyfluorescein. Both TPA and lipopolysaccharide caused a 0.15 unit increase in cytoplasmic pH within 5 min after addition to the cells at 37 °C. The pH change required high extracellular Na⁺ and was inhibited by amiloride. These data suggest a mechanism by which phorbol esters affect cellular growth and differentiation.

For a variety of cell lines, such as Chinese hamster fibroblasts (1), Swiss 3T3 cells (2), MDCK cells (3), and the human epidermoid carcinoma line A431 (4), addition of growth factors results in a rapid elevation of cellular Na⁺ content. This increase in [Na⁺]i appears to be due to activation of an amiloride-sensitive, plasma-membrane Na⁺/H⁺ exchange system; an efflux of H⁺ in exchange for Na⁺ leads to cytoplasmic alkalization. It is unclear whether or not the change in intracellular pH or [Na⁺], (or both) serves as the signal for proliferation. For immature, differentiating cells, a similar mechanism may be operative, although little evidence to date has been offered to support this idea.

The phorbol ester tumor promoters have also been shown to cause an increase in [Na⁺]i in quiescent 3T3 fibroblasts (5). These agents are known to have mitogenic activity in a variety of different cell systems (6) and presumably mediate such effects by binding to and activating a Ca²⁺- and phospho-dependent protein kinase (7). These results suggest that the Na⁺/H⁺ exchange system may be directly or indirectly regulated by this kinase.

We have previously reported that treatment of the immature pre-B-lymphocyte cell line, 70Z/3 with the polyclonal B-cell mitogen LPS, results in a rapid influx of Na⁺ through an amiloride-sensitive uptake system (8, 9). LPS also induces differentiation from a surface IgM negative to a surface IgM positive phenotype (8–10). Other agents which act to raise [Na⁺]i, such as ouabain or the Na⁺ ionophore, monensin, also cause differentiation in 70Z/3 cells (8, 9).

In this report we show that the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate, not only induces differentiation in 70Z/3 cells, but does so in a manner similar to LPS, namely by increasing [Na⁺]i. In addition we also demonstrate that both LPS and TPA do this by enhancing Na⁺/H⁺ exchange, thus leading to an increase in pH. Both the TPA-induced differentiation and pH change are inhibited by amiloride. These data suggest a possible mechanism for the observed biological effects of phorbol esters.

MATERIALS AND METHODS

Cell Line—70Z/3.B3 cells were used for all experiments. They are a subclone of the parent line supplied by Dr. C. Paige (Basei Institute of Immunology, Basel, Switzerland). Cells were maintained in RPMI 1640 medium supplemented with 15% FCS (GIBCO) and 10 mM Na pyruvate, 10 mM HEPES buffer, 50 μM 2-mercaptoethanol, 100 units/ml of penicillin G, and 100 μg/ml of streptomycin sulfate. They were cultured at 37 °C in humidified air, 5% CO₂.

Reagents—Escherichia coli LPS (serotype 055:B5), ouabain, HEPES, MES, Tris base, 4α-phorbol 12,13-didecanoate, and TPA were purchased from Sigma. Dinonyl phthalate was from ICN; silicone oil was from Aldrich; Scintiverse I liquid scintillation mixture was from Fisher. *RbCl and 22NaCl were from New England Nuclear. Amiloride was the gift of Dr. C. Stone of Merck Research Laboratories, West Point, PA. 5,6-Carboxyfluorescein diacetate was from Molecular Probes, Inc. Fluorescein-conjugated goat anti-mouse IgM was from N. L. Cappell Laboratories Inc.

Differentiation Assay—All differentiation experiments were conducted with 70Z/3 cells at a concentration of 1–2 × 10⁶/ml and LPS at 10 μg/ml (diluted from a stock solution of 2 mg/ml in phosphate-buffered saline): this concentration had previously been determined to induce maximal surface IgM expression at 24 h (8). Cell viability was determined by exclusion of trypan blue. Surface IgM positive cells were counted by direct immunofluorescence microscopy using a Zeiss fluorescence microscope with epillumination as previously described (8, 9).

Isotope Flux Measurements—For assays of Na⁺ uptake, a trace quantity of ²²NaCl was added to 70Z/3 cells suspended in RPMI 1640, 15% FCS (pH 7.4) at 2 × 10⁶ cells/ml and allowed to equilibrate for 15 min at 37 °C (isotopic equilibrium of ²²NaCl is complete in less than 1 min: ¹ The abbreviations used are: LPS, lipopolysaccharide; TPA, 12-O-tetradecanoylphorbol 13-acetate; [Na⁺]; intracellular Na⁺ concentration; pH, intracellular pH; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, MES, (N-morpholino)ethanesulfonic acid; FCS, fetal calf serum.

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than 10 min in these cells (see Ref. 8). At various times after the addition of the test inducing agent, quadruplicate 0.5-ml aliquots were removed and rapidly added to 1.5-ml microfuge tubes containing 0.3 ml of ice-cold phosphate-buffered saline and 0.3 ml (1:1 (v/v) dimethyl phthalate: silicone oil. The tubes were centrifuged for 30 s in a Beckman microfuge after which the fluid was drained. Radioactivity was determined in the pellet as described (8, 11).

146Rb+ uptake (as a measure of K+ flux) was determined by measuring the rate of ouabain-sensitive and -resistant 146Rb+ influx in TPA- or LPS-treated 70Z/3 cells. 50 nM TPA or 10 μg/ml of LPS was added to 70Z/3 cells (suspended at 2 × 10⁶ cells/ml in RPMI 1640, 15% FCS) 45 min prior to the addition of trace 32PCl. When used, ouabain was added to a final concentration (from a 10 mM stock in RPMI 1640, 15% FCS) at t = -1 min. 146Rb+ (1 μCi/sample) was added at t = 0 min. All experiments using TPA were carried out in subdued light.

Intracellular pH Measurements—Determination of pH, was made by a modification of the method of Thomas et al. (12) by utilizing the pH-dependent fluorescence of carboxyfluorescein. The permeant ester, 5,6-carboxyfluorescein diacetate was loaded into 5 × 10⁶ 70Z/3 cells/ml in a solution containing 50 μM NaCl, 5 mM KCl, 20 mM MES buffer, 1 mM KH2PO4, 2 mM MgSO4, 1 mM CaCl2, 0.1 mM EDTA, and 10 mM glucose (pH 6.3). This mixture was incubated in the dark at 37 °C for 35-40 min. At the end of the loading period, the cells were washed three times with incubation buffer (130 mM NaCl, 4 mM KCl, 25 mM HEPES buffer, 1.0 mM Na2HPO4, 1.8 mM CaCl2, 0.8 mM MgCl2, and 10 mM glucose, pH 7.4) and then loaded a further 10 min at 37 °C. The cells were washed once more prior to each experiment. The cells were then suspended at 5 × 10⁶ cells/ml in the incubation buffer and absorption spectra were obtained from λmax nm to λmax nm in a Kontron Uvikon 810 dual beam spectrophotometer. Base line spectra for the appropriate buffer containing untreated cells were subtracted. The ratio of absorption at λmax nm was compared to a standard calibration curve to obtain pH: λmax nm is an absorption isosbestic point for carboxyfluorescein above pH 6 (12). A standard calibration curve was generated by loading cells with 5,6-carboxyfluorescein diacetate, then washing and resuspending them in a solution containing 130 mM KCl, 1 mM MgCl2, and one of the following buffers: 25 mM MES (pH 6.3), 25 mM MOPS (pH 6.8), 25 mM HEPES (pH 7.3), 25 mM HEPES (pH 7.5), or 25 mM Tris base (pH 8.2). Cells were permethylated with 5 μg/ml of nigericin and λmax nm absorbance ratios taken. For experiments in which pH was determined with variable [Na+], the standard incubation buffer (pH 7.4) was used but with a change in the ratio of NaCl to choline Cl (while maintaining approximate isotonicity). The ratios used were (a) 146 mM NaCl, 0 mM choline Cl (b) 169 mM NaCl, 36 mM choline Cl (c) 84 mM NaCl, 61 mM choline Cl, and (d) 44 mM NaCl, 101 mM choline Cl.

RESULTS

Phorbol Esters Induce 70Z/3 Cell Differentiation—When 70Z/3 cells were exposed to low concentrations of the potent tumor promoter TPA for 24 h and tested for the presence of surface IgM, 74% of the cells differentiated to the surface IgM positive phenotype (Table I). LPS induced a similar percentage of 70Z/3 cells. The effects of TPA were detected at concentrations as low as 10 nM, a dose comparable to that reported to induce differentiation in HL-60 cells (13). The biologically less active phorbol ester, 4α-phorbol 12,13-didecanoate; failed to induce expression of surface IgM in 70Z/3 cells. Table I also shows that amiloride inhibits both LPS and TPA induction of differentiation in these cells. These results imply that similar Na+ uptake mechanisms may be important to the mode of action of both agents.

TPA Increases Cellular Na+—Since TPA induces differentiation in 70Z/3 cells, we wished to determine if it did so by a mechanism similar to that of LPS, i.e., by causing an increase in [Na+], through an amiloride-sensitive 'uptake system. We equilibrated cells with 32P and measured the cellular Na+ content after addition of 50 nM TPA. The results are shown in Fig. 1. As previously observed with LPS, TPA caused a significant increase in total cellular Na+ within 10 min after addition of the drug. The cellular water content, measured by

FIG. 1. 32P uptake in 70Z/3 cells stimulated by LPS or TPA. 70Z/3 cells were equilibrated with trace 32P in RPMI 1646 medium with 15% FCS then treated with either 10 μg/ml of LPS or 50 nM TPA. Quadruplicate aliquots of cells (10⁶ cells/aliquot) were removed, centrifuged through dimethyl phthalate:silicone oil (1:1) (v/v) and the radioactivity in the pellet determined in a liquid scintillation counter as described under "Materials and Methods." Data points represent means ± S.D. of four experiments.

TABLE I

| Addition | Surface IgM positive |
|----------|----------------------|
| None (control) | 0.15 |
| LPS | 73.4 |
| TPA | 73.5 |
| PDDU | 0.8% |
| TPA/amiloride | 0.5% |
| LPS/amiloride | 2% |

* LPS, TPA, 4α-phorbol 12,13-didecanoate, and amiloride were used at 10 μg/ml, 50 nM, and 60 μM, respectively, and were present for 24 h. Surface IgM positive cells were determined at the end of the 24-h incubation period by immunofluorescence microscopy as described under "Materials and Methods." At least 500 cells were examined in each experiment. Cell viability was greater than 90% as judged by exclusion of trypan blue in all experiments. Results represent means of three experiments.

† PDDU, 4α-phorbol 12,13-didecanoate.
caused stimulation of ouabain-sensitive $^{86}\text{Rb}^+$ uptake (127 and 54%, respectively) with little effect on the ouabain-resistant component (although TPA caused a slight inhibition of ouabain-resistant $^{86}\text{Rb}^+$ uptake). These data indicate that Na+/K+-ATPase activity is increased in these cells in the presence of TPA or LPS, presumably in response to an elevated [Na$^+$]. Thus, the increase in cellular Na$^+$ must result from enhanced uptake rather than a decrease in Na+/K+-pump activity. An uncoupling of the pump is not excluded by these data (however, see below).

If TPA is acting in a manner similar to that of LPS, then its effects should be blocked by amiloride. Both TPA-induced differentiation (Table I) and Na$^+$ uptake (Fig. 3) were inhibited by amiloride. The $K_{1/2}$ of 0.75 mM amiloride for inhibition of TPA-induced Na$^+$ uptake is in close agreement with that observed for inhibition of LPS-stimulated Na$^+$ uptake (8, 9).

**FIG. 2. Effects of LPS and TPA on $^{86}\text{Rb}^+$ (K$^+$) uptake in 70Z/3 cells.** 10 μg/ml of LPS or 50 nM TPA were added to 70Z/3 cells suspended at $2 \times 10^6$ cells/ml in RPMI 1640 medium with 15% FCS 45 min prior to addition of trace $^{86}\text{RbCl}$. At $t = -1$ min, ouabain was added to 1 mM final concentration to some aliquots. Quadruplicate 0.5-ml aliquots were removed at various times and radioactivity determined as described (see "Materials and Methods"). The ordinate is plotted the nanomoles of K$^+$ exchanged into the cells, assuming that $^{86}\text{Rb}$ is a functional isotopic analog for K$^+$. A, ouabain-sensitive $^{86}\text{Rb}^+$ (K$^+$) uptake. B, untreated control; 0, LPS; 1, TPA. Data points represent $^{86}\text{Rb}^+$ (K$^+$) uptake after subtraction of mean of total uptake minus the ouabain-resistant component. B, ouabain-resistant $^{86}\text{Rb}^+$ (K$^+$) uptake. C, control + ouabain; Δ, LFS + ouabain; O, TPA + ouabain. Data expressed as means ± S.D.

**FIG. 3. Inhibition of TPA-stimulated $^{22}\text{Na}^+$ uptake in 70Z/3 cells by amiloride: effects of increasing extracellular amiloride concentration.** 70Z/3 cells were equilibrated with $^{22}\text{Na}^+$, then treated with 50 nM TPA. Various concentrations of amiloride were added at the same time as the TPA. Total cellular Na$^+$ content was measured periodically over 120 min after the addition of the phorbol and amiloride as described in the legend to Fig. 1. The average rate of $^{22}\text{Na}^+$ uptake over the first 45 min was plotted versus amiloride concentration. Each data point was determined by linear regression. The initial uptake was 70 pmol/10$^6$ cells/min in the absence of amiloride; the error is approximately ±15% for each point.

pH is driven by a high external Na$^+$ concentration. Accurate estimate of initial rates of proton fluxes could not be measured by this technique so the $K_{1/2}$ for extracellular NaCl stimulation does not accurately reflect the affinity of the transport system for Na$^+$. In any event, when extracellular Na$^+$ was reduced to 45 mM, this effectively eliminating the Na$^+$ gradient, no TPA or LPS-induced pH change was observed.

**DISCUSSION**

In this report we have shown that both the tumor-promoting phorbol ester TPA and the polyclonal B-cell mitogen LPS...
by the ratio of absorbance at X4w,a
measurement. pHt was determined
the calibration curve. LPS (10 μg/ml), TPA (50 nM), and/or amiloride (1 mM, in 0.1 M) were added at 0 min after drug addition to the cell suspension and expressed as the means of two experiments.

**Fig. 4.** Effects of LPS, TPA ± amiloride on intracellular pH in 70Z/3 cells. Cells were loaded with 5,6-carboxyfluorescein diacetate as described under "Materials and Methods." pHt was determined by the ratio of absorbance at λexc/em, when compared to a standard calibration curve. LPS (10 μg/ml), TPA (50 nM), and/or amiloride (1 mM) were added at t = 0. ● – ●, untreated control; ○ – ○, LPS; ○ – ○, TPA; ○ – ○, LPS + amiloride; ▲ – ▲, TPA + amiloride; ▲ – ▲, amiloride alone. Data points represent means of three experiments.

**Fig. 5.** Effect of varying extracellular [Na+] on TPA- or LPS-induced changes in pHt in 70Z/3 cells. Cells were loaded with 5,6-carboxyfluorescein diacetate and incubated with variable [Na+] buffers for 30 min at 37°C prior to addition of drug ("Materials and Methods"). Plotted is the increase in cytoplasmic pH between 0 and 10 min after drug addition to the cell suspension and expressed as the means of two experiments. ○ – ○, TPA (50 nM); ● – ●, LPS (10 μg/ml).

induce differentiation in a murine pre-B-lymphocyte cell line by enhancing amiloride-sensitive Na+/H+ exchange. In 70Z/3 cells this event is the critical, rate-limiting step for induction to a surface IgM positive phenotype (8, 9). The diuretic amiloride blocks both differentiation and TPA (or LPS)-induced Na+/H+ exchange in this cell line. The biologically less active phorbol ester 4α-phorbol 12,13-didecanoate induces neither differentiation (Table I) nor the rise in pHt that accompanies it (data not shown).

Recently, Burns and Rozengurt (15) reported that phorbol esters, as well as other growth factors, raised intracellular pH in Swiss 3T3 cells. They had previously shown that these agents also produced a rapid influx of Na+ into these cells (5). These data certainly suggest that a Na+/H+ antiport mechanism may mediate phorbol ester stimulation in 3T3 cells, as we have reported here for 70Z/3 cells. Our evidence of the extracellular Na+ requirement for the TPA- and LPS-induced pH change further supports this mechanism.

We also found that TPA decreases ouabain-sensitive 38Rb+ uptake in 70Z/3 cells (Fig. 2). Similar results have been reported for BALB/c 3T3 pre-adipose cells exposed to phorbol esters (16). The increase in ouabain-sensitive 38Rb+ uptake in 70Z/3 cells after treatment with phorbol esters or LPS presumably results from increased activity of the Na+/K+ ATPase in response to the rise in [Na+].

It is still unclear whether the signal required for surface IgM expression in 70Z/3 cells is an increase in [Na+], or pHt, or both. Ouabain induces 70Z/3 cells to express surface IgM at concentrations which elevate cytoplasmic Na+ suggesting that cytoplasmic Na+ levels are important for differentiation (8, 9, 17). However, we found that ouabain also raises pHt by an unknown mechanism (data not shown). Ouabain has also been shown to affect pH regulation in A431 cells (4). Monensin also induces differentiation in these cells presumably by exchanging Na+ for H+, thereby leading to a rise in [Na+], as well as cytoplasmic alkalinization (8). Monensin is also capable of overcoming the amiloride inhibition of differentiation in this system (8, 9), further supporting the importance of the Na+/H+ exchange system for the initiation of differentiation. However, since all agents which induce surface IgM expression cause both a rise in cytoplasmic pH and Na+, it is still not possible to determine which ionic event is critical to this process.

The evidence that amiloride blocks surface IgM expression by inhibiting Na+/H+ exchange should be discussed further. Several reports have suggested that amiloride worked by competitively inhibiting Na+ binding to an antiprotein from the outside of the cell (1, 4, 14). In this paper we show that the K1/2 of amiloride for inhibition of TPA-induced Na+ uptake is 0.75 mM whereas only 66 μM amiloride is needed to block differentiation. However, we have previously shown that when 70Z/3 cells are incubated in 66 μM amiloride at 37°C the drug is concentrated into the cells to a plateau value of approximately 0.8 mM after 2–3 h and this level is maintained for at least 16 h (9). Since LPS-stimulated Na+ uptake is inhibited after several hours of preincubation in 66 μM amiloride we have suggested that amiloride can also block Na+/H+ exchange from inside the cells (9). These data can explain the apparent discrepancy between the amount of amiloride needed to block Na+ uptake from the outside of the cell immediately after addition of drug and that required to inhibit differentiation over a 24-h period. Although amiloride may be having diverse effects in the cell, the fact that the Na+/H+ ionophore, monensin, circumvents the amiloride inhibition of differentiation argues that the important effect is inhibition of the exchange system (8, 9).
activate, protein kinase C, the Ca\(^{2+}\)-activated, phospholipid-dependent protein kinase, by substituting for diacylglycerol (7). Although the physiologically important substrates of this kinase have not been identified, the rapid changes in Na\(^{+}\) and H\(^{+}\) fluxes observed in 70Z/3 cells after treatment with TPA suggest that the Na\(^{+}/H^{+}\) exchange system is directly or indirectly affected by this kinase, the activation of which may be dependent on protein kinase C, the Ca\(^{2+}\)-activated, phospholipid-kinase have not been identified, the rapid changes in Na\(^{+}\) and H\(^{+}\) fluxes observed in triggering differentiation in this cell line.

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