Functional Modulation of Brain Sodium Channels by Protein Kinase C Phosphorylation

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Voltage-gated sodium channels, which are responsible for the generation of action potentials in the brain, are phosphorylated by protein kinase C (PKC) in purified form. Activation of PKC decreases peak sodium current up to 80 percent and slows its inactivation for sodium channels in rat brain neurons and for rat brain type IIA sodium channel α subunits heterologously expressed in Chinese hamster ovary cells. These effects are specific for PKC because they can be blocked by specific peptide inhibitors of PKC and can be reproduced by direct application of PKC to the cytoplasmic surface of sodium channels in excised inside-out membrane patches. Modulation of brain sodium channels by PKC is likely to have important effects on signal transduction and synaptic transmission in the central nervous system.

VOLTAGE-SENSITIVE SODIUM (Na^+) channels are responsible for both initiation and conduction of the neuronal action potential. Modulation of the functional properties of brain Na^+ channels would therefore be expected to exert an important influence on the function of central neurons. PKC modulates the activity of many different ion channels in neurons and other cells (*1*). The Na^+ current in *Xenopus* oocytes injected with chick brain RNA is reduced by the PKC activator β-phorbol 12-myristate 13-acetate (PMA) (*2*), but modulation of Na^+ channel function in neurons or other excitable cells by PKC has not been reported. Brain Na^+ channels consist of an α subunit (260 kD) and two smaller (30 to 40 kD) β 1 and β 2 subunits. PKC phosphorylates the α subunit of rat brain Na^+ channels in purified preparations and in synaptosomal membranes (*3*). Four subtypes of Na^+ channel α subunits, designated types I, II, IIA, and III, are expressed in rat brain (*4–6*). Expression of these α subunits alone is sufficient to produce functional Na^+ channels in *Xenopus* oocytes (*5, 7*) or in mammalian cells (*8*). We have now observed two modulatory effects of PKC phosphorylation on Na^+ channels in brain neurons and in transfected Chinese hamster ovary (CHO) cells.

Rat brain neurons were dissociated from day 20 embryos and maintained for 2 to 6 days in cell culture (*9*). Neurons in similar cultures express primarily type II or IIA α subunits (*10*). We used the whole-cell voltage-clamp technique to record Na^+ currents (*11, 12*). To prevent regenerative action potentials in poorly clamped regions of neuronal processes, we reversed the Na^+ gradient (Na_{out} = 10 mM; Na_{in} = 140 mM), and intracellular Na^+ was perfused into the cell body from the recording pipette. Application of DOG (1,2-dioctanoyl-*rac*-glycerol), a diacylglycerol activator of PKC, to a rat brain neuron under voltage clamp reduced the peak Na^+ current by 50% and substantially slowed the time course of inactivation of the macroscopic Na^+ current (Fig. 1A). Peak Na^+ current was reduced at all stimulus potentials (Fig. 1B), whereas the voltage dependence of steady-state Na^+ channel inactivation was not markedly altered (Fig. 1C). In 18 other cells, DOG or its analog OAG (1-oleoyl-2-acetyl-*sn*-glycerol) at concentrations of 0.25 to 1 μM reduced the Na^+ current by 20 to 70% and slowed the time course of inactivation in each cell. The phorbol ester PMA at 25 nM to 1 μM also reduced the peak Na^+ current and slowed the time course of inactivation in nine additional cells. Thus, activation of PKC reduces peak Na^+ current and slows Na^+ channel inactivation in intact rat brain neurons.

Macroscopic Na^+ currents were recorded in cell-attached patches from CHO cells expressing α subunits of type IIA Na^+ channels [cell line CNaIIA-1 (*13, 14*)]. When OAG was added to the bath solution, the Na^+ current recorded from a typical cell-attached patch was dramatically reduced within 5 to 6 min and reached a new steady-state value in 30 min (Fig. 2A). This reduction was usually accompanied by slowed Na^+ current inactivation as observed in brain neurons. OAG and DOG at concentrations from 1 to 50 μM decreased peak Na^+ current by 20 to 90% in 16 cells, and this was accompanied by a slowing of the time course of inactivation in 81% of the cells. The peak Na^+ current-voltage (*I-V*) relation after PKC treatment can be exactly superimposed on the control values after scaling by a constant factor (Fig. 2B), indicating that the voltage dependence of activation was not significantly changed (<4 mV; *n* = seven cells). The voltage dependence of steady-state inactivation (Fig. 2C) was also unaffected.

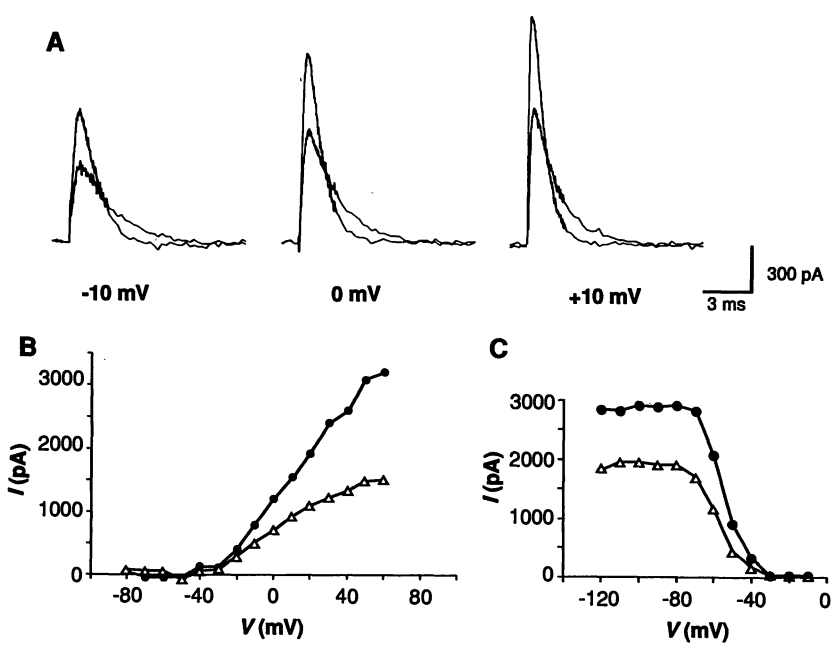

Fig. 1. Na^+ current modulation by DOG in primary cultured rat brain cells. (**A**) The effect of the PKC activator DOG (1 μM, 20 min) on whole-cell voltage-clamp recordings of outward Na^+ currents. Currents were evoked by depolarizations to the indicated potentials after a 100-ms-long prepulse to −120 mV from a holding potential of −80 mV. The smaller and slower current trace in each pair was obtained in the presence of DOG. (**B**) Current-voltage relation for outward Na^+ currents before (●) and during (△) DOG exposure. Peak outward currents were measured from records like those in (A) and are plotted as a function of test pulse voltage. (**C**) Steady-state inactivation curve in control (●) and in the presence of DOG (△). Prepulses 100 ms long were followed by a test pulse to +40 mV. Peak test pulse current is plotted versus prepulse potential. Results in (A), (B), and (C) were obtained from the same cell. Current records were filtered at 10 kHz.

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The effects of activators of PKC were partially reversible in two experiments in which cells were exposed to DOG and then washed with Ringer's solution (Fig. 2, D and E). During washing, the peak of the Na^+ current greatly increased and the time course of inactivation of the Na^+ current accelerated as inactivation returned to normal.

The slowing of the time course of Na^+ channel inactivation was even more apparent when single-channel currents were examined (Fig. 2F). In control solutions, openings of single Na^+ channels in cell-attached patches were clustered near the beginning of the test pulse with only rare openings in the second half of the trace (Fig. 2F,a). After treatment with PKC activators, individual single-channel openings were prolonged and many reopenings occurred throughout the test pulse (Fig. 2F,b), even for pulses as long as 200 ms (Fig. 2F,c). These prolonged openings and reopenings are characteristic of the effects of OAG observed in ten other cells.

The diacylglycerol analog OAG used to activate PKC in the CHO cells can have suppressive effects on neuronal Ca^{2+} currents independent of PKC activation (*15*). To examine the specificity of OAG in our system, we tested the effects of peptide inhibitors of PKC and adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase. CNaIIA-1 cells were microinjected with a 0.25 mM solution of a 13-residue synthetic peptide corresponding to the inhibitory pseudosubstrate domain of PKC (*16*). This peptide specifically blocks the effects of OAG on neuronal Ca^{2+} currents (*17*). In four cells that had been injected with a 250 μM solution of the peptide (Fig. 3, A and B), prolonged application (15 to 60 min) of OAG at high concentrations had no significant effect on the Na^+ current. In three control cells injected with the vehicle (150 mM KCl), OAG caused the usual reduction of Na^+ current and slowing of inactivation, showing that injection alone does not abolish PKC activation. To test the effect of injection of an unrelated peptide, we injected two cells with a 1 mM solution of a 20-residue peptide corresponding to the pseudosubstrate inhibitory site of the cAMP-dependent protein kinase inhibitor (*18*). OAG was still able to reduce the Na^+ current and slow the time course of Na^+ current inactivation (Fig. 3, C and D). This series of experiments demonstrates that, at the concentrations used in this study, the diacylglycerol activators have no nonspecific

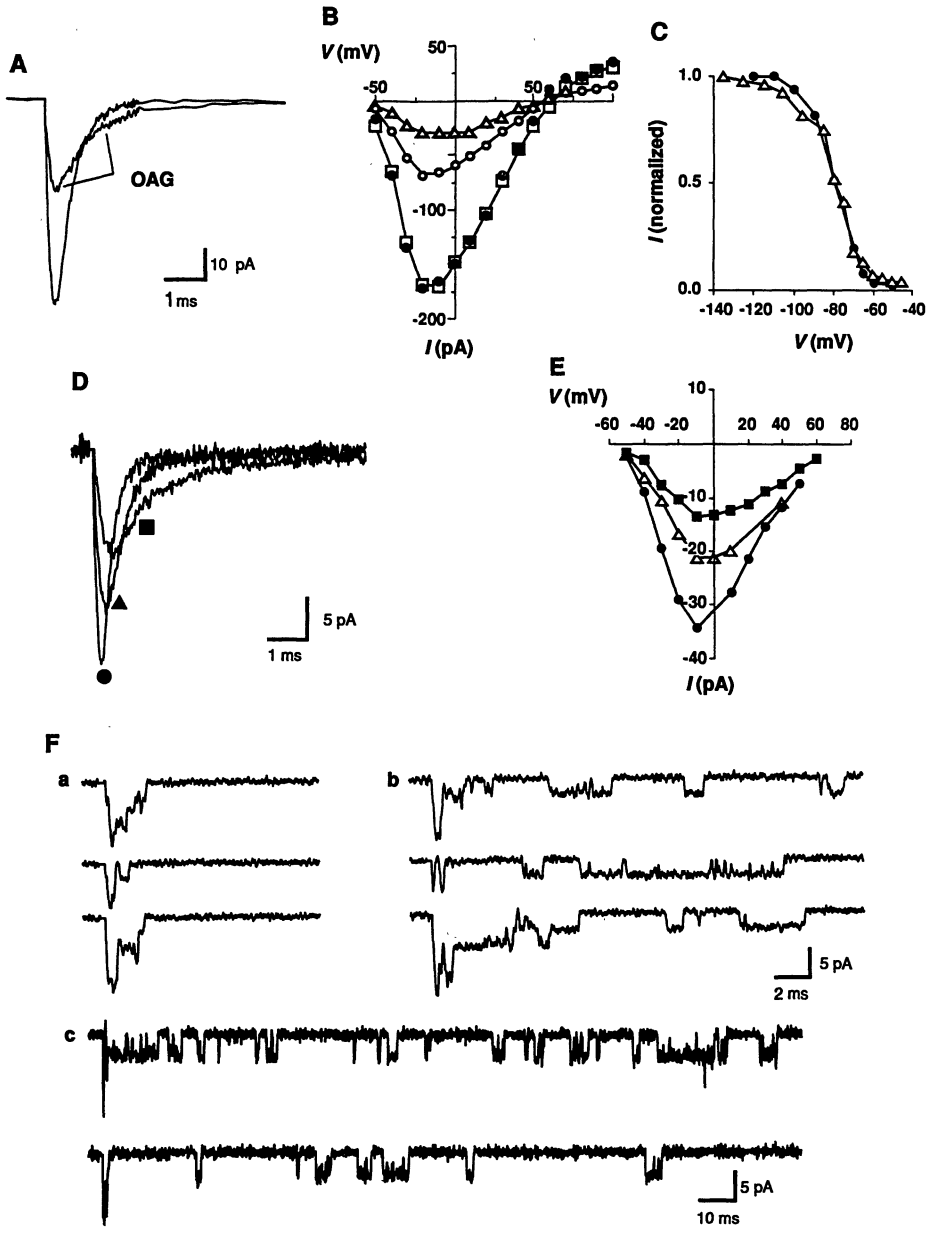

Fig. 2. Effect of OAG on ensemble average currents from cell-attached patches from CNaIIA-1 cells. (**A**) Averaged currents from a cell-attached patch containing a large number of Na^+ channels before and 20 min after addition of 20 μM OAG. Test pulses (12.5 ms) to 0 mV from a holding potential of −110 mV were applied at 0.8 Hz. Averages of 15 to 50 current traces obtained during such test pulses are shown. (**B**) Current-voltage relations before (●) and 10 min (○) or 40 min (△) after addition of 10 μM OAG. The squares show the 10-min OAG data scaled by a factor of 2.5. Averaged currents like those in (A) were measured at a series of voltages, and peak currents were plotted as a function of test pulse potential. (**C**) Steady-state inactivation curve before (●) and after (△) application of 10 μm OAG. Prepulses (150 ms) to a series of potentials were followed by test pulses to 0 mV. Peak ensemble average test pulse currents were normalized and plotted as a function of test pulse potential. The OAG data recorded in the presence of OAG were normalized by a factor of 2.7. (**D**) Reversal of the effect of DOG. Averaged currents from a cell-attached patch from a cell that had been exposed to 30 μM DOG for approximately 1 hour at the beginning of the experiment (■). The cell was then washed repeatedly with DOG-free control solution for 15 min (▲) and 45 min (●). Same pulse protocol as in (A) except that the test pulse was to −10 mV. (**E**) Current-voltage relations at the beginning of the experiment (■) and at 15 min (△) and 45 min (●) after removal of DOG. Same cell as in (D). Current records were filtered at 10 kHz for (A through E). (**F**) Single-channel data from a cell-attached patch containing only a few Na^+ channels. Three examples of representative data in response to voltage steps to −30 mV from a holding potential of −110 mV: a, control; b, after 15 min in the presence of 1 μM OAG; c, data from the same patch 45 min after the addition of OAG. Only one or two Na^+ channels remained active and appeared to have most of their inactivation removed (note change in time bar). The currents in panel (F) were recorded with the 50-gigaohm headstage resistor and 5-kHz filter.

effects on the Na^+ current and the modulation they produce can be directly attributed to activation of PKC.

To provide evidence that additional cytosolic components are not required for Na^+ channel modulation by PKC, we examined the effects of the purified enzyme itself on Na^+ channels in excised patches of membrane from CNaIIA-1 cells. Inside-out patches (*11*) containing multiple Na^+ channels were exposed to PKC purified from rat brain (*19*), and Na^+ currents were recorded during depolarizing pulses. When a mixture of reagents required for PKC activity [diacylglycerol, phosphatidylserine, and adenosine triphosphate (ATP)] was applied to the inner surface of the membrane patch, there was no significant change in the ensemble Na^+ current averaged from 50 depolarizing pulses (Fig. 4A), indicating that these substances by themselves had no effect. When 10 nM purified PKC was then added to the bath solution, there was an immediate reduction of the peak Na^+ current and a slowing of the inactivating phase (Fig. 4A, n = six patches). To establish that other components of the purified PKC preparation were not affecting the Na^+ channel, we applied a three- to fourfold higher PKC concentration in the absence of diacylglycerol, phosphatidylserine, and ATP. The Na^+ current increased slightly (Fig. 4B) or remained the same (n = three patches), with no change in the time course of inactivation. Thus, both the reduction in peak Na^+ current and the slowing of the inactivation time course are due to the enzymatic activity of PKC.

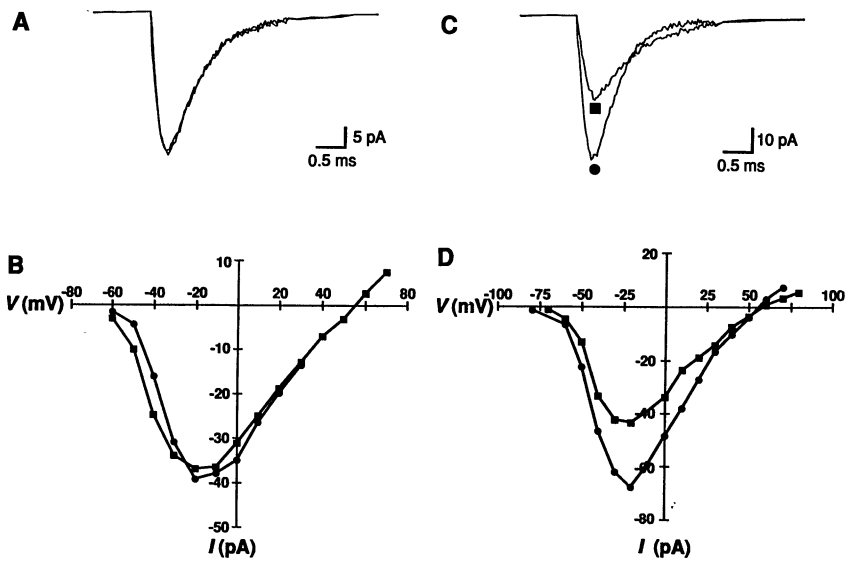

Fig. 3. Effect of specific peptide inhibitors of PKC and cAMP-dependent protein kinase A on the action of PKC activators. (**A**) Cell-attached patch recording from a cell microinjected 5 to 10 min earlier with a 250 μM solution of a synthetic peptide blocker of PKC activity (*16*) in 150 mM KCl. Superimposed ensemble average of 50 current traces in control and after exposure for 30 min to 70 μM OAG. Holding potential −110 mV, test potential 0 mV. (**B**) Current-voltage relation for the cell in (A): control (●) and 70 μM OAG (■). (**C**) Average current traces from a cell injected 5 to 10 min earlier with 150 mM KCl containing 1 mM of a peptide inhibitor of cAMP-dependent protein kinase (*18*) before (●) and after exposure to 20 μM OAG (■). Same pulse protocol as in (A). (**D**) Current-voltage relation for the cell in (C): control (●) and 20 μM OAG (■).

Single Na^+ channels in excised membrane patches respond to treatment with PKC in a manner similar to those in cell-attached patches (Fig. 4, C and D) (n = five patches). The control traces show fast inactivation kinetics with multiple superimposed openings clustered near the beginning of the trace. After treatment with PKC, channels have longer openings and single-channel activity persists throughout prolonged test pulses. Thus, both the reduction of peak Na^+ current and the slowing of Na^+ channel inactivation are observed in excised membrane patches and may not require the action of diffusible cytosolic components other than PKC and its substrates and activators.

Our results show directly that Na^+ channels in intact rat brain neurons are subject to modulation by phosphorylation by PKC. This modulation involves a reduction of peak Na^+ current without a change in the voltage dependence of activation or inactivation of the Na^+ current. Peak Na^+ current is equal to the product NPi, where N is the number of active Na^+ channels, P is the probability of channel opening at the peak of the current, and i is the current contributed by each activated channel. Our single-channel recordings show that i is unchanged by PKC phosphorylation, so the reduction in peak current must result from a reduction in N, P, or both. The reduction of peak Na^+ current is accompanied by a substantial slowing of Na^+ channel inactivation that results from an increased lifetime of single Na^+ channel openings and an increased probability of reopening of Na^+ channels during prolonged depolarizations. It is likely that protein kinase C phosphorylates one or more sites on the α subunit of the Na^+ channel as observed in vitro (*3*) and that this phosphorylation directly mediates the two effects we have observed.

In contrast to our results, the modulation of Na^+ channels expressed in *Xenopus* oocytes by phorbol esters was reported to be due to a shift in the voltage dependence of Na^+ channel activation without any change in the time course of the Na^+ current (*20*). The apparent shift in the voltage dependence of activation observed in those experiments could have resulted from the difficul-

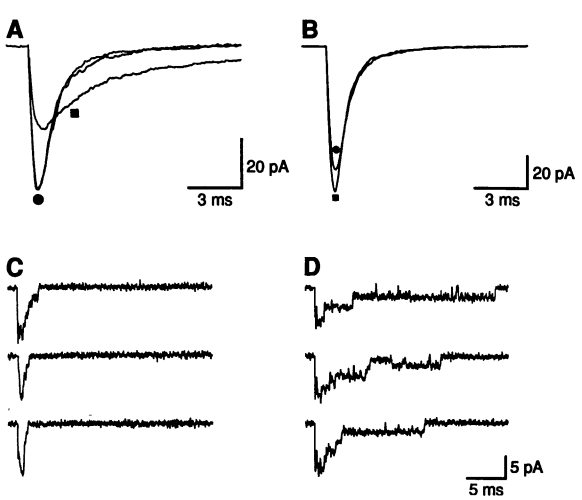

Fig. 4. Direct application of PKC to the cytoplasmic surface of excised inside-out patches. (**A**) Current traces both before and after addition of 1 μM diacylglycerol, 1 μM phosphatidylserine, and 1 mM ATP are shown superimposed (●). Three minutes after addition of 10 nM PKC the final trace was obtained (■). Averages of 50 sweeps. Test pulses were to −30 mV from a holding potential of −110 mV; 50-gigaohm resistor, 5 kHz. (**B**) Inside-out patch from another cell before (●) and after (■) exposure to 30 nM PKC. Diacylglycerol, phosphatidylserine, and ATP were not present. (**C**) Three examples of single-channel recordings from a single inside-out patch exposed to 1 μM diacylglycerol, 1 μM phosphatidylserine, and 1 mM ATP but not PKC. (**D**) Single-channel recordings from the same patch as in (C) after subsequent addition of 10 nM PKC. Recording conditions as in (A).

ty in maintaining adequate voltage control of the large, highly infolded oocyte membrane with a two-microelectrode voltage clamp, and the changes in the time course of Na^+ channel inactivation that we have observed could have been obscured by the inappropriately slow inactivation of the Na^+ channels expressed in *Xenopus* oocytes (*5*).

The physiological significance of modulation of Na^+ channel activity in brain neurons by PKC is unknown. Types II and IIA Na^+ channels are preferentially localized in axons, whereas types I and III Na^+ channels are preferentially localized on neuronal cell bodies in vivo (*21*). Reduction and prolongation of Na^+ currents mediated by type II and IIA Na^+ channels in axons may alter the threshold for initiation of a conducted action potential and the frequency of action potential generation, and similar effects on Na^+ channels in nerve terminals may be expected to alter neurotransmitter release. Reduction of Na^+ currents by activation of PKC may also protect neurons against episodes of hyperexcitability. Further studies of the modulation of Na^+ channel function by PKC will be necessary to critically assess these possible physiological effects.

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9. Primary rat brain cell cultures were prepared from day 20 embryos essentially as described [P. Vassilev, T. Scheuer, W. A. Catterall, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 8147 (1989)]. Bipolar and pyramidal shaped cells were studied 2 to 6 days after plating.
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12. Patch-clamp experiments (*11*) were performed with the Axopatch 1C voltage clamp. The 500-megohm headstage resistor was used unless otherwise noted. Currents were digitally sampled at 40 kHz. Linear leakage and capacitance have been corrected by subtraction of null traces with no channel activity or scaled pulses recorded in voltage ranges where channel activity was absent. Current traces used for leakage subtraction were sometimes fit with a series of exponentials, and the resulting fit trace was used for subtraction to avoid addition of noise to the records. Solutions for generating outward currents in rat brain cells contained 140 mM NaCl, 20 mM CsCl, 5 mM Hepes, pH 7.4 in the recording pipette and 115 mM choline chloride, 20 mM tetraethylammonium chloride, 10 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 5 mM Hepes, pH 7.4 in the bath. Measurement of outward Na^+ currents does not make the voltage homogeneous throughout the length of the neurites of cultured rat brain neurons but does improve the quality of the recorded Na^+ currents by preventing regenerative activity. Solutions for cell-attached and excised patch recording from CNaIIA-1 cells contained 150 mM KCl, 10 mM NaCl, 1.5 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM EGTA, 5 mM Hepes, KOH to pH 7.4 in the bath and 150 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4 in the recording pipette. All recordings were performed at room temperature (approximately 22°C). Peptide inhibitors of protein kinase A [cAMP-dependent protein kinase inhibitor (PKI) 5-24, Peninsula Labs] and of PKC were dissolved in 150 mM KCl before microinjection. PMA, DOG, and OAG were dissolved in dimethyl sulfoxide at concentrations of 10, 8.33, and 8.33 mM, respectively, and stored at −20°C until use. The pseudosubstrate site inhibitor peptide of PKC was a gift of K. Meier and E. G. Krebs. PMA was obtained from Calbiochem. All other chemicals were obtained from Sigma.
13. CNaIIA-1 cells are Chinese hamster ovary cells (CHO-K1, American Type Culture) that have been stably transfected by calcium phosphate precipitation [D. Chen and H. Okayama, *Mol. Cell. Biol.* **7**, 2745 (1987)] with the plasmid pZem228/SP6 containing the coding sequence for the rat brain type IIA Na^+ channel α subunit under control of the mammalian metallothionein promoter. pZem228/SP6 is a variant of Zem228 [C. Clegg *et al.*, *J. Biol. Chem.* **262**, 13111 (1987)] and contains the *neo* gene, conferring G418 resistance. Transfected cells were maintained in RPMI (Gibco), 10% fetal calf serum (Hyclone), and G418 (200 μg/ml) (Gibco). The type IIA Na^+ channel sequence contains the natural leucine at position 860, which confers a normal voltage dependence of activation and inactivation (*5, 6*). Transfected cells express large voltage-dependent Na^+ currents (4 to 25 nA). CNaIIA-1 cells have also been referred to as C81-11 cells, our original laboratory designation (*14*).
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Establishment of the Mesoderm-Neuroectoderm Boundary in the *Drosophila* Embryo

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A gradient of the maternal morphogen *dorsal* establishes asymmetric patterns of gene expression along the dorsal-ventral axis of early embryos and activates the regulatory genes *twist* and *snail*, which are responsible for the differentiation of the ventral mesoderm. Expression of *snail* is restricted to the presumptive mesoderm, and the sharp lateral limits of this expression help to define the mesoderm-neuroectoderm boundary by repressing the expression of regulatory genes that are responsible for the differentiation of the neuroectoderm. The *snail* gene encodes a zinc finger protein, and neuroectodermal genes that are normally restricted to ventral-lateral regions of early embryos are expressed throughout ventral regions of *snail*$^-$ mutants. The formation of the sharp *snail* border involves dosage-sensitive interactions between *dorsal* and *twist*, which encode regulatory proteins that are related to the mammalian transcription factors NF-kB and MyoD, respectively.

A POLYCLONAL ANTIBODY TO *SNAIL* (*sna*) (*1*) was prepared against a *sna* protein made in *Escherichia coli* (*2*). The bacterial protein contains 287 amino acid residues and lacks 102 NH_2-terminal residues present in the native *sna* protein. The resulting antibody (*3*) was used to monitor *sna* expression during embryogenesis (Fig. 1). The protein was detected by the onset of cleavage cycle 14, and, by the middle of cycle 14, its distribution appeared to coincide with the presumptive mesoderm (Fig. 1A). The borders of the *sna* pattern were sharp in lateral regions (arrow) and at the boundary between the presumptive mesoderm and posterior midgut invagination (PMG) (arrowhead). Expression persisted in the differentiating mesoderm throughout the formation of the ventral furrow (Fig. 1B) and the initial phases of germ-band elongation (Fig. 1C). After germ band elon-

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