Responses of Amphibian Embryos and Blastomeres to a Tumor-promoting Phorbol Ester

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ABSTRACT

Phorbol ester tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) alter the morphology and differentiated states of numerous cell types in vitro. It is likely that these molecules are teratogenic for vertebrate embryos in which cytodifferentiation, cell-tissue interactions, and cellular motility are prominent. By examining embryonic effects, I thought that insights into mechanisms might be gained that would not be apparent in adult tissues. The purpose of this study was to characterize the responses of frog (Bombina orientalis) embryos and embryonic cells to TPA.

Effects of 10 to 100 ng TPA per ml on whole embryos included epithelial dissociation, inhibition of muscle segment histogenesis and adhesive organ differentiation, and acceleration of cell sheet movements during gastrulation. Possibly correlated with accelerated gastrulation, TPA also induced circumferential rotations of lobopodial blebs in blastula cells cultured on plastic. This capability is normally not seen until later developmental stages and is most prominent in areas undergoing morphogenetic movements. For blastula cells cultured on agar, TPA (1.0 to 10 ng/ml) inhibited cytokinesis but not karyokinesis, leading to the formation of abnormal multipolar spindles. A similar uncoupling of cytokinesis and karyokinesis in adult tissues could lead to mitotic aneuploidy, possibly an important step in tumor promotion. The amphibian embryo should be a useful organism for further studies on phorbol ester tumor promoters.

INTRODUCTION

In several animal models, carcinogenesis has been shown to consist of at least 2 distinct stages, "initiation" and "promotion" (5, 7, 47). Initiation is irreversible and possibly results from structural changes in DNA caused by mutagenic carcinogens (7). The promotion phase, at intermediate stages, is substantially reversible (7, 44). Tumor promoters, such as the phorbol diester TPA, are nonmutagenic but increase tumor incidence and decrease the lag period to tumor formation in tissues exposed previously to initiating agents (7).

Depending on the cell lines used, phorbol ester tumor promoters have been reported to inhibit (11, 15, 32, 37, 39) and stimulate (23, 31) several types of differentiation in vitro. For chick fibroblasts, TPA has been shown to cause normal cells to undergo some of the changes associated with transformation (6, 13, 16, 48). TPA induces irreversible shifts toward anchorage independence in several mouse epidermal cell lines (12) and, in cells transformed previously, may cause an exaggeration of transformed properties (21). In mouse skin, TPA has been shown to influence numerous biochemical parameters (1, 4, 35, 40, 41). On the basis of the results of these and other experiments, it has been suggested that promoters facilitate the development of neoplasia by altering the differentiated states of initiated cells (14, 49).

In spite of numerous studies on changes in cellular differentiation induced by promoters, little is known about possible teratogenic effects of phorbol esters in early embryos. TPA has been reported to block the differentiation of gut and skeletal cells in sea urchin embryos (9), but similar studies have not been reported for vertebrates. High-affinity membrane receptors for phorbol esters have been demonstrated on mammalian (33), avian (43), and nematode (3) cells. In light of the apparently ubiquitous distribution of these receptors in the animal kingdom, I thought that amphibians, with their numerous advantages for developmental experiments (30), would be suitable for studies on embryonic responses to TPA.

MATERIALS AND METHODS

Chemicals. TPA and the nonpromoting phorbol analog, 4a-PDD (22), were purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.), dissolved in DMSO, and stored in the dark at −20°. The final concentration of DMSO in all test solutions was 0.1%; this concentration of solvent had no effect on the embryos and cells used in these experiments.

Obtaining Eggs and Embryos. Adult Bombina orientalis frogs (Anura: Discoglossidae) were maintained in the Department of Zoology, Southern Illinois University at Carbondale, as described previously (10). Ovulation, spermiation, and amplexus were induced by injecting 250 to 300 IU human chorionic gonadotropin beneath the dorsal skin. Fertilized eggs produced by amplexing couples were reared in 10% protein solution (3). For dejellying, eggs were placed for 3 min in distilled H2O containing 2.0% cysteine-HCl, 0.2% trypsin, and 0.2% papain, adjusted to pH 8.0 with NaOH, followed by 6 rinses in 10% DMSO in Ear's solution (3). For dejellying, eggs were placed for 3 min in distilled H2O containing 2.0% cysteine-HCl, 0.2% trypsin, and 0.2% papain, adjusted to pH 8.0 with NaOH, followed by 6 rinses in 10% DMSO in Ear's solution (3). For dejellying, eggs were placed for 3 min in distilled H2O containing 2.0% cysteine-HCl, 0.2% trypsin, and 0.2% papain, adjusted to pH 8.0 with NaOH, followed by 6 rinses in 10% DMSO in Ear's solution (3). Fertilized eggs produced by amplexing couples were reared in 10% Barth's solution (3). For dejellying, eggs were placed for 3 min in distilled H2O containing 2.0% cysteine-HCl, 0.2% trypsin, and 0.2% papain, adjusted to pH 8.0 with NaOH, followed by 6 rinses in 10% Barth's solution (3). The enzyme treatment provides a more complete removal of jelly material than is feasible with manual methods and does no developmentally significant damage to the eggs (18). With B. orientalis, it has not proved feasible to remove manually the vitelline envelope, prior to neurulation, without damaging the embryo. Nevertheless, the enzyme treatment "softens" the vitelline envelope, allowing, for example, much easier penetration with a micropipet (18).

Treatment and Scoring of Whole Embryos. Four-cell-stage embryos were placed in finger bowls containing 100 ml 10% Barth's solution, 0.1% DMSO, and appropriate concentrations of phorbol ester. Fresh solutions were given at 24-hr intervals until the experiments were terminated. For pulsed exposures, embryos were removed from the phorbol ester solutions at appropriate times, washed 3 times in 10% Barth's solution, then reared in finger bowls containing 100 ml 10% Barth's solution only. In individual experiments, control and experimen-
tual embryos were derived from the same mating. All embryos were staged according to the criteria of Sussman and Betz (46). For estimation of yolk plug size, greatest yolk plug diameters were measured with a Nikon SMZ10 stereomicroscope fitted with a calibrated micrometer.

Preparation and Treatment of Blastomeres. Animal hemispheres of Stage 8.5 embryos (mid-late blastula) were placed in Petri dishes, the bottoms of which were covered with 2.0% nutrient agar (BBL, Becton, Dickinson & Co., Cockeysville, Md.). The dishes contained Solution A, a calcium-free modification of Barth’s solution which promotes dissociation of the animal hemisphere but allows for continuing mitotic activity of the individual blastomeres on nutrient agar (17). Following dissociation, aliquots of approximately 1000 cells were placed into similar agar-bottomed dishes containing Solution A, 0.1% DMSO, and appropriate concentrations of phorbol ester. On agar, the blastomeres showed no attachment and remained mitotically active. Other aliquots were plated into 35-mm Falcon plastic culture dishes containing Solution A, 0.1% DMSO, and phorbol ester. On plastic, the blastomeres underwent attachment and flattening and were inactive mitotically. Behavior of the blastomeres on agar was monitored with a stereomicroscope using incident illumination. Behavior of the blastomeres on plastic was monitored with an inverted-phase tissue culture microscope. On plastic, cells were scored as attached if they did not move when the dish was gently shaken. Cells were scored as flattened if they had become sufficiently spread such that nuclei were visible.

Histology. Whole embryos were fixed for 72 hr at 5°C in Smith’s fluid (45). Specimens were dehydrated in graded alcohols, cleared in xylene, and embedded in paraplast. Transverse and longitudinal sections were cut at 8 μm and stained with Harris’s hematoxylin and 0.5% aqueous eosin Y. For visualization of nuclei in spherical, yolk-laden blastomeres, the cells were fixed in Carnoy’s solution (29), washed in 70% ethanol, air-dried on a glass microscope slide, and then taken to 100% ethanol and then into collodion (Mallinckrodt, Inc., St. Louis, Mo.) solution [collodion:ether:100% ethanol (1:50:50)]. After being air-dried, the cells were stained in an aqueous solution of 22% acetic acid, 22% lactic acid, and 1.0% synthetic orcein (Carolina Biological Supply Co., Burlington, N. C.); destained in acetic acid: lactic acid; taken through a graded series of ethanol (70 to 100%) and then into xylene; and finally mounted in Permount under a glass coverslip.

RESULTS

Effects of TPA on Whole Embryos. Dejellied embryos arrested at mid-to-late cleavage stages when placed in 100 ng TPA per ml at the 4-cell stage. In 25 ng TPA per ml, embryos entered gastrulation but arrested prior to neurulation. Embryos were also adversely affected by 10 ng TPA per ml, with fewer than 25% surviving to the gill bud stage (Chart 1). Development of embryos in 100 ng 4a-PDD per ml was indistinguishable from that of the controls (data not shown).

Arresting embryos in all concentrations of TPA were characterized, initially, by the appearance of loose cells on the surface, followed by progressive disruption of the surface epithelium. Recently dissociated surface cells were viable, based on trypan blue exclusion. Development of postneurula embryos in 10 ng TPA per ml was retarded, and most were foreshortened in comparison to controls (Fig. 1). Many of the TPA-treated embryos also displayed an abnormal ventral flexure (Fig. 1). Notochords of TPA-treated (10 ng/ml) embryos (same stages as those shown in Fig. 1), as revealed by longitudinal sections, appeared normal. In comparison to controls, numbers of somites were reduced by one-third to one-half in the TPA embryos. Muscle segments in the TPA embryos appeared disorganized and poorly differentiated (Fig. 2). Transverse sections revealed that the cavities of the brain, spinal cord, and gut were reduced in the TPA-treated embryos (Fig. 3). Differentiation of the ventral adhesive organ cells was also inhibited in the TPA-treated embryos (Fig. 3).

Results of pulsed exposure to 10 ng TPA per ml indicated that the effects were reversible if the embryos were removed from TPA by Stage 12 (late gastrula), when epithelial dissociation had not yet progressed to advanced stages (Chart 2). The effects of TPA were attenuated when the jelly coats and vitelline envelope were left intact. For example, 10 ng TPA per ml had no effect on nondejellied embryos, and developmental anomalies induced by 100 and 1000 ng TPA per ml did not appear until Stage 14 (neurula) and Stage 17 (tail bud), respectively (data not shown).

In an initial experiment with dejellied embryos in 10 ng TPA per ml, some promoter-treated embryos, at the late gastrula stage, appeared developmentally advanced in comparison to controls. This was not seen in embryos with intact jelly coats. Several dejellied embryos in TPA displayed nearly complete yolk plug internalization (Stage 12.5), when the most advanced
Yolk Plug Diameter (mm)

Chart 3. Acceleration of yolk plug internalization by TPA. Embryos were placed in test solutions at the 4-cell stage. When several of the TPA-treated embryos displayed nearly complete yolk plug internalization, all embryos were fixed, and yolk plug diameters were measured. ■, reared in 10 ng TPA per ml (n = 37); □, controls reared in 0.1% DMSO only (n = 39). The mean yolk plug diameter of TPA-treated embryos (0.81 mm) was less than the mean diameter for controls (1.20 mm) (p < 0.01).

control embryos were still at mid-yolk plug (Stage 11.5) (Fig. 4). This experiment was repeated 3 times with similar results. In one of these trials, all embryos were fixed in 10% formalin when several of the TPA-treated embryos displayed nearly completely internalized yolk plugs. Yolk plug diameters were then measured and compared for the control and promoter-treated groups (Chart 3).

Histological analysis of control and TPA-treated gastrulas indicated that the accelerated disappearance of the yolk plug was due to a normal-appearing involution of cells over the blastopore rims and not, for example, to an abnormal collapse of the endoderm into the blastocoele (Fig. 5). In comparison to controls, yolk plugs of TPA-treated gastrulas were often "sunken" below the blastopore rims and frequently exhibited surface unevenness (Figs. 4 and 5). This somewhat abnormal appearance of the blastopore area did not, however, preclude normal development if the embryos were removed from TPA (Chart 2; see below).

To characterize the developmental potentials of TPA-accelerated gastrulas, 57 dejellied embryos were placed in 10 ng TPA per ml and 57 in 0.1% DMSO. When 15 of the TPA embryos had nearly completed yolk plug internalization, they and 15 of the most advanced controls (which were still at the mid-yolk plug stage) were removed and placed in 10% Barth’s solution for further rearing. At Stage 13 (neural plate), the TPA-treated embryos no longer appeared advanced compared to the controls. Both controls and experimental cells developed normally at the same rate thereafter.

Effects of TPA on Anchorage-independent Cytokinesis of Blastomeres on Agar. Over an interval of 375 min at 21°C, blastomeres in 0.1% DMSO underwent approximately 3.5 population doublings, blastomeres in 1.0 ng TPA per ml underwent less than 2 doublings, while cytokinesis was nearly completely inhibited by 10 ng TPA per ml (Chart 4). Proliferation of blastomeres in 100 ng 4α-PDD per ml did not differ from the controls (data not shown). While cytokinesis was inhibited by TPA, many of the TPA-treated cells had multiple nuclei or abnormal multipolar spindles (Fig. 6), indicating that karyokinesis had continued. At the end of 20.5 hr in TPA (1.0 and 10 ng/ml), 99% of the blastomeres were still viable as determined by trypan blue exclusion.

Effects of TPA on Attachment, Flattening, and Circus Movements of Blastomeres on Plastic. TPA at 10 ng/ml had an inhibitory effect on the ability of blastomeres to attach and flatten on plastic (Table 1). Numerous TPA-treated cells displayed clear lobopodial blebs which moved around the cell periphery (Fig. 7). Such "circus movements" (24) were occasionally seen to exert sufficient force to move a cell away from an adjacent cell with which it had been in contact. The frequencies with which control and TPA-treated cells displayed circus movements are given in Table 1. The blebbing activity of TPA-treated cells was not associated with decreased attachment efficiency, since similar proportions of unattached (26%) and attached (23%) TPA-treated cells displayed circus movements.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of cells</th>
<th>Unattached (%)</th>
<th>Attached (%)</th>
<th>Flattening (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% DMSO</td>
<td>1719*</td>
<td>1.6</td>
<td>98.4</td>
<td>97.6</td>
</tr>
<tr>
<td>4α-PDD (100 ng/ml)</td>
<td>535*</td>
<td>3.6</td>
<td>96.4</td>
<td>92.9</td>
</tr>
<tr>
<td>TPA (10 ng/ml)</td>
<td>1841*</td>
<td>33.6</td>
<td>66.4</td>
<td>10.7</td>
</tr>
</tbody>
</table>

* Combined totals from 2 experiments.
DISCUSSION

Three major responses of embryos to TPA can be identified in these experiments. The first, observed in both dejellied and nondejellied embryos and in concentrations of TPA ranging from 10 to 1000 ng/ml, involved a progressive dissociation of the surface epithelium. The ability of dissociated epithelial cells to exclude trypan blue suggests that TPA-induced epithelial disruption was due to alterations in intercellular adhesive properties and not to simple cell toxicity. A disruption of cell-adhesive properties was also indicated in the in vitro experiments, where TPA had a marked inhibitory effect on the ability of blastomeres to attach and flatten on plastic.

Previous studies have established that TPA changes numerous membrane and cell surface properties, including loss of fibronectin (6), increased choline incorporation (27), decreased binding of epidermal growth factor (28), alteration of cytoskeletal elements and epithelial tight junctions (36), disruption of intercellular communication via gap junctions (20), and alterations in membrane fluidity (8). Enomoto et al. (20) suggested that TPA may inhibit electrical cell coupling by changing the intracellular and extracellular Ca²⁺ concentration balance. In this regard, it should be noted that early amphibian embryos are easily dissociated to single cells by incubation in reduced Ca²⁺ or Ca²⁺-free media (3). The postgastrula surface epithelium is a dynamic, mitotically active tissue, one to 2 cell layers thick, with precursor ciliated cells inserting into it from below (19). Any TPA-induced disruption of cell-cell communication or adhesion, whether by direct interference with cell surface structures or by alteration of calcium ion microenvironments, could lead to rapid loss of epithelial integrity.

A second major effect was the inhibition of histogenesis in some embryonic tissues. This was most notable in the muscle segments, where myogenesis appeared disrupted, and in the anteroventral skin epithelium, where differentiation of the adhesive organ cells was inhibited. This is consistent with the well-documented ability of TPA to inhibit numerous types of differentiation in vitro (14). However, the embryonic effects may result secondarily from an overall TPA-induced retardation of development, which could, in turn, be due to the initial loss of epithelial integrity. Whether or not TPA directly inhibits myogenesis and adhesive cell differentiation could be tested by organ-tissue culture studies.

A third significant response of embryos to TPA was an acceleration of morphogenetic movements during gastrulation. This effect was more dependent on experimental conditions than was epithelial disruption or inhibition of differentiation, being observed only with dejellied embryos reared in 10 ng TPA per ml. To my knowledge, this represents the first example of a chemical accelerating early vertebrate development at the whole-organism level, although TPA has been reported to cause precocious tooth eruption in mice (42). While external cell movements during gastrulation were accelerated by TPA, late gastrula embryos removed from TPA did not form neural plates any sooner than did the controls. Thus, postgastrula developmental "clocks" were not reset by TPA-induced morphogenetic movements during gastrulation.

During anuran gastrulation, yolk-laden endoderm cells of the vegetal hemisphere form a temporary yolk plug which is gradually covered over by the contracting rims of the blastopore. This is, in turn, due to the active involution of cell sheets over the blastopore rims and an accompanying expansion ("epiboly") of the ectoderm to cover the embryonic surface as mesoderm and endoderm are internalized (2). In Xenopus laevis, scanning electron microscopy has been used to demonstrate that epiboly is accompanied by changing cell shapes and cell-cell associations in the animal hemisphere and marginal zone (26). Superficial cells flatten, while cells of the deeper layers extend protrusions inward or outward and move between one another. This "radial interdigitation" of the deeper cells, combined with superficial cell flattening, is thought to be the basis for an overall expansion at the expense of thinning (26). It is possible that 10 ng TPA per ml induced a "sublethal" reduction in cell-cell adhesion, facilitating slippage and interdigitation of the deeper cell layers, thereby contributing to an accelerated expansion of the ectoderm during gastrulation. Scanning electron microscopy studies could shed some light on this.

While reduced intercellular adhesion could contribute to some aspects of accelerated gastrulation, it is difficult to imagine that reduced adhesiveness alone could be responsible for the coordinated cell and tissue movements required to accelerate involution and epiboly. In this regard, the in vitro behavior of individual blastula cells in TPA was noteworthy. On a substrate which normally promoted attachment (tissue culture plastic), TPA caused blastula cells to undergo circus movements, i.e., formation of peripheral hyaline blebs which rotated around the cells in clockwise or counterclockwise directions. Johnson (24) and Johnson and Adelman (25) have demonstrated that the ability of amphibian cells to undergo circus movements in vitro is correlated with their morphogenetic capabilities in vivo. Thus, circus movements normally occur in very few cells taken from blastulas; during gastrulation, there is a progressive increase in the proportion of cells displaying this activity. With gastrula cells, circus movements are most prominent in cells taken from those areas of the embryo involved in morphogenetic movements (24).

When cells capable of circus movements are in contact with adjacent cells, coordinate motions of hyaline blebs and lobopodia could generate large mass movements and may be a significant driving force in gastrulation (25). Hyaline blebs and lobopodia have been observed in amphibian gastrulas in vivo (34). Thus, TPA-induced circus movement activity in vitro may relate directly to TPA-induced acceleration of gastrulation in vivo. TPA may, in fact, have dual effects in these embryos, accelerating mass cell movements such as involution but also decreasing cell-cell adhesion to the point of dissociation and eventual embryonic arrest. Differences in susceptibility of embryos to these 2 effects may explain the variability in responses of embryos to TPA-induced accelerated gastrulation (Chart 3).

Whether induction of hyaline membrane activity in frog blastula cells relates to the mode of action of TPA in tumor promotion remains to be demonstrated. It is significant, however, that the phorbol monoester 4a-PDD, which is inactive as a promoter in mouse skin (22), had no circus movement-inducing activity and also had no effects on whole embryos.

Many of the responses observed in this study are suggestive of alterations in the cytoskeleton or in the association of cytoskeletal elements with the plasma membrane. For blastula cells cultured on agar, where substrate attachment was inhibited and the cells remained mitotically active, TPA (1.0 and 10 ng/ml) had a marked inhibitory effect on cytokinesis. Again, this
ACKNOWLEDGMENTS
I wish to thank James F. Moruzzi for developing the stimulus-staining procedure and for processing the cells. I also wish to thank Dr. R. A. Brandon and Dr. G. M. Malacinski for critically reading the manuscript. The Scientific procedure and for processing the cells. I also wish to thank Dr. R. A. Brandon

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Effects of TPA on Frog Embryo Cells


Fig. 1. Gross morphology of embryos reared in 10 ng TPA per ml (2 embryos at right) and control embryo reared in 0.1% DMSO only (single embryo at left). Note foreshortening of embryonic axes and epithelial disruption on the TPA-treated embryos. × 15.

Fig. 2. Longitudinal sections through muscle segments of Stage 18 embryos (similar to those shown in Fig. 1). a, control embryo, showing well-organized appearance of muscle fibers, and nuclei situated as bands down the middle of each segment. × 140. b, embryo reared in 10 ng TPA per ml; note lack of muscle fiber differentiation and random positions of nuclei. × 140.

Fig. 3. Transverse sections through myelencephalon regions of Stage 18 embryos. a, control embryo; ventral skin epithelium is thickened and constitutes the ventral adhesive organ. × 70. b, embryo reared in 10 ng TPA per ml; note reduced cavities of the brain and gut, lack of adhesive organ differentiation, and loose cells on the lateral skin epithelium. × 70.

Fig. 4. Appearance of yolk plugs in dejellied embryos reared in 10 ng TPA per ml (top) and in 0.1% DMSO (bottom). All 6 eggs were fertilized at the same time. Yolk plug internalization is advanced in the TPA-reared embryos. Each embryo is approximately 2.0 mm in diameter.

Fig. 5. Sagittal sections through yolk plugs, a, control embryo, × 80. b, embryo reared in 10 ng TPA per ml. × 80. Arrows, directions of cell migration over the dorsal (right) and ventral (left) lips of the blastopore.

Fig. 6. Multipolar mitosis in a blastomere exposed to 1.0 ng TPA per ml. × 1900.

Fig. 7. Effects of 10 ng TPA per ml on blastomeres cultured on plastic. a, controls, showing attachment filopodia and flattening. b, blastomeres in TPA, showing clear lobopodial blebs and no flattening. c, same cells as b, 30 sec later, showing changed locations of blebs, indicative of active circus movements.
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