may be replaced by normal constituents of QT-6 fibroblasts. We propose that 43-kD protein serves as the key link between AChR and the cytoskeleton, and that aggregation of 43-kD protein drives AChR clustering rather than simply stabilizing AChR clusters formed by other means.

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16. Single-channel currents were recorded with standard methods [M. Covarrubias, C. Kopta, J. H. Steinbach, *J. Gen. Physiol.* **93**, 765 (1989)]. Outside-out patches were formed with a pipette solution containing 140 mM CsCl, 1 mM $MgCl_2$, 2 mM EGTA, and 20 mM Hepes (pH 7.35), osmolarity adjusted to 290 mosm with glucose. The bath contained 140 mM NaCl, 2 mM KCl, 0.5 mM $CaCl_2$, and 20 mM Hepes (pH 7.35), osmolarity adjusted to 320 mosm with glucose. The perfusion pipette (tip diameter about 1 μm) contained bath solution plus 1 μM acetylcholine chloride and 100 nM atropine sulfate. Parent QT-6 cells showed relatively few membrane channels; the dominant channel appeared to be a high-conductance calcium-activated potassium conductance. There were muscarinic responses, blockable by 100 nM atropine, which increased openings of this type of channel. The majority (more than 95%) of openings elicited by ACh application were of the conductance classes shown in Fig. 1. A few openings of lower conductance were seen [O. P. Hamill and B. Sakmann, *Nature* **294**, 462 (1981); R. Kullberg *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2067 (1990)].
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24. On the day after plating onto cover slips, cells were rinsed in warm, serum-free medium and fixed in 1% paraformaldehyde, 100 mM L-lysine, 10 mM sodium *m*-periodate, and 0.1% saponin in PBS. After they were washed three times in PBS, cover slips were incubated for 1 hour at room temperature with a cocktail containing the rat monoclonal anti–α-subunit, MAb 210 [M. Ratnam *et al.*, *Biochemistry* **75**, 2621 (1986)] (1:500) together with the two mouse monoclonal anti–43-kD protein antibodies MAb 1234 and 1579 [S. C. Froehner, *J. Cell Biol.* **99**, 88 (1984)] (supernatants 1:4). Cover slips were washed three times over at least 30 min, incubated for 1 hour with affinity-purified fluorescein isothiocyanate (FITC) goat anti–rat-IgG (Boehringer) and biotin goat anti–mouse-IgG (Sigma) from which species cross-reactive antibodies had been adsorbed by the manufacturers. After they were washed again, cover slips were incubated for 1 hour with Streptavidin–Texas red (BRL), washed, mounted, and examined with a Bio-Rad MRC 500 laser scanning microscope (a gift of the Lucille P. Markey Charitable Trust).

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Protein Kinase C and Regulation of the Local Competence of *Xenopus* Ectoderm

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The limited competence of embryonic tissue to respond to an inductive signal has an essential, regulatory function in embryonic induction. The molecular basis for the competence of *Xenopus* ectoderm to differentiate into neural tissue was investigated. Dorsal mesoderm or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) caused in vivo activation of protein kinase C (PKC) and neural differentiation mainly in dorsal ectoderm and to a lesser extent in ventral ectoderm. These data correlate with the observations that PKC preparations from dorsal and ventral ectoderm differ, the dorsal PKC preparation being more susceptible to activation by TPA and diolein than is the ventral PKC preparation. Monoclonal antibodies against the bovine PKC α plus β or γ isozymes immunostained dorsal and ventral ectoderm, respectively, which suggests different localizations of PKC isozymes. These results suggest that PKC participates in the establishment of embryonic competence.

AMPHIBIAN BLASTULA-STAGE ECTODERM can be induced to form mesoderm, whereas early gastrula-stage ectoderm cannot, but can be induced to neural tissue instead. This phenomenon is called embryonic competence; its molecular mechanisms remain obscure (*1*). Dorsal mesoderm, which induces overlying dorsal ectoderm to differentiate to neural tissue during normal gastrulation, is able to induce ventral ectoderm to differentiate to neural tissue (*2*), although it induces the expression of neural markers strongly in dorsal ectoderm and only weakly, if at all, in ventral ectoderm (*3*), indicating that dorsal and

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ventral ectoderm differ in competence. In the face of a constant strength-inducing signal, the lower capacity of ventral ectoderm to express neural markers must depend on a difference in its capacity to receive, transduce, or respond to the inducing signals. In the present study, we investigated whether PKC, which becomes activated in response to neural-inducing signals (*4*), participates in the establishment of the difference in competence between dorsal and ventral ectoderm.

We first examined whether the in vivo activation of PKC differs between dorsal and ventral ectoderm. We used recombinates made of dorsal mesoderm and dorsal or ventral stage 10 ectoderm, which were then cultured to the equivalent of stage 13 (*5*). PKC normally becomes activated in the neurectoderm in vivo between stages 10 and 13 (*4*). We measured translocation of PKC from the soluble (cytosolic) to the particulate (membrane) fraction, which is a measure for PKC activation (*4, 6, 7*), and found more translocation in recombinates with dorsal rather than ventral ectoderm (Fig. 1A). Dorsal mesoderm also induced more in vivo phosphorylation of an 80-kD PKC substrate in dorsal than in ventral ectoderm (Fig. 1C) (*6, 8*), while the amount of the 80-kD substrate did not differ between dorsal and ventral ectoderm (Fig. 1E) (*8*). We also examined the in vivo activation of PKC using 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) instead of mesoderm as the PKC activator (*4*) and found that TPA induced PKC translocation in dorsal stage 10 ectoderm, but virtually none in ventral stage 10 ectoderm (Fig. 1B). Similarly, TPA induced phosphorylation of the 80-kD PKC substrate in dorsal but little in ventral ectoderm (Fig. 1D). These results indicate that both dorsal mesoderm and TPA induce more PKC activation in dorsal than in ventral ectoderm.

We also measured the activatable adenylate cyclase (AC) activity in the different explants and recombinates. Because the activatable AC activity increase during neural induction depends on PKC activation (*9*), we were able to use this parameter as an indirect measure for PKC activation. We found that dorsal mesoderm and TPA induced a larger increase in activatable AC activity in dorsal ectoderm than in ventral ectoderm, indicating a stronger PKC activation in dorsal ectoderm (*9*).

We next examined to what extent dorsal mesoderm and TPA are able to induce neural differentiation in dorsal and ventral ectoderm. Histology revealed that whereas all ectoderm-mesoderm recombinates showed some neural differentiation, the volume of induced neural tissue was much larger (81 ± 5%; mean ± SEM) (Fig. 2A) in recombinates made with dorsal ectoderm than in those made with ventral ectoderm (33 ± 5%; mean ± SEM) (Fig. 2B). This difference indicates that dorsal ectoderm can more easily be induced to neural tissue by stage 10 dorsal mesoderm than can ventral ectoderm. The total amount of mRNA for the XIF-6 neurofilament gene (*9, 10*) also reached a factor 3.0 ± 0.3 (n = 5) more in dorsal ectoderm recombinates than in ventral ectoderm recombinates. The difference in competence between dorsal and ventral ectoderm was revealed more strikingly when TPA was used as neural inducer. TPA (i) induced neural differentiation in dorsal ectoderm only (*4, 11*), (ii) gave no histologically detectable neural differentiation in ventral ectoderm (Fig. 2C), and (iii) also gave no expression of the XIF-6 neurofilament gene probe. This difference in competence to differentiate into neural tissue correlates closely with the difference between dorsal and ventral ectoderm in the in vivo activation of PKC. This difference is more pronounced when TPA is used to activate PKC or to induce neural differentiation. Because TPA bypasses receptors and activates PKC directly, we then wondered whether the difference in competence might involve PKC itself.

Because we found differences in the in vivo translocation of PKC between dorsal and ventral ectoderm, we examined whether DEAE isolates of PKC from dorsal and ventral ectoderm also differed in susceptibility to various activation conditions and substrates. The choice of substrate greatly influences which cofactors (such as Ca^{2+} and phospholipids) are required by PKC (*12*). We selected three general substrates: the nonapeptide from the EGF receptor, the peptide from glycogen synthase (*7*), histone III S, and a neurospecific substrate, B-50 (*13*). We found that partially purified dorsal and ventral PKC preparations phosphorylate histone III S (Fig. 3A) and the two other general substrates with the same kinetics under maximal activation conditions (*7*), which confirms that the amount of PKC does not differ between dorsal and ventral ectoderm. However, under maximal activation conditions, dorsal PKC preparations phosphorylated B-50 to a greater extent than did ventral PKC preparations. B-50 is, apparently, a better substrate for the dorsal PKC preparation.

Next, we examined the influence of different activation conditions on dorsal and ventral PKC preparations. To do this, we determined the relative contributions of the PKC activators diolein, TPA, and phosphatidylserine (PS) to the activation of dorsal and ventral PKC preparations, by lowering the concentration of PS from 8 μg/ml (100%) to 0 μg/ml, while keeping the diolein or TPA concentration constant. We found that at lower PS concentrations,

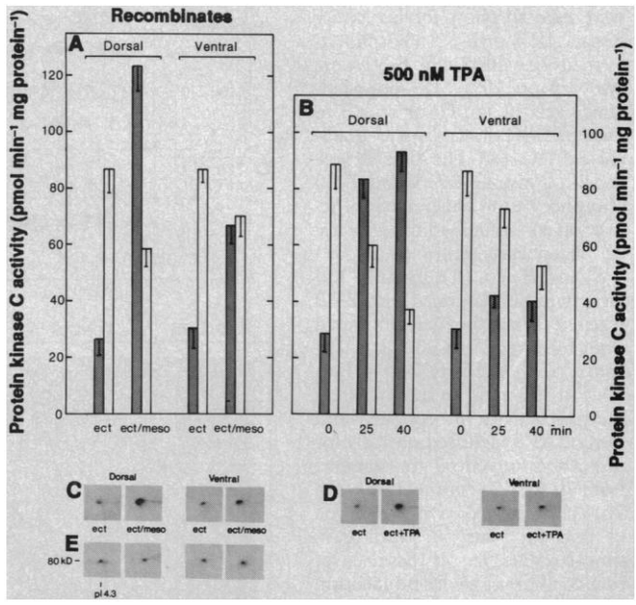

Fig. 1. Protein kinase C activation in dorsal and ventral ectoderm. (**A**) The change in distribution of PKC in the cytosolic (soluble, open bars) and membrane (particulate, stippled bars) fractions relative to the distribution in cultured ectoderm alone was taken as a measure for PKC activation (*4, 6*). Stage 10 ectoderm explants alone (ect) or recombined with dorsal mesoderm (ect/meso) were cultured until control stage 10 embryos reached stage 13 (*5*), at which time the tissue was processed for the PKC assay, with the EGF-receptor nonapeptide as a substrate (*7*). Values are means ± SEM of six independent experiments. (**B**) The distribution of PKC in the cytosolic (open bars) and the membrane (stippled bars) fraction was measured in dorsal and ventral stage 10 ectoderm explants, which were incubated with 500 nM TPA for 0, 25, or 40 min, the time period in which maximal PKC translocation is found in entire stage 10 ectoderm when this is treated with TPA (*4*). Values are means ± SEM of ten independent experiments. (**C** and **D**) Dorsal and ventral stage 10 ectoderm was incubated in [^{32}P]orthophosphate-containing medium for 3 hours, either alone (ect) or as a recombinate (ect/meso) with dorsal mesoderm or in the presence of 350 nM TPA (ect + TPA). The 80-kD phosphoprotein was analyzed by two-dimensional gel electrophoresis (*8*). The experiment was repeated four times with the same result. We analyzed [^{35}S]methionine-labeled 80-kD protein to quantify the amount of protein (**E**).

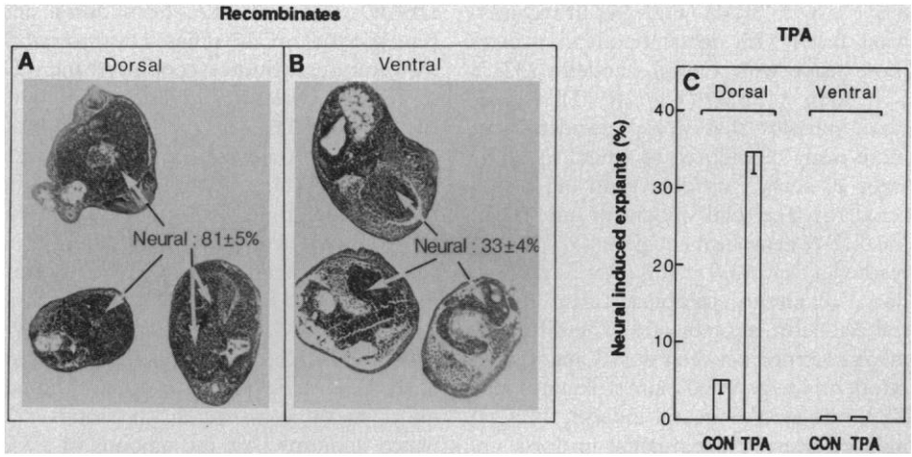

Fig. 2. Neural differentiation in dorsal and ventral ectoderm. (**A** and **B**) Dorsal (A) and ventral (B) ectoderm recombinates were cultured to stage 41 and were then processed histologically. The cross-sectional area of the neural tissue was measured with a digitizer, and the volume of neural tissue was calculated. The volume of neural tissue, measured in 15 recombinates, is indicated. (**C**) Dissected stage 10 dorsal and ventral ectoderm was incubated without (CON) or with 250 or 350 nM TPA (TPA) for 4 or 16 hours (*4, 11*). Explants were cultured for 2 days, prepared for histology, and scored for neural differentiation. Values are means ± SEM of at least 15 independent experiments. Addition of the cAMP analogs dibutyryl-cAMP or 8-bromo-cAMP (1 mM each) after 4 hours of treatment with TPA, which enhances the neural differentiation–including ability of TPA (*9*), also failed to induce neural tissue in ventral ectoderm (*18*).

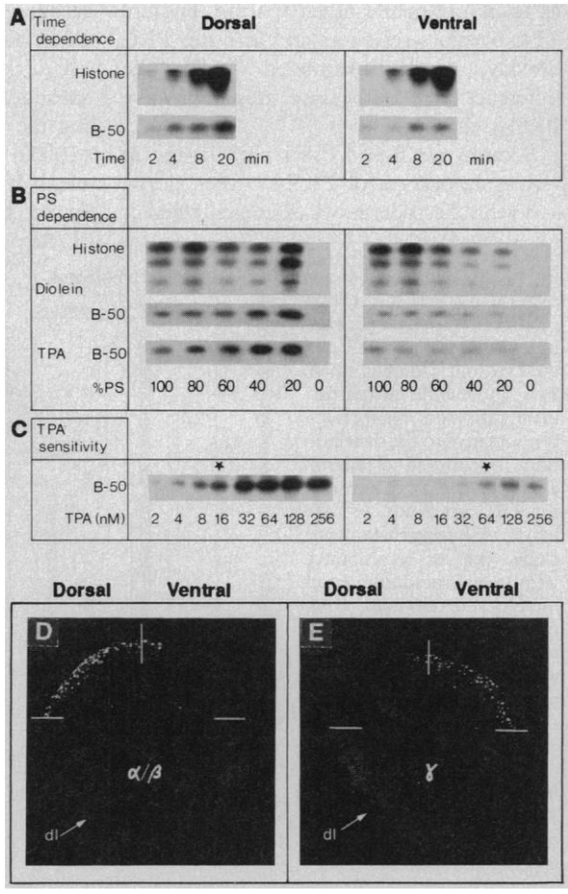

Fig. 3. Different characteristics of PKC preparations from dorsal and ventral stage 10 ectoderm. Total PKC (cytosolic and membrane fraction) was isolated from ectoderm explants. Equal activities of the dorsal and ventral PKC [assayed with using histone III S, or peptides from the EGF receptor or glycogen synthase as substrate under maximal stimulation conditions (*7*)] were used to phosphorylate either histone III S or B-50. Phosphorylated histone III S and B-50 were analyzed on 10% SDS–polyacrylamide gels (B and C) or on a 5 to 15% (SDS–polyacrylamide gradient gel (A). (**A**) The time dependence of histone III S and B-50 phosphorylation under maximal activation conditions [in the presence of phosphatidylserine (8 μg/ml) (PS) and diolein (0.8 μg/ml)]. Radioactivity incorporated into B-50 (excised from the gel) increased from 50 (t = 2) to 350 (t = 20) cpm (dorsal) and from 35 (t = 2) to 90 (t = 20) cpm (ventral), whereas the radioactivity incorporated into histone III S increased similarly for dorsal and for ventral, for example, from 70 (t = 2) cpm to 1200 (t = 20) cpm. These data are from one of four experiments, which gave similar results. Under these activation conditions, we found identical results using the three different general PKC substrates, histone III S, the nonapeptide from the EGF receptor (compare with Fig. 1, A and B) or the peptide from glycogen synthase. (**B**) Histone III S and B-50 phosphorylation were tested for a range of PS concentrations from 100% (8 μg/ml), through 80, 60, 40, 20, and 0% in the presence of diolein (0.8 μg/ml) or 200 nM TPA. (**C**) B-50 phosphorylation in the presence of 20% PS and a range of TPA concentrations as indicated. The TPA concentration that gave half-maximal B-50 phosphorylation is indicated with an asterisk. In (B) and (C) the incubation time was 20 min. (**D** and **E**) Stage 10 embryos were whole-mount immunostained with monoclonal antibodies against bovine PKC isozymes α and β (D) or γ (E); dl, dorsal lip.

phosphorylation of either substrate by the dorsal PKC preparation increased, but that phosphorylation by the ventral PKC preparation decreased (Fig. 3B). These differences were greater for phosphorylation of B-50 and were also greater when TPA instead of diolein was used to activate PKC (Fig. 3B). Because the differences in response to diolein or TPA were most noticeable at the lower PS concentrations, these results indicate that the dorsal PKC preparation is better activated by diolein and TPA than is the ventral PKC preparation. At a low PS concentration, the TPA concentration that gave half-maximal B-50 phosphorylation was much lower for the dorsal (16 nM) than for the ventral PKC preparation (64 nM) (Fig. 3C).

These results indicate that PKC preparations from dorsal and ventral ectoderm have different biochemical properties in vitro, the dorsal PKC preparation being more responsive to activation by diolein and TPA. The PKC preparation derived from dorsal ectoderm, which is induced to become neural tissue during normal development, is also better able to phosphorylate the neurospecific PKC substrate B-50 than is the ventral PKC preparation. These results suggest that the differences between dorsal and ventral PKC could reflect differences in isozymes. It has been shown that various mammalian PKC isozymes have subtly different biochemical properties, such as their responses to phospholipids or TPA and their substrate specificities, and that they are expressed in tissue-specific patterns (*14*). To investigate this possibility, we immunostained whole-mount stage 10 embryos with monoclonal antibodies against the bovine PKC α plus β or γ isozymes (*15*). We found immunostaining in dorsal ectoderm with the antibody against PKC α plus β (Fig. 3D), and immunostaining in ventral ectoderm with the antibody against PKC γ (Fig. 3E). These results indicate more directly that different PKC isozymes are localized in dorsal and ventral ectoderm. Bovine PKC α and β phosphorylate B-50 three- to sixfold better than does PKC γ (*16*), which fits well with our findings here.

Finally, we treated fertilized eggs with D_2O or with 254-nm ultraviolet (UV) light during the first cell cycle to generate hyperdorsalized or hyperventralized aneural (*17*) embryos, respectively. PKC preparations, which had been isolated from entire stage 10 ectoderm in D_2O-treated and UV-treated embryos and then activated in vitro (as in Fig. 3B), exhibited the same B-50 phosphorylation characteristics as normal dorsal or normal ventral PKC preparations, respectively (*18*). This finding indicates that manipulating the first cell cycle, which leads

to a change in the relative amounts of dorsal versus ventral tissue, also changes the in vitro activation characteristics of PKC in the entire ectoderm.

In this study we investigated whether PKC has a function in the neural competence of dorsal and ventral ectoderm. We conclude that dorsal and ventral ectoderm have different competences to be induced to differentiate to neural tissue. This difference in competence is shown most clearly by the observation that TPA is able to induce neural differentiation in dorsal ectoderm but none in ventral ectoderm and confirms the findings of Sharpe *et al.* (*3, 19*). However, our results also agree with other studies (*2*) that show ventral ectoderm can be induced to form neural tissue when placed in contact with dorsal mesoderm (*20*).

The abilities of dorsal mesoderm and TPA to induce more neural tissue in dorsal than in ventral ectoderm correlate with their relative abilities to activate PKC in vivo in dorsal and in ventral ectoderm. This indicates that the PKC signal transduction pathway has a role in regulating the difference in competence to differentiate to neural tissue.

In vitro activation of PKC shows that dorsal and ventral PKC preparations have different biochemical properties, which parallel the differences in in vivo PKC activation. The more extreme difference between the dorsal and ventral PKC preparations in in vitro PKC activation with TPA than with the natural PKC activator diolein correlates with the more extreme difference between the in vivo response of dorsal and ventral ectoderm to TPA than to the natural inducer, dorsal mesoderm. This again suggests that the difference in neural induction competence between dorsal and ventral ectoderm may be regulated by PKC itself. Finally, the differences between dorsal and ventral PKC reflect differences at the level of PKC isozymes, as suggested by the observations that monoclonal antibodies against the bovine PKC isozymes α plus β or γ selectively immunostain dorsal and ventral ectoderm, respectively. Although the precise identities of the *Xenopus* PKC isozymes have not been determined yet, the striking similarity in their B-50 phosphorylation characteristics between the bovine PKC α plus β and *Xenopus* dorsal PKC and between bovine PKC γ and the *Xenopus* ventral PKC, respectively, suggest that these are related enzymes. Our results suggest that these differences in localization of the different PKC isozymes may reflect distinct physiological functions and may be related to the types of tissue in which the isozymes are found.

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5. Recombinates were made between dorsal mesoderm and dorsal or ventral stage 10 ectoderm, which was prepared by cutting the whole animal cap into two equal pieces. For dorsal mesoderm, only the inner, mesodermal cell layers from the blastopore region of a stage 10, early gastrula were used. Equal-sized pieces of dorsal mesoderm were recombined with the dorsal and ventral halves of the animal cap, respectively. Staging was according to P. D. Nieuwkoop and J. Faber [*The Normal Table of* Xenopus laevis (North-Holland, Amsterdam, ed. 2, 1969].
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7. Partial purification of PKC with DEAE-cellulose and the PKC assay in which histone III S was used as a substrate were performed exactly as described previously (*4*). The PKC assay in which a nonapeptide, derived from the epidermal growth factor (EGF) receptor (Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-NH_2), or the peptide from glycogen synthase (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys-NH_2) (Auspep, South Melbourne, Australia) was used as substrate, was performed as described [C. House, R. E. H. Westenhall, B. E. Kemp, *J. Biol. Chem.* **262**, 772 (1987); C. House and B. E. Kemp, *Science* **238**, 1726 (1987)]. In experiments where the concentration of phosphatidylserine was lowered, phosphatidylcholine was added to obtain stable liposomes (*12*). We obtained similar results using histone III S, the EGF receptor nonapeptide, or the glycogen synthase peptide as substrate. Only the absolute value of the specific activities differs [compare with (*4*)], due to a different number of phosphorylation sites in histone III S and the peptides. B-50 was phosphorylated under exactly the same conditions as when histone III S or the nonapeptide from the EGF receptor were used as PKC substrates. The kinase fractions are entirely phospholipid- and Ca^{2+}-dependent, indicating they contain only protein kinase C. Further, when the fractions are incubated with ^{32}P-labeled adenosine triphosphate, with or without the exogenous PKC substrates, or in the presence of cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate, no phosphorylation of either of these substrates or of endogenous substrates can be detected when analyzed on gel autoradiography, unless Ca^{2+} and phospholipids were added to the reaction mixture.
8. Explants were incubated for 3 hours in 5 ml of Holtfreter solution (59 mM NaCl, 0.7 mM KCl, and 0.9 mM $CaCl_2$) containing 80 μCi [^{32}P]orthophosphate. Two-dimensional electrophoresis was carried out according to P. H. O'Farrell [*J. Biol. Chem.* **250**, 4007 (1975)] with isoelectric focusing (4% ampholytes pH 3 to 10; Sigma) in the first dimension. The 80-kD PKC substrate has a highly acidic character (*p*I 4.3). We previously found that this protein is an abundant PKC substrate in membranes of induced neuroectoderm [A. P. Otte, I. J. M. Kramer, M. Mannesse, A. C. Lambrechts, A. J. Durston, *Development* **110**, 461 (1990)]. The characteristics of this protein resembles those of the 80-kD PKC substrate previously described [A. A. Aderem *et al.*, *Nature* **332**, 362 (1988); J. K. T. Wang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 2253 (1989); D. J. Stumpo *et al.*, *ibid.*, p. 4012; S. F. Brooks, J. D. Erusalimsky, N. F. Totty, E. Rozengurt, *FEBS Lett.* **268**, 291 (1990); J. M. Graff, D. J. Stumpo, P. J. Blackshear, *J. Biol. Chem.* **264**, 11912 (1989)]. To investigate the amount of the 80-kD protein, we incubated fertilized eggs for 2 hours in Ca^{2+}- and Mg^{2+}-free [^{35}S]methionine-containing media, and then added Ca^{2+} and Mg^{2+}. At stage 10, dorsal and ventral ectoderm were dissected and cultured as explant or recombinate. At stage 13, the 80-kD protein was analyzed by two-dimensional electrophoresis. The amount of 80-kD protein in TPA-treated explants was similar between dorsal and ventral ectoderm.
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19. Our use of different types of dorsal mesoderm probably caused the considerable amount of neural tissue in recombinates of dorsal stage 10 mesoderm with ventral ectoderm. We used the stage 10 dorsal mesoderm (*5*), whereas Sharpe *et al.* (*3*) used the most anterior part of the involuted dorsal mesoderm from the stage 11 gastrula, which may contain a considerable amount of pharyngeal endoderm [R. E. Keller, *Dev. Biol.* **42**, 222 (1975); R. E. Keller, *ibid.* **51**, 118 (1976)].
20. Dorsal mesoderm induces neural induction much more efficiently than TPA, both in dorsal and in ventral ectoderm. This may be due to the greater effectiveness of dorsal mesoderm compared to TPA as an in vivo PKC activator (Fig. 1) (*4, 9*). Also, TPA activates only one branch of the phosphatidyl inositol (PI) pathway, and the neural-inducing ability of dorsal mesoderm is mediated not only by the PI pathway, but by the cAMP pathway as well (*9*).

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