Effect of TPA on Ion Fluxes and DNA Synthesis In Vascular Smooth Muscle Cells

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ABSTRACT Previous reports have suggested that phorbol esters can decrease the affinity of epidermal growth factor (EGF) for its cellular receptors. Investigations of the consequences of the interaction between phorbol esters and EGF, however, have been limited to EGF-stimulated Na/H exchange in A431 cells (Whitely, B., D. Cassel, Y.-X. Ziang, and U. Glaser, 1984, J. Cell Biol., 99:1162-1166). In the present study, the effect of the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) on EGF-stimulated ion transport and DNA synthesis was determined in cultured vascular smooth muscle cells (A7r5). It was found that TPA stimulated Na/H exchange when added alone (half-maximal stimulatory concentration, 25 nM). However, when cells were pretreated with TPA and then challenged with EGF, TPA significantly inhibited EGF-stimulated Na/H exchange (78%; half-maximal inhibition [Kd] at 2.5 nM). Subsequently the effects of TPA on Na/K/Cl co-transport were measured. TPA was observed to inhibit Na/K/Cl co-transport (half-maximal inhibitory concentration, 50 nM) and also to inhibit EGF-stimulated Na/K/Cl co-transport (100%; Kd at 5 nM). Finally, the effects of TPA on DNA synthesis were assessed. TPA had a modest stimulatory effect on DNA synthesis (half-maximal stimulatory concentration, 6 nM), but had a significant inhibitory effect on EGF-stimulated DNA synthesis (56%; Kd at 5 nM). These findings suggest that the inhibitory effect of TPA on EGF-receptor functions goes beyond previously reported effects on Na/H exchange in A431 cells and extends to EGF-stimulation of Na/K/Cl co-transport and DNA synthesis in vascular smooth muscle cells.

The tumor-promoting phorbol esters have recently been demonstrated to exhibit a wide range of biological effects. In this regard, the phorbol esters have been shown to stimulate Na/H exchange in human epidermoid carcinoma cells A431 (1), in the human leukemic cell line HL-60 (2), in the pre-B-lymphocyte cell line 70Z/3 (3), in Swiss 3T3 cells (4), and in HeLa cells, human fibroblasts, and N1E-115 neuroblastoma cells (5). Furthermore, phorbol esters have been found to inhibit Na/K/Cl co-transport in BALB/c-3T3 preadipose cells (6). In addition to these effects upon cellular ion fluxes, many laboratories have observed that phorbol esters are mitogenic for a variety of cell types (7, 8).

Although the mechanism by which phorbol esters have these effects is not clear, it is known that phorbol esters bind to a specific high affinity membrane receptor (9, 10). This receptor has been postulated to be the calcium- and phospholipid-dependent protein kinase known as protein kinase C (11-12). At present, the physiological substrate for protein kinase C has not been identified; however, recent reports from several laboratories suggest that the epidermal growth factor (EGF) receptor may be a prominent substrate (13-16). These recent observations were not entirely unexpected as previous work by Shoyab et al. indicated that phorbol ester binding to cells resulted in a decreased affinity of EGF for its receptor (17). One interpretation of these above results is that phorbol esters via protein kinase C cause a phosphorylation of the EGF receptor which results in a decreased affinity for EGF. The binding of EGF to its membrane receptor has been demonstrated to have consequences similar to binding to phorbol esters to their membrane receptors. In this regard, EGF has been shown to also cause changes in Na/H exchange.

1Abbreviations used in this paper: DME, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; PBS, fetal bovine serum; MEM, minimal essential medium; TCA, trichloroacetic acid; TPA, 12-O-tetradecanoyl phorbol-13-acetate.
(18) and in Na/K/Cl co-transport (19, 20) as well as to result in mitogenesis (21).

Although phorbol esters have been shown to cause a decreased affinity of EGF for its receptor, the result of this interaction has not been well characterized. Whiteley et al., however, recently reported that the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) inhibits EGF-stimulated Na/H exchange in A431 cells (1). It is not known whether this inhibitory effect of TPA extends to other cell types or to other EGF-mediated activities.

On the basis of these observations, we investigated the effect of TPA on EGF-stimulated ion fluxes (i.e., Na/H exchange and Na/K/Cl co-transport) and on EGF-stimulated DNA synthesis in cultured vascular smooth muscle cells (A7r5). A7r5 cells were used since both the Na/H exchange system (22) and the Na/K/Cl co-transport system (20) (transport systems which mediate phorbol ester-sensitive activity) have been shown to be present and have been characterized. It was observed that TPA significantly inhibited EGF-stimulated Na/H exchange as well as EGF-stimulated Na/K/Cl co-transport. Addition of TPA also significantly inhibited EGF-stimulated DNA synthesis. These results indicate that the inhibitory effect of TPA on EGF receptor functions goes beyond previously reported effects on Na/H exchange (1) and extends to inhibition of Na/K/Cl co-transport and EGF-stimulated DNA synthesis.

MATERIALS AND METHODS

Materials

Amiloride was the generous gift of Merck Sharp & Dohme Div., West Point, PA. Bumetanide was provided by Dr. H. C. Palfrey, University of Chicago, Chicago, IL (originally from Dr. P. Fei of Leo Pharmaceuticals, Copenhagen, Denmark). Ouabain and insulin were purchased from Sigma Chemical Co., St. Louis, MO. EGF was from Collaborative Research Inc., Lexington, MA. 86Rb (1 mCi/5 umol) and [3H]thymidine were purchased from Amersham Corp., Arlington Heights, IL.

Cells

A7r5 cells originally derived from embryonic rat thoracic aorta were purchased from the American Type Culture Collection, Bethesda, MD. A7r5 cells were originally characterized as smooth muscle on the basis of enzyme activity (myokinase and creatine kinase), electrophysiological measurements, and electron microscopy (23). Cells were cultured in Dulbecco's modified Eagle's medium (DME) (KC Biological, Inc., Lenexa, KS) that contained 10% fetal bovine serum (FBS) (KC Biological, Inc.).

Transport Studies

NA/H EXCHANGE MEASUREMENTS. Cells were removed from stock flasks by trypsinization and were seeded onto 60-mm culture dishes. The cells were used 3-5 d after subculture while they were in the logarithmic phase of growth. Net Na influx was assayed after incubating cells in amino acid-free HEPES-buffered minimal essential medium (MEM) for 4 h at 37°C in an air atmosphere in the presence of 0.1% FBS. We have shown previously that this treatment reduces Na influx to the same level as measured in quiescent, serum-deprived cells (24). At time zero, the preincubation medium was aspirated, and 3 ml of 37°C amino acid-free HEPES-buffered MEM that contained 2 mM ouabain was added. Ouabain was present to prevent Na taken up by the cell membrane from entering the cell. Cells were preincubated at 37°C in a gyratory bath at 100 rpm. After a 5-min assay, uptake was terminated by aspirating the assay medium and washing the dishes in ice-cold isotonic MgCl2 as described above. Cells were allowed to air dry and were then extracted with 0.2% SDS. Aliquots of the SDS extract were counted in a liquid scintillation counter (Packard model 2300) (Packard Instrument Co., Inc., Downers Grove, IL) in order to quantitate 86Rb influx. Protein concentration of the SDS extract was measured as described above. The 86Rb uptake was plotted as cpm/g protein versus time. Initial influx, expressed as mol K/g protein/min was calculated from

\[ \text{influx} = \frac{\text{dRc/dt}}{\text{SAex}^{-1}} \]

where dRc/dt represents the slope of the linear phase of the uptake curve (cpm of 86Rb taken up per g protein in 1 min), and SAex is the specific activity of the extracellular phase (cpm/μmol K).

DNA Synthesis Studies

Cells were plated in 24-well cluster dishes (Costar, Cambridge, MA) at a sparse density of 5,000 cells per well, and incubated in DME with 10% FBS. After 24 h, the cells were serum deprived for 4 d in DME that contained 0.1% FBS. After serum deprivation, phorbol ester + EGF was added to cells in the presence of 10 μg/ml insulin and 1 μCi/ml [3H]thymidine. Cells were treated with the above mixture for 40 h. To assess [3H]thymidine incorporation, the medium was aspirated and cells were washed with Tris-buffered saline (three times), fixed with 10% TCA (twice, washed with 95% ethanol (twice), and finally extracted with 0.1 N NaOH. Aliquots were counted in a liquid scintillation counter (Packard model 2300) (Packard Instrument Co., Inc.) to determine [3H]thymidine incorporation. Cellular protein was determined in parallel samples by fluorometric assay as described above. Six replicate protein samples and six replicate [3H]thymidine samples were taken for each condition. The [3H]thymidine incorporation is expressed as cpm [3H]thymidine per g cellular protein.

Calculations

The concentration of an agent causing half-maximal stimulation (K0) was calculated from double reciprocal plots. The concentration of an agent causing half-maximal inhibition (KI) was calculated from fractional inhibition analysis (26). Variations in results are expressed as the SEM. Tests for significance of differences were made by a two-tailed Student's t test (unpaired means solution).

RESULTS

Effect of TPA on EGF-stimulated Net Na+ Influx

Previous experiments have established that vascular smooth muscle cells have an amiloride-sensitive Na+ influx pathway which can be activated (approximately twofold) by serum or platelet-derived growth factor (22). This Na+ influx pathway appears to be analogous to the amiloride-sensitive Na/H exchange system which has been described in a wide variety of cell types (27-30). In addition to platelet-derived growth factor and 10% serum, TPA has been found, in this study, to enhance Na uptake in serum-deprived vascular smooth muscle cells (i.e., 1,3-fold over control (Table I)). The half-maximal stimulatory concentration (K0) for TPA was found to be 25 nM (data not shown). In addition, the TPA-stimulated Na

acetic acid (TCA) for Na determination. Sodium concentration was measured on the TCA extract using a Varian Model 1275 atomic absorption spectrophotometer. Initial net Na influx was taken as the slope of the linear phase of a plot of Na content (μmol/g protein) versus time.

NA/K/CL CO-TRANSPORT MEASUREMENTS. Cells were subcultured as described above for Na/H exchange measurements. Before Na/K/Cl co-transport measurement, cells were serum deprived in amino acid-free HEPES-buffered MEM that contained 0.1% FBS for 4 h at 37°C in an air atmosphere. Na/K/Cl co-transport was assayed by measuring K influx and K influx was assayed by measuring 86Rb uptake. Rb was found to substitute quantitatively for K as previously described (19, 20). After serum deprivation, dishes were incubated at 37°C in a gyratory bath at 100 rpm. Cells were preincubated with agents to be tested for 5 min in fresh amino acid-free HEPES-buffered MEM. This medium was then removed and identical fresh medium that contained 1 μCi/ml 86Rb was added. After a 5-min incubation (86Rb uptake was previously found to be linear over a 5-min time course (19)), uptake was terminated by aspirating the assay medium and washing the dishes in ice-cold isotonic MgCl2 as described above. Cells were allowed to air dry and then were extracted with 0.2% SDS. Aliquots of the SDS extract were counted in a liquid scintillation counter (Packard model 2302) (Packard Instrument Co., Inc., Downers Grove, IL) in order to quantitate 86Rb influx. Protein concentration of the SDS extract was measured as described above. The 86Rb uptake was plotted as cpm/g protein versus time. Initial influx, expressed as mol K/g protein/min was calculated from

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influx pathway was also found to be amiloride sensitive (Table I). On the basis of these findings, it appears that TPA can stimulate an amiloride-sensitive Na influx pathway in A7r5 cells.

EGF has been reported to stimulate an amiloride-sensitive Na influx pathway in a variety of cell types. Consistent with findings in other cells, EGF was found to stimulate amiloride-sensitive Na influx in vascular smooth muscle cells. It was found that EGF caused a 1.8-fold increase in net Na influx (top curve, Fig. 1). The Kₐ value for EGF was calculated to be ~5 nM. When A7r5 cells were incubated for 15 min with 0.16 μM TPA and then challenged with varying concentrations of EGF, net Na influx was inhibited (lower curve, Fig. 1). The maximal flux stimulated by EGF dropped from 16.3 ± 0.6 to 10.0 ± 0.5 μmol/g protein/min representing a 78% inhibition (in the absence and presence of TPA, respectively). The Kₐ value for EGF was unchanged. Thus, it appears that pretreatment of vascular smooth muscle cells with TPA can inhibit the ability of EGF to stimulate amiloride-sensitive Na influx. This observation is in agreement with previously reported findings by Whiteley et al. using A431 cells (1), and suggests that TPA inhibition of EGF-stimulated Na/H exchange is not exclusive to one cell type.

The concentration dependence of TPA inhibition of EGF-stimulated Na influx is shown in Fig. 2. Cells were pretreated for 15 min with varying concentrations of TPA and then challenged with 10 nM EGF (a saturating concentration). The concentration of TPA which caused half-maximal inhibition (Kᵯ) was calculated to be 2.5 nM, and the TPA effect saturated at ~7.5 nM. These values are both approximately one order of magnitude lower than those reported above for stimulation of Na influx by TPA. However, they agree closely with the Kᵯ values for TPA binding to specific receptors (9, 10) and with Kᵯ values reported by Whiteley et al. for phorbol ester inhibition of EGF-stimulated Na/H exchange in A431 cells (1).

**TABLE I. Effect of TPA on Ion Fluxes and DNA Synthesis in Vascular Smooth Muscle Cells**

| Parameter          | Effect       | Half-maximal effective concentration |
|--------------------|--------------|--------------------------------------|
| Net Na⁺ influx     | 25% stimulation | 25 nM                               |
| Na/K/Cl co-transport | 40% inhibition | 50 nM                               |
| DNA synthesis      | 25% stimulation | 6 nM                                |

Values were obtained as described in Figs. 1-4 and Table II. All conditions were as described in Materials and Methods. Values are the mean of six quadruplicate determinations.

**Effect of TPA on EGF-stimulated Na/K/Cl Co-transport**

To determine whether the TPA inhibition of EGF-stimulated cellular function was specific for Na/H exchange, the effects of TPA were tested on another ion transport system, namely, Na/K/Cl co-transport. The Na/K/Cl co-transport system has been well characterized in a wide variety of cells and tissues (31-33) and has recently been reported to occur in A7r5 cultured vascular smooth muscle cells (20). The Na/K/Cl co-transport system in A7r5 cells is most conveniently assayed by determining the bumetanide-sensitive K influx. Since K is quantitatively substituted for by Rb (19, 20), ⁸⁶Rb is used as a tracer for K influx measurements.

When A7r5 cells were pretreated with 0.16 μM TPA for 15 min, Na/K/Cl co-transport was inhibited by ~38% (Table I). The Kᵯ was found to be 50 nM and the inhibitory effect saturated at 75 nM (data not shown). This finding is in agreement with a previous report by O'Brien et al. who measured the effect of phorbol esters on Na/K/Cl co-transport in BALB/c-3T3 preadipose cells (6). However this finding disagrees with our previously published report ([20], see Discussion).

Previous work from our laboratory has shown that a saturating concentration (10 μM) of EGF can activate Na/K/Cl co-transport in A7r5 cells (20) (top curve, Fig. 3). When A7r5...
cells were assayed in identical medium that contained 10 μCi/ml arabinose for 5 min in identical medium containing 10 μM bumetanide. Finally, cells were assayed in identical medium containing 100 μM bumetanide. Values represent mean ± SEM from six quadruplicate determinations.

**Figure 3** EGF-stimulated K influx in A7r5 cells: effect of TPA. A7r5 cells were cultured, subcultured, and serum deprived as described in Materials and Methods. Cells were then preincubated for 15 min in amino acid-free HEPES-buffered MEM that contained 0.01% dimethyl sulfoxide (DMSO) or 0.16 μM TPA. Cells were incubated for 5 min in identical medium ± 10 μM bumetanide. Finally, cells were assayed in identical medium that contained 100 μCi/ml 86Rb for 5 min. Data is expressed as bumetanide-sensitive flux (total-flux in the presence of bumetanide). Values represent mean ± SEM from six quadruplicate determinations.

Since TPA is mitogenic in many different cell types (7, 8), we measured the effects of TPA on EGF-stimulated DNA synthesis in A7r5 cells.

When A7r5 cells were subcultured onto 24-well cluster dishes and serum deprived for 4 d, growth was arrested at G1/G0 and they were rendered quiescent (data not shown). At the end of the 4-d serum-deprivation, cells were treated with 10 μg/ml insulin and DNA synthesis was assayed by measuring [3H]thymidine-incorporation for 40 h in response to 10% FBS or varying concentrations of TPA. As shown in Table I, TPA stimulated DNA synthesis slightly (25%) and the half-maximal stimulatory concentration was calculated to be 6 nM (data not shown). Thus, in A7r5 cells, it appears that TPA is mitogenic, as has been previously shown in other cell types.

Subsequently, the effects of TPA on EGF-stimulated DNA synthesis were determined. As described above, A7r5 cells were serum deprived for 4 d and then treated with 10 μg/ml insulin. DNA synthesis was assayed by measuring [3H]thymidine-incorporation for 40 h in response to 10% FBS, or 10 nM EGF ± varying concentrations of TPA. The K values for EGF was found to be about 5 nM (data not shown) and 10 nM EGF was a saturating concentration. As depicted in Table II, TPA strongly inhibited EGF-stimulated DNA synthesis. The half-maximal inhibitory concentration of TPA was calculated to be 5 nM and the inhibitory effect saturated at 10 nM. These data suggest that TPA can also inhibit EGF-stimulated DNA synthesis in A7r5 cells.

**DISCUSSION**

In the present study the effect of the phorbol ester, TPA, on EGF-stimulated cellular processes was measured in vascular smooth muscle cells. It was found that TPA can inhibit EGF-stimulated Na influx (78% maximal inhibition; K at 2.5 nM), ATPase activity (75% inhibition at 10 nM), and EGF-stimulated Na/K/Cl co-transport.

It is important to point out that with higher concentrations of TPA (i.e., > 20 nM), not only was EGF-stimulated Na/K/Cl co-transport inhibited, but unstimulated Na/K/Cl co-transport was also inhibited as reported above. This finding suggests that TPA inhibits EGF-stimulated Na/K/Cl co-transport in a biphasic pattern. Thus, at TPA concentrations of < 25 nM, the inhibition appears to be specific for the stimulated portion of Na/K/Cl co-transport. On the other hand, at TPA concentrations of > 25 nM, TPA inhibits both stimulated co-transport as well as 40% of the unstimulated (basal) flux. It is possible that the effects measured using high concentrations of TPA are nonspecific and reflect toxic side effects of the compound (see Discussion).

**Effect of TPA on EGF-stimulated DNA Synthesis**

The above results provide support for the hypothesis that the effects of TPA on EGF-stimulated cellular processes do not appear to be specific for A431 cells or to Na/H exchange.
EGF-stimulated Na/K/Cl co-transport (100% maximal inhibition; $K_i$ at 5 nM), and EGF-stimulated DNA synthesis (56% maximal inhibition; $K_i$ at 5 nM). These results on Na influx confirm the findings of Whiteley et al. using A431 cells (human epidermoid carcinoma cells) (1). Those authors observed that another phorbol ester, phorbol 12-myristate-13-acetate, had marked inhibitory effects on EGF-stimulated Na/H exchange. However, the results on Na/K/Cl and DNA synthesis suggest that the inhibitory effects of TPA on EGF-stimulated Na/H exchange extends beyond a specific cell type and ion transport system to include Na/K/Cl co-transport and DNA synthesis in vascular smooth muscle cells.

The mechanism by which TPA is affecting ion transport processes and DNA synthesis is presumably via binding to a membrane receptor and subsequent activation of protein kinase C (11, 12). The calculated $K_i$ values for TPA inhibition of EGF-stimulated cellular processes in the present study (i.e., 2.5–5 nM) correlate closely with the previously reported binding constants of 5–25 nM (9, 10). This contention is supported by our previous finding that phorbol esters which do not cause activation of protein kinase C had no effect on ion transport processes or on DNA synthesis (34).

In previous studies, Shoyab et al. demonstrated that TPA did not compete with EGF for receptor binding (17), but rather that TPA binding causes a decrease in the affinity of EGF binding. This claim has been countered by other workers who argue that TPA causes a decrease in the number of EGF receptors (35). Whatever the mechanism, it is clear from the present studies, that TPA has a profound effect on EGF-stimulated activity. In this regard, a large body of evidence from several different laboratories has recently been published which strongly suggests that activation of protein kinase C causes changes in the phosphorylation pattern of the EGF receptor (13–16). While these changes in receptor phosphorylation pattern have not to date been directly correlated with specific changes in cellular activity, it is tempting to speculate that this is the case. Thus, it is possible that TPA via activation of protein kinase C causes changes in the pattern of phosphorylation of the EGF receptor, which in turn alters EGF receptor affinity or EGF receptor number and subsequent EGF-stimulated cellular processes.

In addition to effects of EGF-stimulated cellular processes, TPA alone was found (a) to stimulate amiloride-sensitive Na influx (25% maximal stimulation; $K_i = 25$ nM), (b) to inhibit Na/K/Cl co-transport (40% maximal inhibition; $K_i = 50$ nM), and (c) to stimulate DNA synthesis (25% maximal stimulation; $K_i = 6$ nM). The finding that TPA stimulated Na influx and DNA synthesis are in agreement with previous reports using a variety of cell types (1–5). However, the observation that TPA inhibits Na/K/Cl co-transport has only been reported using BALB/c-3T3 adipose cells (6). Our previous reports using vascular smooth muscle cells have suggested that TPA enhanced Na/K/Cl co-transport rather than inhibited it (20). However these results could not be reproduced with subsequent lots of TPA. As the present studies have been reproduced using three different lots of TPA, we must agree with O'Brien et al. (6) that TPA does, in fact, cause inhibition of Na/K/Cl co-transport.

The inhibitory effects of TPA on EGF-stimulated cellular processes were observed at lower concentrations than the effect of TPA alone. Since the lower concentrations agree more closely with the reported binding constant, it is possible that the effects of TPA alone were the results of nonspecific or toxic effects of the compound. While toxicity cannot be totally eliminated, the finding that EGF-stimulated Na influx was amiloride sensitive argues against this position. If Na influx were increased as a result of TPA-induced membrane damage, then it would probably not have been amiloride sensitive. Thus the possibility exists that TPA via protein kinase C activation may be stimulating phosphorylation of different sites when added alone than when added in the presence of EGF. Experiments are in progress to test this hypothesis.

The author thanks Kosthy Chacko, Cynthia Galin, and Joanne Knapiuk for their excellent technical assistance, and Cheryll Johnson and Mary Jo Lindgren for their help in manuscript preparation.

This work was supported by grant HI. 31959 from National Institutes of Health, as well as The Chicago Heart Association, and the Schweppes Foundation.

Received for publication 19 February 1985, and in revised form 5 April 1985.

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