

Phorbol Esters Alter Cell Fate during Development of Sea Urchin Embryos

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Abstract. Protein kinase C (PKC) has been implicated as important in controlling cell differentiation during embryonic development. We have examined the ability of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), an activator of PKC, to alter the differentiation of cells during sea urchin development. Addition of TPA to embryos for 10–15 min during early cleavage caused dramatic changes in their development during gastrulation. Using tissue-specific antibodies, we have shown that TPA causes the number of cells that differentiate as endoderm and mesoderm to increase relative to the number that differentiate as ectoderm. cDNA probes

show that treatment with TPA causes an increase in accumulation of RNAs specific to endoderm and mesoderm with a concomitant decrease in RNAs specific to ectoderm. Treatment of isolated prospective ectodermal cells with TPA causes them to differentiate into endoderm and mesoderm. The critical period for TPA to alter development is during early to mid cleavage, and treatment of embryos with TPA after that time has little effect. These results indicate that PKC may play a key role in determining the fate of cells during sea urchin development.

KNOWLEDGE of the mechanisms involved in determining cell fate during development is central to understanding how embryos are formed. The roles of signal transduction pathways in these processes have recently begun to be examined, and it has become clear that such pathways play a role during the development of a number of organisms (Otte et al., 1989; Greenwald and Rubin, 1992; Jessel and Melton, 1992).

Cell-cell interactions have long been known to play a role in sea urchin development (Horstadius, 1973). This suggests that signaling mediated by cell membrane interactions may be important in instructing cells to differentiate into tissues appropriate to their position in the embryo. The fates of sea urchin blastomeres are initially determined by their position along an animal-vegetal axis. Cells from the animal hemisphere of cleavage stage embryos give rise solely to ectoderm; cells of the vegetal hemisphere give rise to ectoderm, endoderm, and mesoderm, respectively, ranging from the equator to the vegetal pole (Cameron et al., 1987). Treatment with lithium causes sea urchin embryos to form exaggerated guts (endoderm) and spicules (mesoderm), and can cause isolated animal blastomeres to differentiate into vegetal tissue (von Ubisch, 1929; Livingston and Wilt, 1989). This suggests that lithium enhances the type of differentiation normally found in cells derived from the vegetal pole of the embryo.

Lithium has been shown to alter the development of several types of embryos (von Ubisch, 1929; Nieuwkoop, 1970; Livingston and Wilt, 1989; Peters et al., 1989) and is

thought to block signaling through the inositol phosphate-protein kinase C (PKC)¹ second messenger pathway (Berridge et al., 1989). This idea is supported by findings in amphibian embryos showing that *myo*-inositol can reverse the effect of lithium (Busa and Gimlich, 1989), and that inositol phosphate levels are altered by lithium treatment (Maslanski et al., 1992). More recently, PKC has been shown to participate in controlling differentiation along an embryonic axis in *Xenopus* (Otte et al., 1988), and implicated in determining cell fate in *Dictyostelium* (Ginsburg and Kimmel, 1989) and *Hydra* (Muller, 1989). In this study, we examine the effect of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) stimulation of PKC on differentiation of blastomeres along the animal-vegetal axis of sea urchin embryos. We show that TPA causes cells committed to form ectoderm and its derivatives to change fate and form tissues normally derived from endoderm and mesoderm, implicating PKC in the mechanisms that establish cell fate.

Materials and Methods

Embryo Culture

Gametes of *S. purpuratus* were obtained and fertilized by standard methods (Hinegardner, 1967; Hall, 1978; Lutz and Inoue, 1986). TPA or an inactive derivative of TPA was added to a final concentration of 5 nM–150 ml of

1. *Abbreviations used in this paper:* PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

a 1% embryo culture (1 ml of packed eggs in 100 ml of sea water) at the 16 cell stage and incubated 10–15 min at 15°C. The range of TPA concentrations that has an effect with killing the embryos is very narrow. As a result, varying the dose of TPA provided no useful information. The embryos were then settled and resuspended twice in 200 ml sea water and cultured at 15°C. Blastomere pairs were obtained and treated with lithium using methods described previously (Livingston and Wilt, 1989). Mesomeres isolated at the 16 cell stage were immediately treated with 5 nM TPA in 5 ml sea water for 30 min, and then transferred to 5 ml sea water and cultured at 15°C.

Antibody Staining

Fixation of embryos and antibody staining were done using methods described by Ettensohn and McClay (1988), with modifications by Livingston and Wilt (1990). FITC-conjugated secondary antibodies (Sigma Chemical Co., St. Louis, MO) were used to visualize the location of the primary antibody. Nuclei were stained with 10 µg/ml propidium iodide (Sigma Chemical Co.) in PBS after antibody staining. Confocal microscopy was used to visualize the FITC antibody staining and the propidium iodide stained nuclei simultaneously. 5-µm serial optical sections were used to determine the number of antibody-positive cells in individual embryos.

Northern Blot Analysis

RNA was isolated from 24–48-h embryos by methods described by Childs et al. (1979). RNA blotting and hybridization were carried out as described by Benson et al. (1987). Single-stranded ³²P-labeled RNA probes were prepared as described by Melton et al. (1984). The mesoderm-specific cDNA clone, pHS72, was isolated by Benson et al. (1987). The endodermal cDNA, Endo16, was isolated by Nocente-McGrath et al. (1989). The two ectoderm-specific cDNAs were isolated by Lynn et al. (1983) (Spec 1a) and Yang et al. (1989) (arylsulfatase). Amounts of RNA were quantitated using a laser scanning densitometer and a probe to rRNA as a control.

DNA Determinations

Control embryos and embryos treated with TPA were cultured at a concentration of 1% in sea water at 15°C. At various times after treatment, three 1-ml samples of each culture were removed and centrifuged briefly, and the pellet of embryos was frozen at –20°C. DNA concentration was determined by the fluorometric method of Brunk et al. (1979).

Results

Lithium has been shown to have its greatest effect during cleavage stages of amphibia (Nieuwkoop, 1970) and sea urchins (Livingston and Wilt, 1990). For this reason, we treated embryos with TPA for a short period at the 16-cell stage. Treated embryos cleaved normally, although the cells of treated embryos were more rounded and adhered to one another less tightly than controls. 20 h after treatment, when the three embryonic germ layers begin to become distinct from one another, control embryos had formed hollow spheres composed of a thin, tight epithelial layer (Fig. 1 A). TPA-treated embryos formed spheres, but the epithelial layer was less well organized and possessed many gaps in the epithelium, and a large number of cells had migrated into the blastocoel (Fig. 1 D). When control embryos had completed gastrulation, at 48 h after fertilization (Fig. 1 B), TPA-treated embryos had also formed the three germ layers. However, gut formation occurred both by invagination, as in con-

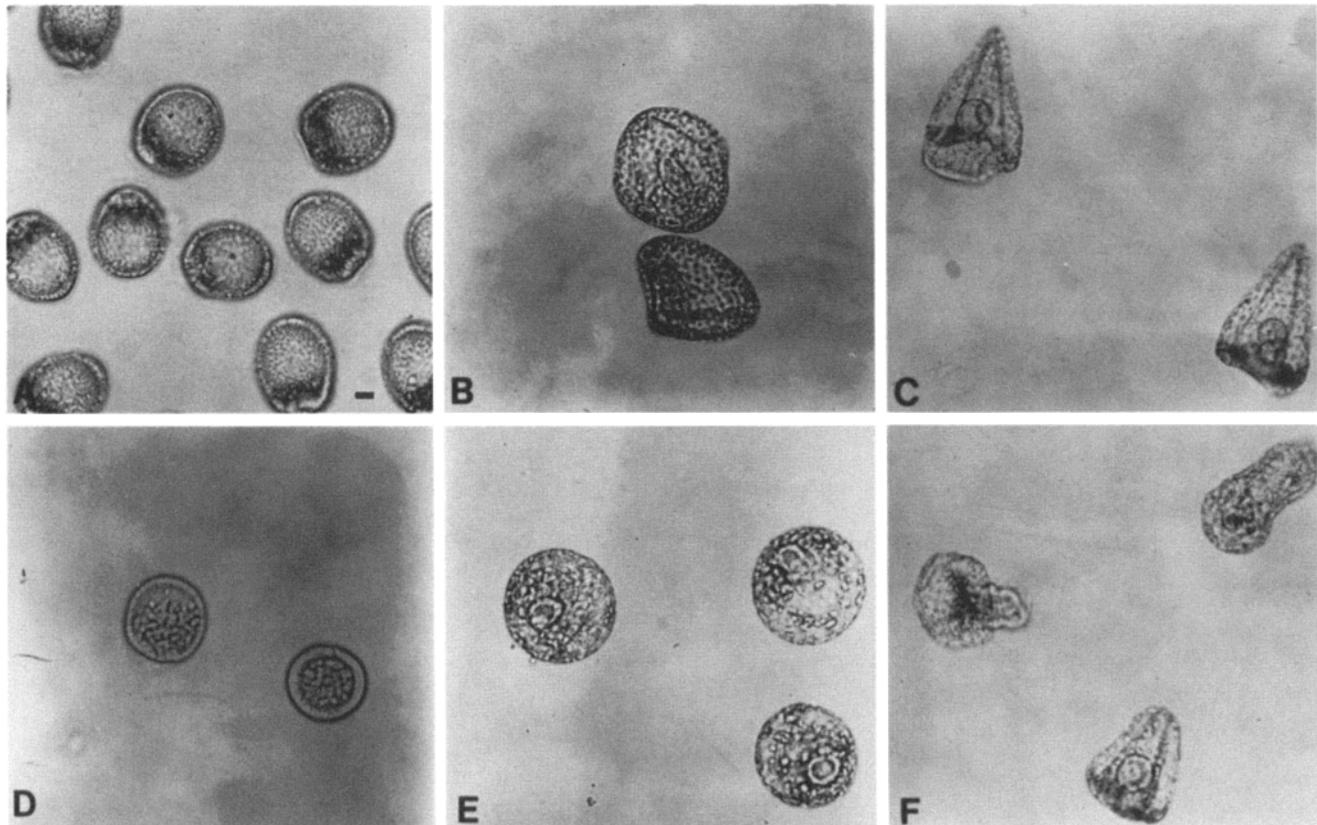


Figure 1. Morphology of TPA-treated embryos. Control embryos and embryos treated with TPA at the 16-cell stage were fixed and examined over the course of development. A–C, control. D–F, TPA-treated. A and D, 24 h; B and E, 48 h; C and F, 72 h. Bar, 25 µM.

trols, and by aggregation of cells that had previously migrated into the blastocoel (Fig. 1 E).

By 72 h, TPA-treated embryos exhibited enlarged, often exogastrulated guts, and a depleted surface epithelium (Fig. 1 F). In treated embryos skeletal spicules were often enlarged and present in much greater numbers than normal (Figs. 1 F and 2 C). In many cases TPA-treated embryos formed condensed spheres, with thickened columnar walls lacking the expanded epithelial cells that normally make up the ectoderm (Fig. 2 E). Such embryos did not seem to be the result of separation of embryos into guts and ectodermal vesicles, since very few hollow ciliated vesicles were observed in the cultures, and, with very few exceptions, all embryos contained a gut and spicules. It is possible that in extreme cases TPA causes ectodermal vesicles to break off and subsequently degenerate. However, this was never observed in the cultures that were examined. The alternative is that in extreme cases, the morphogenetic movements that give rise to normal morphology are altered or never occur, and that endodermal and mesodermal cells simply differentiate into characteristic tissues in the positions they originally occupy. Variability was observed between egg batches in the degree of their response to treatment with TPA, although the response of embryos within a batch was mostly uniform. Fig. 1 F was chosen to show the range of response to TPA. The inactive phorbol ester (4 α -phorbol-12,13-didecanoate) had no effect whatsoever at similar concentrations.

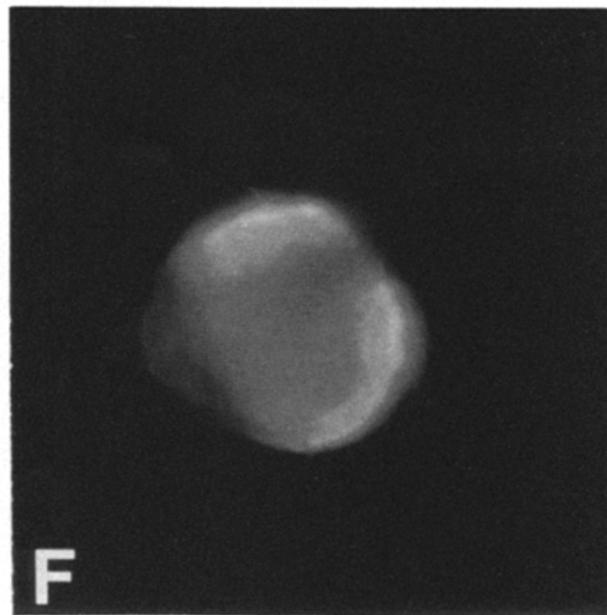
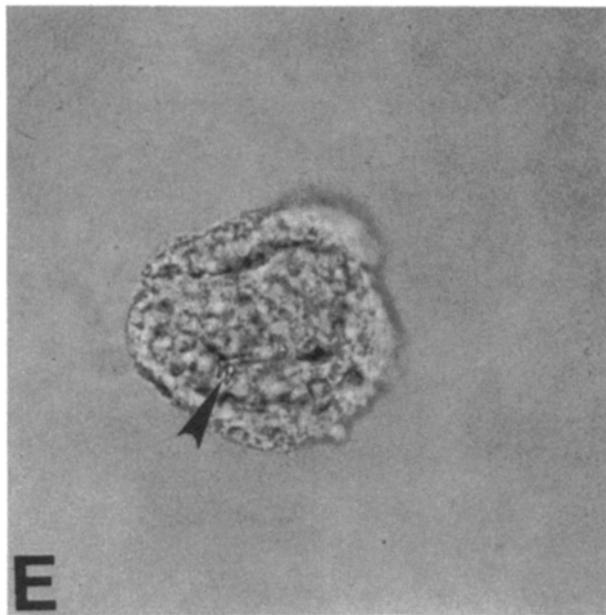
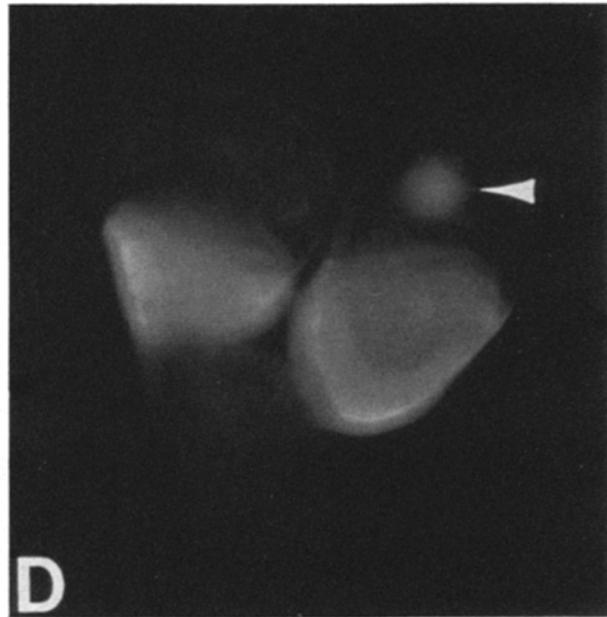
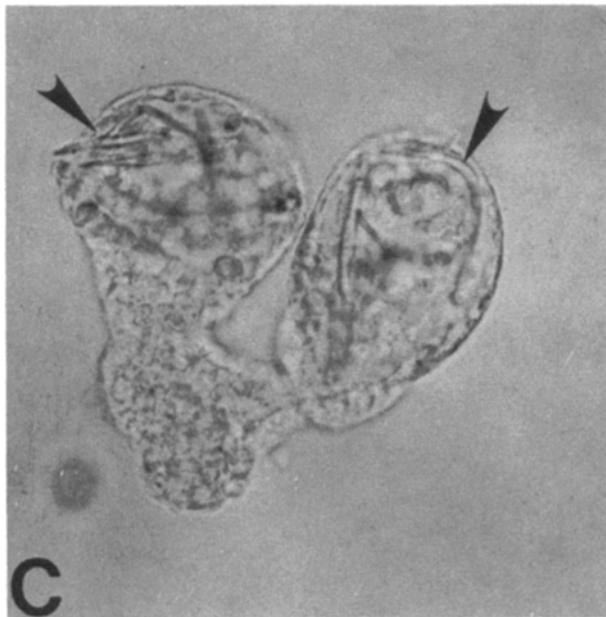
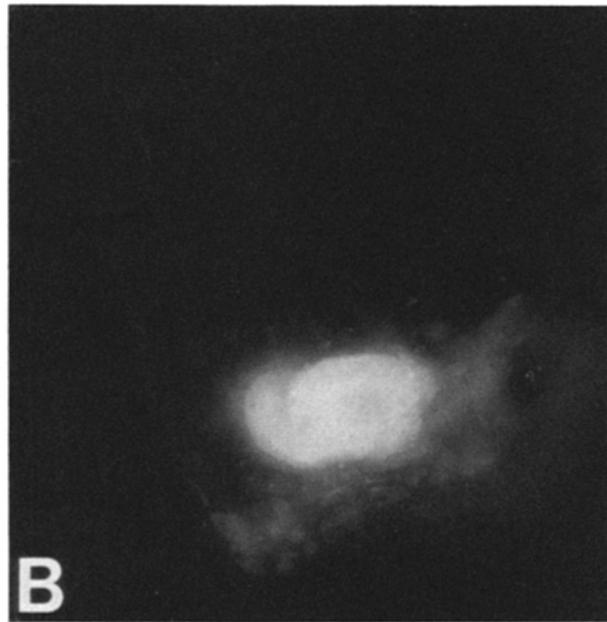
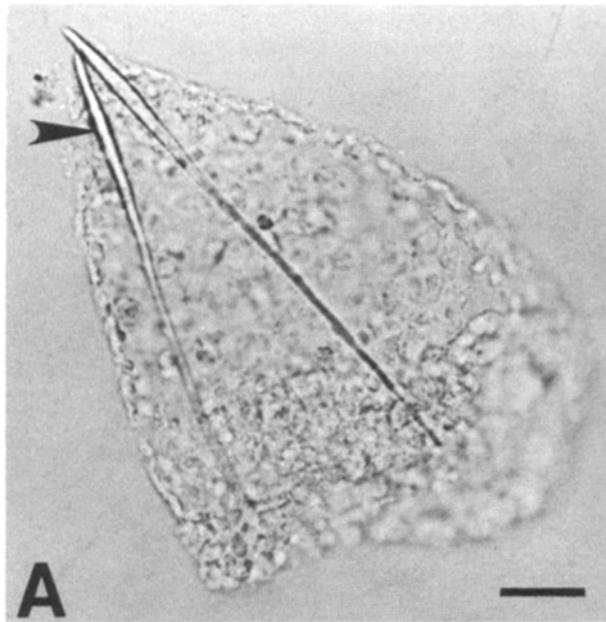
Because the altered morphology of TPA-treated embryos made it difficult to define cell types unequivocally on morphological grounds, we used antibodies that bind specifically to gut and skeletogenic mesenchyme (1 $^{\circ}$ mesenchyme) to assess the tissue types that were formed. In normal development, 1 $^{\circ}$ mesenchyme cells ingress into the blastocoel cavity as the first step in gastrulation. Lineage tracing has shown that after ingress there are 32 distinct 1 $^{\circ}$ mesenchyme cells (Cameron et al., 1987). By 48 h, the number of 1 $^{\circ}$ mesenchyme cells in control embryos has increased to 60. During this time the cells fuse to form an elongated syncytial cytoplasmic cable, and two triradiate skeletal spicules have been formed within an extracellular space formed by the cable (Decker and Lennarz, 1987; Fig. 3 C). The number of cells that react with the antibody to 1 $^{\circ}$ mesenchyme can be determined by staining nuclei with propidium iodide and using a confocal microscope to produce optical sections of the embryos (Fig. 3). Control embryos were shown to contain 31.3 ± 0.6 cells after ingress, while TPA-treated embryos had 46 ± 11.1 1 $^{\circ}$ mesenchyme cells at the same stage. The staining pattern in TPA-treated embryos indicated that cell membranes had become irregular (Fig. 3 B), suggesting that the 1 $^{\circ}$ mesenchyme had undergone precocious fusion to form a syncytium. At the 48-h prism gastrula stage, control embryos had 57 ± 5.0 1 $^{\circ}$ mesenchyme cells, while in TPA-treated embryos the number of cells staining increased to 75 ± 11.3 ($n = 7$, $P < 0.01$ for comparisons of TPA vs. control at 24 and 48 h). The embryos treated with TPA exhibited a range of spicule morphology and 1 $^{\circ}$ mesenchyme-specific antibody staining that correlated with the amount of surface epithelium present. Those embryos that maintained sufficient number of surface epithelial cells formed elongated syncytia and spicules that were longer, thicker, and more numerous than controls. More often, the reduction in the number of surface epithelial cells and the corresponding in-

crease in 1 $^{\circ}$ mesenchyme in TPA-treated embryos resulted in the formation of a large, compressed syncytial mass containing a large number of skeletal spicules twisted within it (Figs. 2 C and 3 D). Additional 1 $^{\circ}$ mesenchyme cells and spicules often appeared at the tip of the gut in exogastrula (Fig. 3 D). In extreme cases in which the embryos were condensed and consisted almost entirely of columnar cells that stained with a gut-specific antibody, a large number of small, triradiate spicules were formed in the blastocoel (Fig. 2 E).

Gut formation in sea urchin normally occurs by the invagination of prospective endoderm at the vegetal pole. In TPA-treated embryos, gut tissue can be formed by invagination (or by exogastrulation), but is sometimes formed by the aggregation of single cells that have moved into the blastocoel before invagination, as determined by staining with a gut-specific antibody. Such aggregations often resulted in a group of cells distinct from the organized gut that also expressed markers of gut differentiation (Fig. 2 D). This type of gut formation is reminiscent of that seen in aggregates of dissociated embryos (Bernacki and McClay, 1989). Furthermore, in TPA-treated embryos that form thick-walled spheres, almost all of the cells of the embryo stain with the gut-specific antibody (Fig. 2 F).

The morphological changes observed during gastrulation as a result of treatment with phorbol ester during cleavage led us to investigate the changes in gene expression in TPA-treated embryos, as assessed by RNA accumulation. A number of genes have been isolated in sea urchins that by the early gastrula stage are restricted in expression to mesoderm (Benson et al., 1987), endoderm (Nocente-McGrath et al., 1989), or ectoderm (Lynn et al., 1983; Yang et al., 1989). Examination of the expression of such genes showed that TPA caused the accumulation of an RNA specific to gut (Endo16) to increase 2.5–5-fold, and a primary mesenchyme-specific RNA (SM50) to increase by 2.5–25-fold in the three embryo cultures examined (Fig. 4). TPA caused the accumulation of two RNAs specific to ectoderm (SpArs, Spec1A) to decrease 2.5–14.8-fold in these same cultures (Fig. 4). The inactive phorbol ester had no effect on expression of the ectodermal genes, and the same inactive control caused only an average 1.2- and 1.36-fold increase in accumulation of gut and 1 $^{\circ}$ mesenchyme-specific RNAs, respectively. The observed degree of change in gene expression correlated with the extent of change in morphology in each batch of embryos examined. For example, in TPA-treated cultures where the morphology of the embryos more closely resembled controls, the increase in both gut-specific and mesoderm-specific RNA was 2.5-fold. In TPA-treated cultures that had greatly exaggerated guts and skeletal spicules, the increase in gut-specific RNA was 5-fold, and in mesoderm-specific RNA was 25-fold. The degree of increase in gut and 1 $^{\circ}$ mesenchyme-specific RNAs in each batch of embryos also correlated well with the degree of decrease in ectoderm-specific RNAs. The largest decrease in ectoderm-specific RNA accumulation was seen in the cultures that exhibited the largest increase in endoderm- and mesoderm-specific RNAs.

To directly examine the effect of TPA on animal ectodermal precursor cells, we investigated the effect of TPA on blastomeres isolated from the animal half of the embryo. We also examined the effects of lithium, and lithium in conjunction with TPA, on the differentiation of these animal blastomeres. Untreated blastomeres formed hollow epithelial



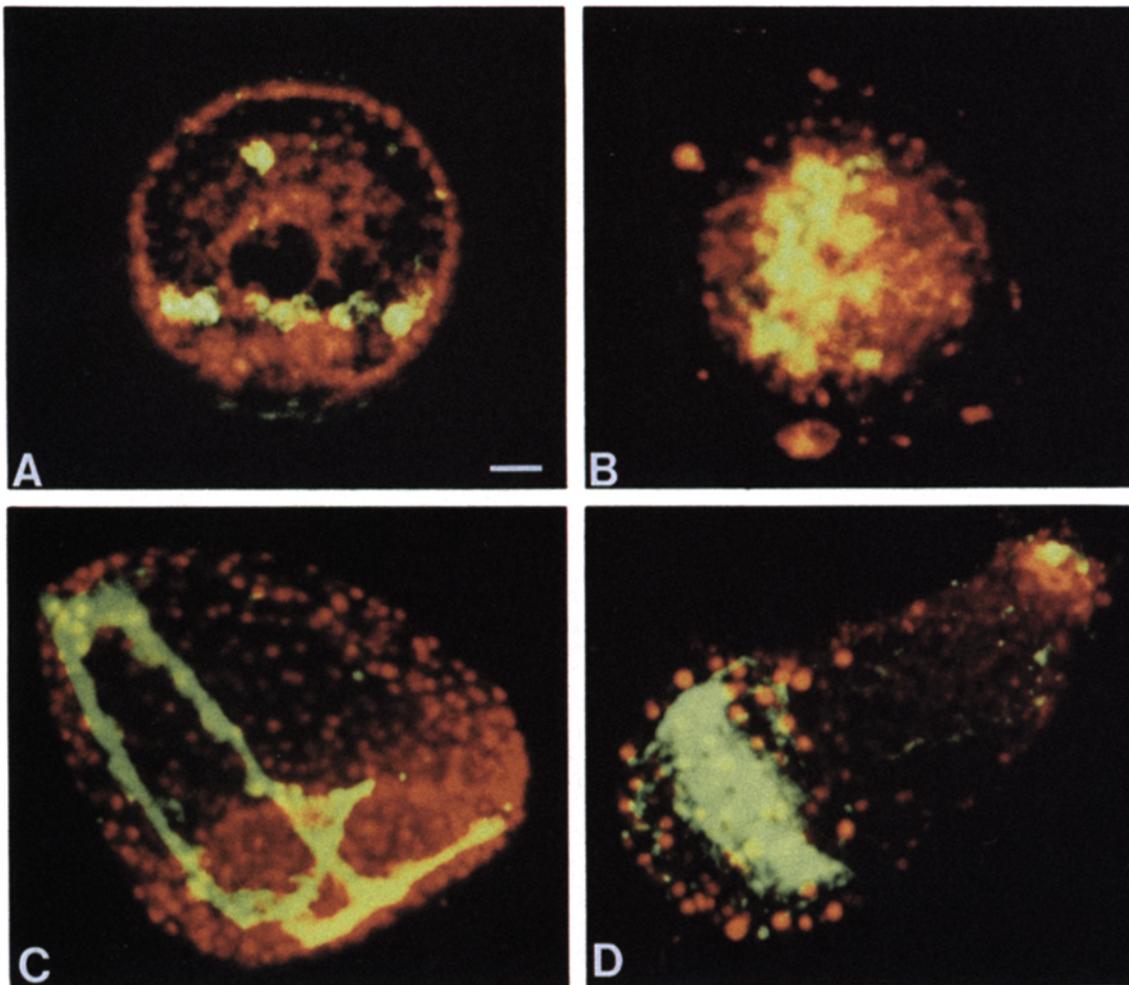


Figure 3. 1° mesenchyme differentiation in control and TPA-treated embryos. Control and TPA-treated embryos were cultured for 24 or 48 h, fixed, and reacted with an antibody specific to 1° mesenchyme (4C12; provided by M. Solorsh, Department of Biology, University of Iowa, Iowa City, IA), followed by an FITC-conjugated secondary antibody (Sigma Chemical Co.). After reaction with antibody, nuclei were stained with propidium iodide. Antibody and nuclei were visualized by confocal microscopy using a fluorescein (antibody) and rhodamine (nuclei) filter. Panels in this figure represent three 5- μ m stacked sections of each embryo. (A) Control 24-h embryo; (B) TPA-treated 24-h embryo; (C) control 48-h embryo; (D) TPA-treated 48-h embryo. Bar, 25 μ m.

balls, while guts and spicules were caused by lithium treatment, as reported previously (Livingston and Wilt, 1989). TPA also caused the blastomeres from the animal hemisphere to form guts and spicules, though some surface epithelium formed as well, just as with lithium treatment. Survival of embryos from TPA-treated animal blastomeres was between 40 and 60%, a survival \sim 10% lower than controls. Treatment of isolated animal blastomeres with both lithium and TPA markedly increased spicule formation compared with treatment with either agent alone (Fig. 5). Hence, TPA causes cell type conversion in isolated animal blastomeres, and its effect is additive to lithium for spicule formation. The fact that lithium and TPA together are not additive with re-

spect to gut formation suggests that endoderm formation differs somewhat from mesoderm formation. This is consistent with what has been seen with dose-response curves for the effects of lithium on animal blastomeres (Livingston and Wilt, 1990).

We have defined the time during development that TPA can alter cell fate by examining the effect of TPA added at different times during cleavage. TPA was added at the same concentration and for the same duration to separate cultures at the 16-, 64-, 128-, and 256-cell stages. RNA was harvested from aliquots of the treated cultures, as well as controls, at 27 h of development (early gastrula) and the levels of SM50 (mesoderm) and Spec la (ectoderm) transcripts were deter-

Figure 2. Differentiation of gut in control and TPA-treated embryos. 72-h-old embryos were stained with an antibody-specific to mid and hind gut (Endo 1; provided by D. McClay, Department of Zoology, Duke University, Durham, NC). A-E are bright field images; B-F are fluorescent images. A and B show control embryos. C and D show two different TPA-treated embryos. E and F show a TPA-treated embryo composed almost totally of gut and mesenchyme. Black arrows indicate skeletal spicules; the white arrow indicates ectopic gut formation. Bar, 25 μ m.

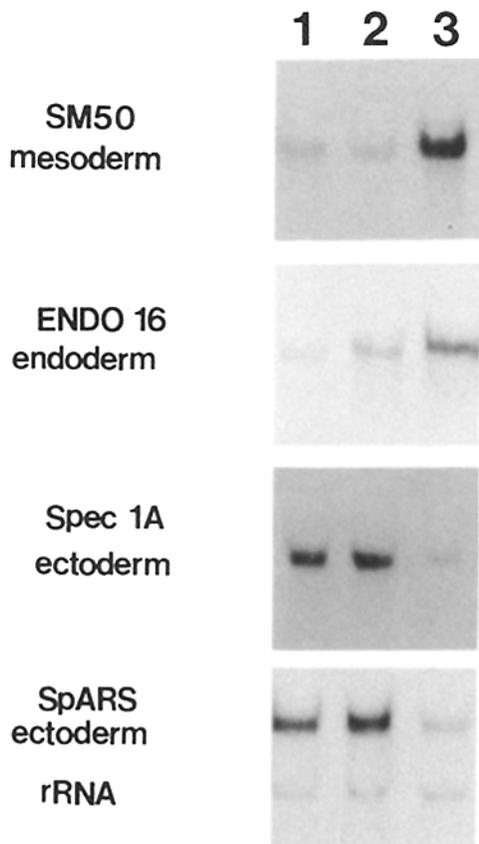


Figure 4. Effect of TPA on tissue-specific gene expression. RNA blots of RNA from 24-h-old embryos were probed with cDNAs homologous to different tissue-specific RNAs as indicated in the figure. 1, control embryos; 2, embryos treated with an inactive ester; 3, TPA-treated embryos. In all cases, the amount of RNA loaded in each lane was determined using a probe to 18s rRNA. An example of this is shown in the SpARS blot. The intensity of each band was measured using a laser densitometer (Bio-Rad Laboratories, Richmond, CA), and the relative signal for the tissue-specific RNA was normalized using the intensity of the rDNA signal in the corresponding lane.

mined using RNA blotting analysis (Fig. 6 A). The change in transcript levels was greatest in cultures that were treated between the 16- and 128-cell stages, while the effect decreased when TPA was added later during cleavage. Examination of the treated cultures after 3 d of development showed that the morphology of the embryos mirrored what was seen with the levels of tissue-specific transcripts (Fig. 6 B). Embryos treated during early cleavage stages (Fig. 6 B, 1 and 2) exhibited enlarged, exogastrulated guts, exaggerated spicule formation, and reduced epithelium. TPA had less effect as embryos were treated at later stages (Fig. 6 B, 3 and 4). The majority of the embryos in the culture treated at the 256-cell stage, one cell division before the cessation of rapid early cleavage, closely resembled control embryos (Fig. 6 B, Control).

Discussion

The results support the hypothesis that stimulation of PKC activity with TPA during cleavage stages of sea urchin devel-

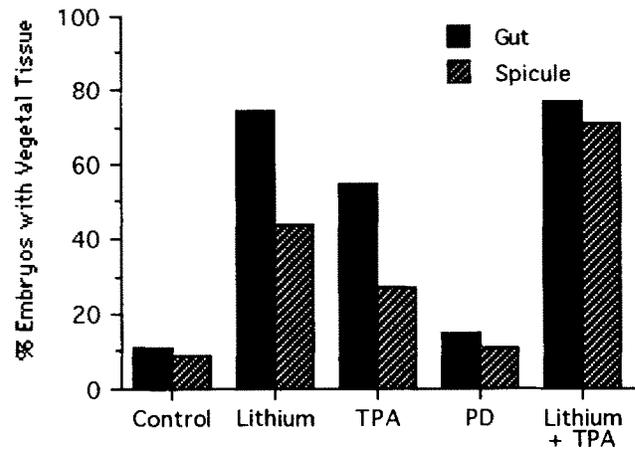


Figure 5. Effects of lithium and TPA on isolated animal blastomeres. Animal blastomeres were isolated as described previously (Livingston and Wilt, 1989) and cultured in sea water (control), sea water + lithium (Lithium), sea water + TPA (TPA), sea water + inactive ester (PD), or sea water + lithium and TPA (Lithium + TPA). After 3 d in culture, the resulting embryos were scored for the presence of guts and spicules. The number of embryos examined is as follows: control, 82; lithium, 79; TPA, 87; PD, 52; lithium + TPA, 49.

opment results in changes in cell adhesion, morphology, and differentiation during gastrulation. The increase in the number of 1° mesenchyme cells, the enlarged gut, and altered accumulation of tissue-specific RNAs are consistent with the proposition that TPA causes a conversion of prospective surface ectoderm to mesoderm or endoderm.

One might wonder whether TPA has either toxic or mitogenic effects that could contribute to the results. TPA-treated embryos often fail to expand their blastocoel, are smaller than controls, and have an apparent depletion of surface epithelium. A selective toxicity of TPA on ectodermal cells could be invoked to explain these results. However, even if this were so, the absolute number of 1° mesenchyme cells per embryo did significantly increase, so toxicity alone cannot explain the results seen with whole embryos. DNA determinations show that TPA-treated embryos have somewhat lower levels of DNA than controls (Table I). However, the small decrease in cell division is not enough to account for the changes in gene expression that TPA produces, and only serves to make the increase in the number of 1° mesenchyme cells even more remarkable.

On the other hand, it can be argued that the increase in endoderm and mesoderm differentiation is due to selective mitogenic effects, not cell conversion. This is difficult to completely eliminate from consideration; indeed, a selective increase in cell division and cell type conversion do not exclude each other. The stimulation of endoderm- and mesoderm-specific gene expression, and the decrease in ectodermal gene expression is so great in many cases that it strongly supports the idea of cell type conversion.

The decisive experiments involve the effects of TPA on isolated mesomeres, which, when untreated, give rise almost exclusively to surface ectoderm under the conditions of these experiments. There is clearly cell type conversion of these mesomeres that is caused by TPA. Possible toxic and mitogenic effects notwithstanding, TPA changes cell fate.

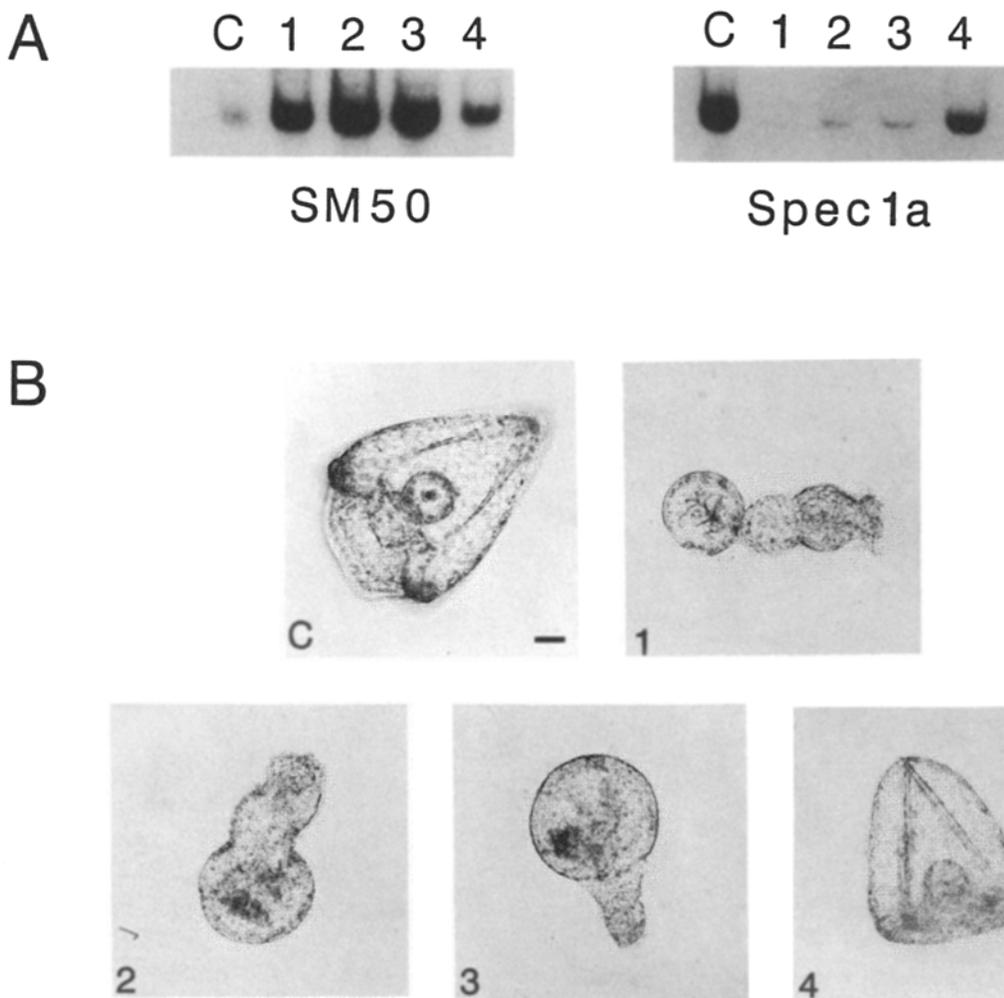


Figure 6. Treatment with TPA at different times during cleavage. Embryos were treated with TPA at different cleavage stages. *A* shows the effects of TPA treatment on accumulation of SM50 (mesoderm) and Spec 1a (ectoderm) transcripts. *B* shows the effects on embryo morphology. C, control; 1, treatment at 5 h (16 cell); 2, treatment at 7 h (64 cell); 3, treatment at 9 h (~128 cell); 4, treatment at 12 h (~256-cell stage). Bar, 25 μ M.

The results suggest that levels of PKC activity are directly involved with determination of cell fate along the animal-vegetal axis of the sea urchin embryo. This is consistent with what has been seen in several other developing organisms. In amphibia, treatment of cleavage stage embryos with lithium causes dorsalization (Nieuwkoop, 1970), and PKC has been shown to be involved in induction of the nervous system (Otte et al., 1988). Lithium has been shown to affect and PKC has been shown to be involved in the differentiation of *Dictyostelium* into stalk and spore (Ginsburg and Kim-

mel, 1989; Peters et al., 1989). PKC activity has been implicated in head/foot polarity in Hydra (Muller, 1989) and in development of vertebrate heart (Runyan et al., 1990). This raises the possibility that this signaling pathway is a general mechanism involved in differentiation along embryonic axes in multicellular animals.

The enhanced effects of combined treatments with lithium and TPA in sea urchins suggest that lithium may indeed be exerting its effects through the inositol phosphate-PKC second messenger pathway. However, this differs from results in amphibia, where TPA can relieve the effects of lithium (Busa and Gimlich, 1989), and in *Dictyostelium*, where lithium and phorbol esters act antagonistically (Ginsburg and Kimmel, 1989; Peters et al., 1989). In these systems it is thought that lithium acts by blocking dephosphorylation of inositol phosphates, which lowers the level of signaling through the inositol phosphate-PKC pathway. This depletion of signaling is thought to result in the observed change in cell fate (Berridge et al., 1989). However, lithium has also been shown to increase levels of diacylglycerol in the membrane (Drummond and Raeburn, 1984), and consequently increase PKC activity (Nishizuka, 1986).

Table I. Effect of TPA on DNA Synthesis (Percent of Control)

	Time after TPA		
	1 h	6 h	22 h
Experiment 1	92	85	81
Experiment 2	92	82	95
Experiment 3	ND	ND	80

Time points were taken in triplicate and averaged to give the data listed above.

This raises the question of whether the effects of TPA on sea urchin development are due to an immediate increase in PKC activity at or shortly after TPA treatment, or to a subsequent downregulation of PKC activity at a somewhat later time (Rodrigues-Pena and Rozengurt, 1984; Young et al., 1987). Although the short time of treatment with TPA would argue that downregulation is not involved, the most compelling evidence that stimulation of PKC activity is how TPA exerts its effects is the brief window during development when TPA can act. The results of these experiments are most consistent with the existence of a critical period during early cleavage when an increase in PKC activity can alter the normal assignment of cell fates.

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