cGMP Enhances the Sonic Hedgehog Response in Neural Plate Cells

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The elaboration of distinct cell types during development is dependent on a small number of inductive molecules. Among these inducers is Sonic hedgehog (Shh), which, in combination with other factors, patterns the dorsoventral (DV) axis of the nervous system. The response of a cell is dependent in part on its complement of cyclic nucleotides. cAMP antagonizes Shh signaling, and we examined the influence of cGMP on the Shh response. Cells in chick neural plate explants respond to Shh by differentiating into ventral neural-cell types. Exposure of intermediate-zone explants to cGMP analogs enhanced their response to Shh in a dose-dependent manner. The Shh response was also enhanced in dorsal-zone explants exposed to chick natriuretic peptide (chNP), which stimulates cGMP production by membrane-bound guanylate cyclase (mGC). Addition of chNP to intermediate-zone explants did not enhance the Shh response, consistent with a reported lack of mGC in this region of the neural tube. Finally, the presence of a nitric oxide (NO)-sensitive guanylate cyclase (GC) was established by demonstrating cGMP immunoreactivity in neural tissue following NO stimulation of whole chick embryos. Intracellular levels of cGMP and cAMP may thus provide a mechanism through which other factors modulate the Shh response during neural development.© 2001 Academic Press

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INTRODUCTION

Inductive interactions between extra-neural tissues and the developing neural tube are critical for correct patterning of the nervous system. Sonic hedgehog (Shh), produced initially in the notochord and later in the neural midline of vertebrate embryos, is one of the factors mediating such inductive interactions. Shh signaling is involved in the differentiation of several ventral cell types in the developing neural tube (Briscoe et al., 1999; Ericson et al., 1995; Roelink et al., 1994, 1995), and Shh is required for the correct dorsoventral (DV) patterning of the nervous system (Chiang et al., 1996). The earliest actions mediated by Shh include repression of the Paired-box domain transcription factors Pax3, -6, and -7 from the medial neural plate and induction of floor plate cells in neural plate tissue abutting the notochord; subsequent effects include the induction of motor neurons and ventral interneurons (Briscoe et al., 1999; Ericson et al., 1997). Each of these responses can be elicited from chick neural plate explants exposed to different concentrations of an amino-terminal, truncated form of Shh (ShhN) that is soluble in tissue culture media (Porter et al., 1995; Roelink et al., 1995), indicating that Shh can act as a morphogen.

Shh is not the only inducer involved in normal DV patterning of the spinal cord. Rather, DV patterning is accomplished by Shh acting in concert with other distinct inductive signals. For example, bone morphogenetic proteins (BMPs), which are initially released from the dorsal ectoderm adjacent to the neural plate and later from the dorsal neural tube itself, induce dorsal cell types while antagonizing the effects of Shh on receptive cells (Liem et al., 1995, 2000; Zhu et al., 1999). BMP antagonists secreted from the notochord and somites, such as Noggin, enhance Shh signaling (McMahon et al., 1995). In addition, other inductive molecules are present in the neural tube, such as transforming Wnt family proteins, which are expressed alongside BMPs (Münterberg et al., 1995; Roelink and Nusse, 1991). Although it remains to be determined whether there is a crucial role for Wnts in DV patterning of...
of the spinal cord, their function is essential for normal formation of the somite, which is patterned by Wnts in conjunction with Shh and BMPs (Borycki et al., 2000; Marcelle et al., 1997). Altogether, a picture emerges in which the final identity of a cell in the developing spinal cord is determined only after integration of a variety of signals. This raises the question of how the intracellular pathways interact.

cAMP, a well-characterized secondary messenger that acts through the cAMP-dependent protein kinase (PKA), can modulate the Hedgehog (Hh) response. In Drosophila, loss of PKA results in the ectopic activation of Hh-responsive genes (Lepage et al., 1995), and PKA activation blocks the consequences of ectopic Hh signaling in the limb imaginal disc (Jiang and Struhl, 1995). In vertebrates, a similar relationship between PKA and the Shh response is observed. Misexpression of a dominant-negative form of PKA in the dorsal neural tube results in the ectopic differentiation of ventral cells normally induced by Shh (Epstein et al., 1996; Hammerschmidt et al., 1996), while activation of cAMP production inhibits the response of neural plate explants to Shh (Ericson et al., 1996). The zinc-finger transcription factor Ci mediates all the effects of Hh signaling in Drosophila (Methot and Basler, 2001) and provides a mechanism by which cAMP can inhibit Hh signaling. The phosphorylation by PKA of Ci and its vertebrate homolog GLI3 facilitates their cleavage to an amino-terminal form that acts as a transcriptional repressor (Chen et al., 1999; Price and Kalderon, 1999; Wang et al., 2000).

Like cAMP, cGMP is a cyclic nucleotide second messenger that can influence the physiological response of a cell to other signals. cGMP is produced by guanylate cyclases (GCs) and degraded by phosphodiesterases (PDEs). A protein kinase (PKG) paralogous to PKA is activated by the binding of cGMP. There are at least two distinct classes of GC that can be activated in response to exogenous signaling. Members of the membrane guanylate cyclase (mGC) class have a single transmembrane domain and are stimulated by the extracellular binding of natriuretic peptides (NPs) (Ardaillou et al., 1985). Another class, the soluble GCs (sGC), consists of cytoplasmic proteins that produce cGMP after association with the free radicals nitric oxide (NO) (Arnold et al., 1977) and carbon monoxide (Brune and Ullrich, 1987), as well as with metabolites of arachidonic acid (Barber, 1976). In addition, three families of orphan GC receptors have been found in vertebrates (Schulz et al., 1998; for review see Garbers, 1999). Finally, a number of putative GCs have been identified in the C. elegans genome that do not share significant homology with other known families (Bargmann, 1998).

While cGMP-based signaling has been more extensively documented and investigated in adult animals, there is some evidence for its activity early in development. In the adult, atrial NP (ANP) and brain NP (BNP) are released from the heart (Ogawa et al., 1991) and regulate blood pressure through effects on kidneys and vasculature (Espiner et al., 1995). NPs may also play a role early in the development of the nervous system, since radiolabeled ANP preferentially binds to cells in the dorsal and ventral neural tube (Scott and Jennes, 1991), indicating the presence of an mGC-based NP receptor in these domains. One of the two major cGMP-dependent protein kinases, PKG-I, is expressed alongside ANP binding sites in the dorsal and ventral neural tube (Qian et al., 1996), implicating PKG as a downstream effector of ANP signaling in these cells. NPs (Cameron et al., 1996) and PKG-I (Qian et al., 1996) have also been detected in developing dorsal root ganglion (DRG) cells, indicating that cGMP signaling may be playing a role in the development of these cells as well. In addition, cGMP signaling may regulate bone development, as mGCs and PKG-II are both abundant in areas of bone growth (Pfeifer et al., 1996; Yamashita et al., 2000). Overexpression of BNP results in bone overgrowth (Suda et al., 1998), while the loss of PKG-II results in mice with shortened limbs (Pfeifer et al., 1996).

Cyclic nucleotides can directly impact each other's synthesis and degradation (for review see Pelligrino and Wang, 1998). For example, PKA activity has been shown to decrease the production of cGMP by mGC in vascular cells (Ledoux et al., 1997), while addition of ANP to lung cells in vitro can reduce the cAMP concentration while increasing the concentration of cGMP (Panchenko et al., 1998). Alternatively, the cyclic nucleotide complement of a cell can determine its responses to exogenous signals, as in the directional tuning of an axon, which is altered by the balance of cyclic nucleotides in the axon when it is presented with a chemotropic cue (Song et al., 1998). There are other instances in which cyclic nucleotides have consequences opposite each other, such as in cultured neuroblastoma cells, where intracellular injections of cGMP cause the motile structures of growth cones to freeze and retract, whereas injections of cAMP promote outgrowth (Bolsover et al., 1992). cAMP and cGMP also have opposite effects on Ca\(^{2+}\) current in heart cells (Hartzell and Fischmeister, 1986). The documented opposing activities of cAMP and cGMP raise the question of whether cGMP might influence Shh signaling in a manner opposite that of cAMP in the developing neural tube.

Here, we present evidence that cGMP enhances the response of cultured neural tissue to Shh. We show that chick N(1) (cNP) elicits the same enhancement of the Shh response, presumably by activating mGC endogenous to neural tissue. Furthermore, by stimulating whole chick embryos with an NO donor prior to cGMP immunocytochemistry, we reveal the presence of NO-activated sGC in neural tissue. We propose that downstream signaling by cGMP aids in the coordination of DV neural tube patterning by enhancing the inductive effects of Shh, an effect that is in opposition to the known inhibitory effects of cAMP.

**MATERIALS AND METHODS**

**Neural Plate Explants**

White Leghorn and Buff Orpington chicken eggs were obtained from H&N Farms (Redmond, WA) and EarlyBird Farms (Grants
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Pass, OR). Dorsal- and intermediate-zone neural plate explants were dissected from posterior stage-10 (Hamburger and Hamilton, 1951) chick embryos as previously described (Yamada et al., 1993). Briefly, the posterior neural plate was removed and divided into dorsal, ventral, and intermediate regions. Dorsal explants likely included ectoderm. Ten to twenty explants were grown in each well of a 4-well plate in serum-free Neurobasal medium (Gibco BRL/Life Technologies, Rockville, MD) supplemented with N3 (described in Yamada et al., 1993). MEM nonessential amino acids (Gibco BRL), L-Glutamine (Gibco BRL), Penicillin/Streptomycin (Gibco BRL), and Dextrose (EM Science, Darmstadt, Germany) in a Vitrogen 100 collagen matrix (Cohesion Technologies, Palo Alto, CA). At the end of the culture period, explants were fixed in 4% paraformaldehyde (JT Baker, Phillipsburg, NJ) in MEM buffer. Explants assayed for Shh response were cultured for 30 h for most effective quantification of Isl1/2- and HNF3β-positive cells. Explants analyzed for Dorsalin response were cultured for 18 h, for most effective quantification of Slug-positive cells.

ShhN supernatant was obtained from High-Five (Invitrogen, Carlsbad, CA) insect cells infected with a recombinant baculovirus construct (Incardona et al., 2000) encoding an amino-truncated form of Shh that is diffusible (Roelink et al., 1995). The supernatant was titrated on explants to determine a concentration that induced both Isl1/2 motor neurons and HNF3β floor plate cells.

Dorsalin1 supernatant was generated by transfecting 293T cells (American Type Culture Collection, Manassas, VA) with a chick Dorsalin1-myc construct that has been previously described (Basler et al., 1993). Control explants were exposed to supernatant from mock-transfected 293T cells.

cGMP Reagents

8-pCPT-cGMP (BioLog, Hayward, CA) and 8-Br-cGMP (Tocris, St. Louis, MO) are membrane-permeable cGMP analogs that are more stabilized and are not broken down as quickly by PDEs. Chick natriuretic peptide (chNP) was obtained from Sigma. These compounds were resuspended in water, and concentrated stock solutions were stored at −20°C and diluted into medium before addition to explants.

Sodium nitroprusside (SNP) (Sigma) degrades to form nitric oxide in solution, thereby stimulating the production of cGMP by endogenous sGCs. SNP was stored as a stock solution of 100 mM in phosphate buffered saline (PBS). 3-Isobutyl-1-methylxanthine (IBMX) (Sigma) inhibits cAMP- and cGMP-dependent phosphodiesterases, allowing the accumulation of cGMP that is being produced. IBMX was stored in a stock solution of 10 mM in PBS. Embryos were extracted from eggs, incubated in 1 mM SNP and 1 mM IBMX in PBS for 30 min at room temperature, and immediately fixed with 4% paraformaldehyde in MEM salts. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Calbiochem, La Jolla, CA), a potent inhibitor of sGC, was maintained as a stock solution at 100 mM IBMX in PBS. 3-Isobutyl-1-methylxanthine (IBMX) (Sigma) inhibits cAMP- and cGMP-dependent phosphodiesterases, allowing the accumulation of cGMP that is being produced. IBMX was stored in a stock solution of 10 mM in PBS. Embryos were extracted from eggs, incubated in 1 mM SNP and 1 mM IBMX in PBS for 30 min at room temperature, and immediately fixed with 4% paraformaldehyde in MEM salts. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Calbiochem, La Jolla, CA), a potent inhibitor of sGC, was maintained as a stock solution at 100 mM in PBS.

Cryosections of these embryos were blocked for 1 h in 10% heat-inactivated goat serum in PBS with 0.01% Triton (Sigma) (PBT) and incubated overnight at 4°C. In a polyclonal cGMP antiserum (1:4,000) made in sheep, a generous gift of Dr. J. de Vente (de Vente et al., 1987). Slides were then incubated in goat anti-sheep IgG antibody (1:200) (Jackson ImmunoResearch Laboratories, West Grove, PA) conjugated to Texas Red for 2 h at room temperature. Alternatively, cGMP primary was detected using a biotin-conjugated donkey anti-sheep IgG antibody (ICN Biochemicals, Aurora, OH), the Vectastain Elite ABC kit (Vector, Burlingame, CA) and diaminobenzidine (DAB) (Sigma).

Explant Antibody Staining

The collagen gels containing explants were fixed in 4% paraformaldehyde in MEM buffer for 20 min at 4°C, removed from the bottom of the well using a spatula, and rinsed four times for 20 min in PBS. Gels were blocked overnight at 4°C in PBT with 10% heat-inactivated goat serum. Primary and secondary antibodies were diluted in PBT and incubated at 4°C overnight. In between incubations, the gels were rinsed in PBT for 4 times for 1 h at 4°C. After staining, gels were rinsed four times for 30 min in PBT and mounted in a solution containing 1 mg/ml p-phenylenediamine (PPDA) (Sigma) in 50% glycerol, 50% 0.2 M sodium carbonate buffer, pH 9.4.

The response of an explant to Shh was measured by counting Isl1/2’ and HNF3β’ nuclei. Isl1/2’ nuclei were identified by using a rabbit polyclonal antibody (K5) at 1:500, a guinea pig antibody at 1:50,000 (both kindly provided by T. Jessell and S. Morton), or a mouse monoclonal antibody (4D5) at 1:25, all directed against a conserved region present in Isl1 and Isl2. HNF3β’ cells were identified by using a monoclonal antibody (4C7) used at 1:10. A mouse monoclonal antibody against Slug (62.1E.6), which marks migratory and premigratory neural crest (Nieto et al., 1994), was used at 1:25. All monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA).

Fluorescent secondary antibodies used were Cy3-coupled Fab fragment goat anti-mouse IgG, FITC-coupled donkey anti-Rabbit IgG, and Texas Red-conjugated donkey anti-guinea pig IgG, all from Jackson ImmunoResearch Laboratories. 4',6-Diamidino-2-phenylindole dihydrochloride hydrate (DAPI) (Boehringer-Mannheim) was used to visualize nuclei in order to painstakingly count the number of cells in each explant. DAPI was added at a 1:10,000 concentration to the secondary antibody solution.

Documentation and Analysis

Images were collected by using a Microphot-SA epifluorescence microscope (Nikon, Melville, NJ), a video camera with frame capture software, and, more recently, a Spot Digital Camera and software (Diagnostic Instruments, Sterling Heights, MI), and analyzed with Photoshop 5.5 (Adobe, San Jose, CA) and Statview 5.0 (SAS Institute, Cary, NC).

PKG and PKA consensus phosphorylation sequences were located by using a Perl script (http://students.washington.edu/cpr/px/pxkixsites.cgi) to find the sequences (R/K)(2-3)-X-S/T-X for PKG and R-(X(1–2)-S/T-X for PKA, as noted in Kennelly and Krebs (1991), and confirmed for accuracy using http://www.cbs.dtu.dk/databases/PhosphoBase/).

RESULTS

cGMP Potentiates the Effects of Shh on Neural Plate Explants

A cell’s response to Shh is inhibited by an increased intracellular CAMP concentration (Ericson et al., 1996). As CAMP and cGMP have been shown to modulate the response of cells to extracellular signals by operating in
concert (Jiang et al., 1992; Ledoux et al., 1997) or in opposition (Panchenko et al., 1998; Song et al., 1998), we investigated whether cGMP could similarly enhance or inhibit Shh signaling. To this end, we examined the effect of the stabilized, membrane-permeable cGMP analogs 8-Br-cGMP or 8-pCPT-cGMP on the response of stage-10 (Hamburger and Hamilton, 1951) chick neural plate explants to Shh. When cultured in the presence of ShhN for 30 h, cells in these explants differentiate and express Islet1/2 and HNF3β, proteins that characterize motor neurons and floor plate cells, respectively. The relative numbers of cells that express these proteins provide a sensitive and reliable assay for Shh signaling in a relevant tissue.

In the absence of Shh, no HNF3β and Islet1/2 cells differentiated, regardless of the presence of cGMP analogs. In the presence of Shh, however, cGMP analogs increased the number of HNF3β and Islet1/2 cells in a dose-dependent manner, resulting in a more than 3-fold induction of HNF3β cells, and a more than 2.5-fold induction of Islet1/2 cells at the maximal concentration of 30 μM (Fig. 1). The overall number of cells in each explant, as assayed by nuclear DAPI staining, was unchanged upon addition of the cGMP analogs (unpaired t test, P > 0.1). The Shh-mediated repression of Pax7 was also unchanged (not shown). These data indicate that cGMP facilitates the induction of ventral neuronal cell types by Shh, which is in contrast to the inhibition of Shh response elicited by cAMP.

cGMP Analogs Have No Effect on Dorsal Induction

One potential mechanism through which cGMP could enhance the Shh response would be through an inhibition of BMP signaling, since these two pathways act in opposition during early spinal cord development (Liem et al., 1995, 2000; Zhu et al., 1999). In order to determine the effect of cGMP on the BMP response, intermediate-zone explants were grown in the presence of the BMP family member Dorsalin, which induces the differentiation of cells expressing Slug, a protein characteristic of migratory and premigratory neural crest (Nieto et al., 1994) (Fig. 2A). The number of Slug-expressing cells was unchanged in the presence of 8-pCPT-cGMP (Fig. 2B), demonstrating that cGMP does not alter Dorsalin-mediated induction and suggesting that the effects of cGMP on the Shh response are not mediated through the BMP response pathway.

ChNP Mimics the Effects of cGMP Analogs on Neural Plate Explants

The enhancement of the Shh response in neural plate explants by cGMP analogs raises the question of whether GCs, which produce cGMP, are present in the neural plate. mGCs are activated by the binding of NP ligands (Ardaillou et al., 1985). I125-ANP binding has been described in broad regions of the ventral and the dorsal neural tube at stages of development shortly after neural tube closure (Scott and
Jennes, 1991), implying that an ANP-binding mGC is present at this time and place, and that NPs could play a role in neural patterning.

Because ANP binding was not observed in intermediate regions of the neural tube (Scott and Jennes, 1991), we decided to use a dorsolateral region to assay the effects of chNP, a peptide with homology to both ANP and BNP (Akizuki et al., 1991; Miyata et al., 1988; Toshimori et al., 1990). Higher concentrations of Shh were required to induce ventral cell types in dorsal-zone neural plate explants, yet Islet1/2+ and HNF3β+ cells were produced in a concentration-dependent manner, comparable to intermediate-zone explants. In dorsal explants, chNP elicited an almost identical enhancement of the Shh response (Figs. 3A and 3B), resulting in more than three times as many HNF3β+ and Islet1/2+ cells over explants exposed to Shh alone. Interestingly, intermediate-zone explants exposed to chNP exhibited no significant change in the number of HNF3β+ or Islet1/2+ cells induced by Shh (Fig. 3C), consistent with the putative lack of ANP binding in this region of the neural tube.

The Developing Neural Tube Produces cGMP When Exposed to Nitric Oxide

sGCs produce cGMP in response to NO (Arnold et al., 1977), an activity that can be exploited to visualize the presence of sGC in situ. If an sGC is present and has been activated by NO, and PDE-mediated degradation is blocked, cGMP accumulates and is detectable by immunocytochemistry (de Vente and Steinbusch, 1992). We exposed whole chick embryos to a combination of SNP, an NO donor, and IBMX, an inhibitor of the PDEs that catalyze the degradation of cGMP and cAMP. cGMP-immunoreactivity (cGMP-
IR) was observed in the ventral and dorsal neural tube of H&H stage-14 embryos exposed to SNP and IBMX for 15 min. (Fig. 4A). In H&H stage-10 embryos exposed to SNP and IBMX, cGMP-IR was distributed more uniformly in the neural plate, with stronger staining in the dorsal folds (Fig. 4B). Low cGMP-IR was observed in the presence of IBMX alone, although more staining was retained in the dorsal neural folds (Fig. 4C). This dorsal staining was almost identical in the presence of an agent that prevents sGC activity, ODQ (Fig. 4D). Together, these data demonstrate the presence of NO-sensitive sGC somewhat ubiquitously in the neural plate and non-NO-sensitive mGC in the dorsolateral neural plate.

**DISCUSSION**

The differentiation of a neural plate cell is regulated through the coordinated action of inductive signals, which direct the activation and repression of transcription factors in a manner appropriate to the cell’s position along the DV axis of the neural tube. Shh, and the molecules that modify its effects are of critical importance in the DV patterning of the neural tube. Among the modifiers of the Shh response are cyclic nucleotides. It has previously been shown that the stimulation of cAMP production inhibits the Shh-mediated induction of ventral cell types in neural explants (Ericson et al., 1996). Our results show that cGMP has the
opposite effect: raising the intracellular cGMP concentration through application of either a membrane-permeable cGMP analog or the mGC ligand chNP enhanced the Shh response. In our hands, membrane-permeable analogs of cAMP comparable to the cGMP analogs we used had no effect on the Shh response (not shown). Still, there is ample indication that cAMP and PKA inhibit the Hh response (Ericson et al., 1996; Price and Kalderon, 1999; Wang et al., 2000). We propose a model wherein the Shh response of a cell is shifted by its intracellular cyclic nucleotide complement. Cyclic nucleotides may thereby provide a mechanism through which other signals impinge on the Shh response pathway and modify its effects on developing tissue.

Although the effects of cAMP/PKA on the Hh response have been widely studied, the nature of the cyclase or cyclases responsible for changing the cAMP concentration in Shh-responsive cells in the embryo is unknown. The cyclases that might control cGMP concentrations in Shh-responsive cells are also not known, but the data presented here show that chNP mimics the effect of cGMP on the Shh response, indicating the presence of an NP-binding mGC in neural plate explants. The enhancement of Shh induction by chNP was observed in dorsal, but not intermediate, neural plate explants, which is consistent with earlier observations demonstrating NP binding in ventral and dorsal regions of the developing spinal cord (Scott and Jennes, 1991). This mGC could serve as an endogenous regulator of cGMP concentrations, and its spatial restriction could provide a mechanism of local Shh response modulation. In vivo, it is more likely that the mGC present in cells in the ventral tube would enhance Shh signaling, since cells in the dorsal neural tube express BMPs which render them refractory to the influence of Shh.

In the adult, NPs are produced mainly by the heart and circulated systemically, but they have also been localized in the developing spinal column and in subsets of dorsal root ganglion cells (Cameron et al., 1996). It remains unclear...
whether chNP is the homolog of mammalian ANP or BNP, an issue that is not resolved by the phenotype of ANP mutant mice, which exhibit no obvious neural tube defects (John et al., 1995; Kishimoto et al., 1996). The overgrowth of bones observed in mice over-expressing BNP (Suda et al., 1998), however, is potentially consistent with a modulation of response to Indian hedgehog (Ihh), which is critical for bone growth (St-Jacques et al., 1999). The idea that cGMP might be involved in Ihh signaling in vivo is strengthened by the observation that the PKG-II knockout mouse displays shortened bones and dwarfism (Pfeifer et al., 1996). Demonstration of an absolute requirement for NP signaling in neural patterning awaits more precise analysis of the developing neural tube in single and compound NP mutant animals.

Besides mGC, our data demonstrate the presence of sGC, another potential source of cGMP in the developing spinal cord. sGC is activated by NO (Arnold et al., 1977), produced by NO synthase (NOS). The presence of NOS has been demonstrated in the developing neural tube (Blottner and Luck, 1998), but elimination of each of the three murine NOS genes genetically has resulted in few developmental defects (eNOS, Huang et al., 1995; iNOS, Laubach et al., 1995; MacMicking et al., 1995; Wei et al., 1995; nNOS, Huang et al., 1993). The eNOS mutant is the exception, exhibiting limb defects and shortened bones (Heffler et al., 2001). While the overall phenotypes argue against a critical role for NO in neural patterning, further examination of multiple compound phenotypes will be necessary to determine the role of NO signaling on early development.

The induction of ventral cell types in explants was not accomplished by cGMP alone, but required concomitant activation of Shh signaling. The observed enhancement of the Shh response may be distinct from the outcome of a simple increase in Shh concentration, as no decrease was detected in the number of cells stained for Pax7, a transcription factor normally down-regulated by Shh.

The Hh signal is transduced by a receptor complex composed of Patched (Ptc) and Smoothened (Smo) (Alcedo et al., 1996; Chen and Struhl, 1996; Stone et al., 1996). Ptc represses the activity of smo, and this repression is released upon binding of Hh to Ptc (Chen and Struhl, 1998). Subsequently, the repression of Hh target genes by the zinc-finger transcription factor Ci (Hepker et al., 1997; Methot and Basler, 2001) or its mammalian homolog Gli3 (Sasaki et al., 1999; Shin et al., 1999) is relieved by intracellular interactions that take place as the result of Smo activity. The cAMP-dependent kinase PKA acts to block Hh transduction by directly phosphorylating Ci or Gli3, causing it to be cleaved to an amino-terminal, repressor form (Chen et al., 1998, 1999; Dai et al., 1999; Price and Kalderon, 1999; Wang et al., 2000). PKA may affect other members of the Hh signaling pathway as well, since putative PKA phosphorylation sites (Kennelly and Krebs, 1991) are present also on Gli1 and Gli2, other Ci homologs that are involved in the activation of Shh target genes (Dahmane et al., 1997; Ding et al., 1998; Hynes et al., 1997; Lee et al., 1997; Matise et al., 1998), and on Ptc and Smo.

The cGMP-dependent protein kinase PKG is paralogous to PKA in form and function, raising the possibility that the enhancement of Shh sensitivity by cGMP is mediated through the analogous but opposite activity of PKG on Shh pathway proteins. PKG-I is expressed in the dorsal and ventral neural tube and a subset of DRG cells during neural development (Qian et al., 1996), yet mice lacking both known isoforms of cGMP-dependent protein kinase have no ostensible developmental defects (Kleppisch et al., 1999), arguing against a critical role for PKGs in development. However, early neural patterning has not been investigated, and subtler or transient defects may not have been detected.

Subsets of PKA consensus phosphorylation sites are also consistent with phosphorylation by PKG (Kennelly and Krebs, 1991; Xue et al., 2000). Sequence analysis of Shh pathway proteins demonstrated that there are nine PKG consensus phosphorylation sites on Gli3, which are conserved in all known vertebrate sequences (Fig. 4A). Six of these sites are present in the sequence of Ci, and several are also conserved in Gli1 and Gli2. Since Gli3 is phosphorylated by PKA, PKG could enhance Shh signaling either by phosphorylating Gli3 or by interfering with phosphorylation of Gli3 by PKA. Furthermore, even if repression of Ci is blocked, an additional activation step is required in order for the protein to serve as a transcriptional activator (Methot and Basler, 1999; Wang and Holmgren, 1999), so the augmentation of the Shh response by cGMP could be explained by either enhanced activation or decreased repression of Gli3.

There are also PKG consensus phosphorylation sequences on Fused, a serine/threonine kinase involved in Ihh signal transduction (Forbes et al., 1993; Robbins et al., 1997). In addition, PKG consensus phosphorylation sites are present on Drosophila Smo, but not in any of the vertebrate Smos examined (Fig. 5B), making Smo the only Shh pathway protein consistently lacking such sites. All but one of the consensus PKG phosphorylation sites on Drosophila Smo are on the intracellular domain of the protein (Fig. 5B), indicating a nonrandom distribution of such phosphorylation sites. Drosophila Smo is phosphorylated and stabilized in the presence of Hh, and there is evidence that PKA is involved in this phosphorylation (Deneuf et al., 2000). Interestingly, on Smo most of the PKA consensus phosphorylation sequences are poorly conserved across species.

We hypothesize that cGMP modifies the cellular response to Shh through PKG, just as cAMP inhibits the response via PKA (Fig. 5C). However, we cannot exclude a potential role for other molecular targets of cGMP, such as cyclic nucleotide-gated ion channels (Johnson et al., 1986; Nawy and Jahr, 1990) or phosphodiesterases (Hartzell and Fischmeister, 1986).

Our data indicate that components of cGMP signaling are present at the place and time that Shh is acting to influence the differentiation of neural tube cells. We also present the
novel finding that cGMP can influence the inductive effects of Shh on neural tissue. We show that cGMP and a ligand that stimulates its production both augment the response of this tissue to Shh. Our findings suggest that the balance of cyclic nucleotides at different DV levels of the developing spinal cord helps refine the inductive influence of Shh.

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