Modulation of *Xenopus* embryo mesoderm-specific gene expression and dorsoanterior patterning by receptors that activate the phosphatidylinositol cycle signal transduction pathway

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SUMMARY

A role for the phosphatidylinositol (PI) cycle signal transduction pathway in *Xenopus* mesoderm induction has been revealed by observations of PI cycle activation coincident with this process, combined with the demonstration that Li⁺ (a PI cycle inhibitor) blocks this response and hyperdorsalizes mesoderm induction in intact embryos or augments growth factor-mediated induction in animal caps. It has been suggested that spatially restricted PI cycle activity in the marginal zone might modulate (but not, itself, activate) mesoderm induction. To better characterize the ability of PI cycle activity to modulate the pattern of mesoderm-specific gene expression elicited by mesoderm-inducing growth factors we have expressed in the embryo exogenous 5-hydroxytryptamine receptors that activate the PI cycle. In embryos, ventral expression and activation of these receptors during mesoderm induction are without obvious effect, whereas dorsal expression and activation yield dorsoanterior-deficient tadpoles. In animal caps induced with activin, simultaneous activation of exogenous 5-hydroxytryptamine receptors inhibits both convergent extension movements associated with dorsal mesoderm induction and the expression of *goosecoid*, a dorsal-specific gene, but is without effect on expression of a generic mesodermal marker, *Xbra*. All of these effects of a PI cycle-stimulating receptor are the opposites of those previously reported for the PI cycle inhibitor, Li⁺. PI cycle activity thus proves able to modulate the dorsal/ventral character of early mesodermal gene expression elicited by growth factor, suggesting a model for mesodermal patterning.

Key words: mesoderm, induction, gene expression, *goosecoid*, *Xbra*, *Xenopus*, phosphatidylinositol cycle, calcium, activin, PCR

INTRODUCTION

From the time of Nieuwkoop’s studies, demonstrating that vegetal cells of the amphibian blastula can cause superjacent blastomeres to produce mesodermal lineages (Nieuwkoop, 1969a,b), determination of the mesodermal germ layer has been viewed as a process of one or more inductions – vectorial intercellular signaling events leading cells of the equatorial region to form mesoderm in a precisely ordered spatial pattern ranging from the dorsal-most derivatives (prechordal mesoderm and notochord) through more intermediate derivatives such as muscle, to the ventral-most forms (mesenchyme, mesothelium, blood, etc.). This patterning underlies the establishment of the dorsal-ventral body axis, and is thus a central event in vertebrate embryogenesis.

Models of mesoderm induction (see Kimelman et al., 1992 for review), concerned with specifying the number, sequence and origin of the intercellular inductive signals necessary to account for the patterning of mesodermal lineages, have led to a concerted search for the extracellular molecules that constitute these signals. Members of the fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) families are now clearly implicated (see Kimelman, 1993 for review). In assays employing animal pole explants (animal caps), which differentiate only as atypical epidermis when cultured in isolation, both FGF and activin (a member of the TGF-β superfamily) induce mesoderm formation, and these factors and their receptors have been detected in the embryo at the appropriate developmental stage. In animal caps, FGF induces ventral and intermediate mesodermal derivatives, whereas activin, over a range of concentrations, can induce the entire spectrum of ventral to dorsal-most derivatives. That these factors (or closely related proteins) play roles in mesoderm induction in the intact embryo is suggested by the ability of dominant negative mutant forms of their receptors to yield embryos deficient in either posterior (FGF) or all (activin) mesodermal derivatives. However, how these signaling molecules achieve the observed dorsal-ventral patterning of mesodermal derivatives remains unclear, as no mesoderm-inducing ligand or receptor has yet been proved to be distrib-
uted in a graded manner along the prospective dorsal-ventral axis.

The recent discovery of intercellular signaling proteins that are able to modulate mesoderm induction (rather than mediate it) may provide a framework within which to explain some aspects of mesodermal patterning. Secreted proteins of the Wnt family, such as Xwnt-8, do not, themselves, induce mesoderm in animal cap assays, but synergize with FGF to elicit formation of dorsal mesoderm (Christian et al., 1992). Similarly, noggin protein cannot itself induce mesoderm, but can dorsalize ventral marginal zone explants (Smith et al., 1993). Such observations imply the existence of pathways that are able to modulate the activity of those other pathways employed by true mediators of mesoderm induction, such as FGF and activin, to alter the types of mesoderm induced by the latter. This has led to the notion that mesoderm induction is a multi-step process composed of synergistic events, involving both induction per se and distinct events that modify that inductive process (see, e.g., Christian and Moon, 1993).

Of equal importance to the question of the identities of the intercellular signaling molecules that mediate and modulate mesoderm induction is the issue of the intracellular signal transduction pathways they employ to set in motion the differential gene expression that is the end product of induction. Knowledge gained from the dissection of the signal transduction pathways employed by mitogenic growth factors in mammalian somatic cells (for reviews see Ullrich and Schlessinger, 1990 and Ruderman, 1993) has begun to increase our understanding of at least some of the pathways employed. Activin and FGF receptors are integral membrane proteins with intrinsic protein kinase activities (serine/threonine or tyrosine kinases, respectively; Dionne et al., 1990; Mathews and Vale, 1991), and in mammals the mitogenic effects of receptor-linked tyrosine kinases such as the FGF receptor appear to involve a cascade of proto-oncogene products including p21ras, Raf-1 kinase and MAP kinase (see Ruderman, 1993 for review). p21ras also appears to be involved in transducing the mesoderm-inducing signals of both FGF and activin in Xenopus, as well (Whitman and Melton, 1992), and both Raf-1 kinase and MAP kinase have been implicated in FGF-mediated mesoderm induction (MacNichol et al., 1993; Graves et al., 1994; Hartley et al., 1994).

In contrast, less is known regarding the details of intracellular signaling pathways employed by modulators of mesoderm induction, such as noggin and Wnts. However, embryological and biochemical studies have combined to implicate the phosphatidylinositol (PI) cycle signal transduction pathway in modulation of the dorsal-ventral character of mesodermal lineages induced by FGF or activin. In mammalian somatic cells, activation of the PI cycle by a variety of G protein-coupled or tyrosine kinase-linked receptors triggers hydrolysis by phospholipase C-β or γ (respectively) of the phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP2), producing inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG; see Berridge, 1984 for review). IP3 triggers release of Ca2+ from the endoplasmic reticulum into the cytosol via IP3 receptors, and DAG is the endogenous activator of protein kinase C. IP3’s second messenger action is terminated by its sequential dephosphorylations to inositol bis- and monophosphates, and finally to free myo-inositol, the substrate for the resynthesis of PIP2. A useful tool in studies of PI cycle function is Li+, an uncompetitive inhibitor of inositol monophosphatase (Hallerer and Sherman, 1980). When cells undergoing PI cycle-mediated signaling are treated with Li+, inositol monophosphates accumulate and myo-inositol levels decline, halting the resynthesis of PIP2 and bringing the PI cycle to a standstill. Because it is an uncompetitive enzyme inhibitor (i.e., one that acts upon the enzyme-substrate complex rather than the free enzyme), Li+ is most effective in inhibiting PI cycle-mediated signaling in cells with the highest levels of activity of this pathway (Nahorski et al., 1991).

Li+ has long been known to be teratogenic for the embryos of many organisms. When microinjected into ventral blastomeres of the 32-cell Xenopus embryo it elicits dorsoanterior duplication, whereas dorsal microinjections are without phenotypic effect (Kao et al., 1986). The dorsalizing action of Li+ causes ventral marginal zone blastomeres to differentiate as notochord and other dorsal mesodermal derivatives, rather than the lateral plate mesoderm that is their normal fate (Busa and Gimlich, 1989). Lithium-induced teratogenesis in Xenopus is prevented by the co-injection of exogenous myo-inositol (but not epi-inositol, an isomer not employed in the PI cycle; Busa and Gimlich, 1989), a treatment that prevents PI cycle inhibition due to substrate depletion. The PI cycle undergoes a pronounced and prolonged activation in normal Xenopus embryos at the 32- to 64-cell stage, coincident with the onset of mesoderm induction, and teratogenic doses of Li+ inhibit this response (Maslanski et al., 1992). We and others have interpreted these data as implicating PI cycle-mediated signaling as a modulator of early mesoderm induction, suggesting that lithium’s differing effects when injected dorsally or ventrally reflect a dorsal-ventral gradient of PI cycle activity across the marginal zone (high activity ventrally, low dorsally), that plays a role in restricting the spatial extent and quantity of dorsal-mesoderm induction (Berridge et al., 1989; Maslanski et al., 1992). In keeping with this, Li+ treatments similar to those we have shown to inhibit PI cycle activity in whole embryos will not themselves induce mesoderm formation in animal caps, but will dorsalize animal caps artificially induced with FGF or activin as judged by enhancement of explant elongation, induction of muscle and notochord at doses of growth factor that would otherwise induce more ventral derivatives, and augmentation of the expression of dorsal marker genes such as goosecoid (Slack et al., 1988; Cooke et al., 1989; Tadano et al., 1993). Zebrafish embryos also hyperdorsalize and overexpress goosecoid in response to Li+ (Stachel et al., 1993).

Unfortunately, Li+ is a relatively non-specific reagent with which to experimentally modify PI cycle activity, and only permits depression of PI cycle-mediated signaling, not its augmentation, as is required to test directly the hypothesis that elevated PI cycle activity ventralizes mesoderm induction. Additionally, it is not yet possible to assay PI cycle activity in restricted regions of the embryo, as will be required in order to unambiguously demonstrate that its activity is graded along the dorsal-ventral axis. Therefore we have adopted the alternative approach of assessing the developmental effects of spatially restricted alterations of PI cycle activity during mesoderm induction by expressing, in embryos, mammalian 5-hydroxytryptamine (5-HT; serotonin) type 1C receptors (5-HT1cR; Julius et al., 1988). These G protein-coupled receptors
activate the PI cycle in a ligand-dependent fashion both in their native tissue (Conn et al., 1986) and in *Xenopus* oocytes (Nomura et al., 1987), and previously have been employed to test the roles of the PI cycle in egg activation (Kline et al., 1991) and sea urchin morphogenesis (Cameron et al., 1994). The precise spatial and temporal control of these receptors’ activity afforded by this approach has enabled us to demonstrate that dorsal PI cycle activation via 5-HT_{1c}R during mesoderm induction has a phenotypic effect – hypodorsalization – opposite that of ventral PI cycle inhibition via Li^+, and inhibits the activin-induced convergent extension movements characteristic of dorsal induction in animal caps. At the molecular level, 5-HT_{1c}R stimulation inhibits goosecoid expression in activin-induced animal caps, indicating suppression of dorsal-most mesoderm induction, but is without effect on expression of the generic mesodermal marker, *Xbra*. These observations suggest a mechanism by which spatially restricted activation of the PI cycle during normal mesoderm induction may delimit the spatial extent of dorsalization.

**MATERIALS AND METHODS**

Preparation and treatment of embryos

In vitro fertilization, rearing of embryos and microinjection were essentially as described by Busa and Gimlich (1989). Briefly, eggs were expressed from females ovulated with human chorionic gonadotropin and sperm were prepared from testes diced in a drop of F1 medium. These were mixed in a dish and immediately flooded with F1. Ten minutes postinsemination, zygotes were dejellied in F1 + 2% cysteine, washed, then cultured at room temperature (approx. 24°C). RNase-free glass micropipettes (tip diameter approx. 1-10 μm) connected to a Picospritzer (General Valve Corp.) were pre-calibrated by injections into silicon oil, and embryos were microinjected while bathed in F1 + 5% polysucrose (Sigma). Microinjections into defined regions along the prospective dorsal-ventral axis were oriented with respect to both the sperm entry site (sometimes marked with Nile Blue Sulfate as described by Busa and Gimlich, 1989) and with respect to the pigment gradient along the animal hemisphere. Only embryos with unambiguous axes were selected for microinjection.

UV-irradiated embryos were exposed in a fused silica dish 10 cm above a UV transilluminator (model TS-15, UVP, Inc.) for 2 minutes. Animal caps were excised with sharpened forceps between approximately the 512- and 1K-cell stages, rinsed in MMR, then transferred to agar-coated dishes containing MMR, 0.1% gentamicin and the ligand(s) specified in the text. After treatment with ligand for the duration specified in the text, caps were rinsed and then cultured to the specified stage in MMR + gentamicin supplemented with 10^{-7} M mianserin. Mianserin was employed subsequent to 5-HT but not treated with 5-HT; mianserin prevented these effects but itself had no apparent effect on normal development.

Animal caps expressing 5-HT_{1c}R were treated with the ligands indicated in the text for 1.5 hours beginning immediately after excision, then washed and cultured in MMR + gentamicin containing 10^{-7} M mianserin. Explant elongation was quantified approximately 24 hours after treatment by measuring each explant’s axial ratio with an eyepiece micrometer in a stereoscope. Axial ratio is defined as the length of the longest axis of the explant divided by the length of the widest section of the explant perpendicular to the long axis.

Recombinant human activin A was a gift from Genentech, Inc.

**Observation of intracellular Ca^{2+} responses with furaductran**

For Ca^{2+} imaging, zygotes were microinjected prior to first cleavage with furaductran (10,000 M, Molecular Probes, Inc.) to a final concentration of 50 μM, then injected at the 4-cell stage, just below the equator at the midlines, with 5-HT_{1c}R mRNA. At the 64-cell stage embryos were transferred to a perfusion chamber with a cover-slip floor on an inverted epi-fluorescence microscope. Explants were alternately excited with 350 and 380 nm light and fluorescence micrographs were collected with an image intensifier and CCD camera. The imaging chamber was continuously perfused, either with F1 or with F1 + 5-HT. 350/380 nm ratio images (in which the ratio is proportional to intracellular [Ca^{2+}]; Grynkiewicz et al., 1985) were calculated by the imaging software (Inovision Corp.), regions of intracellular Ca^{2+} response to ligand were manually defined with a mouse, and average values of the 350/380 ratio for these regions were calculated.

**RT-PCR assay**

At the times indicated, groups of 5 to 8 animal caps were removed from the treatment dishes and RNA was extracted by homogenizing in RNAzol (20 μl per 1 mg tissue; Cinna/Biotecx) in a glass homogenizer. One-tenth volume of chloroform was added, the samples were vortexed, incubated on ice for 15 minutes, and centrifuged at 13,000 g for 15 minutes. The upper, aqueous phase was removed, the RNA precipitated with an equal volume of isopropanol at −20°C, and pelleted by centrifugation at 13,000 g. Pellets were washed twice with 75% ethanol, dried in a vacuum centrifuge, and dissolved in sterile water. The RNA content of these suspensions was determined spectrophotometrically.

For reverse transcription (RT), total RNA was incubated with MMLV reverse transcriptase (50 U; Stratagene) for 45 minutes at 37°C and then 10 minutes at 95°C. The total reaction volume was 20 μl and contained 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl\_2, 1 mM dithiothreitol, 20 U RNAsin, 5 μM random hexamers and 500 μM each of deoxynucleotides. Approximately one-tenth volume of the RT reaction was employed for PCR, in a 50 μl total volume overlaid with mineral oil and containing 20 mM Tris–HCl (pH 8.2), 10 mM KCl, 6 mM (NH\_4)\_2SO\_4, 2 mM MgCl\_2, 0.1% Triton X-100, 10 ng/μl nuclelease-free BSA, 50 μM dNTPs, 2.5 μl Pfu DNA polymerase (Stratagene), 1 μCi ([γ-32P]dTTP (3000 Ci/mmol) and 25 pmol each of either goosecoid + EF-1α or Xbra + EF-1α primers (see below). The thermal cycler program was 94°C for 1 minute (denaturation), 60°C for 1 minute (annealing), 60°C for 1 minute (extension).

One third of each PCR reaction was electrophoresed on a 6% polyacrylamide gel, which was dried and autoradiographed with an intensifier screen. PCR products were quantified using a Molecular Dynamics Phosphorimagex by dividing the background-subtracted intensity of each goosecoid or Xbra band by the background-subtracted intensity of the EF-1α band in the same lane.

Initial studies determined the range of input RNA over which the
RT-PCR assay provides a linear response. Duplex PCR employing primers for the gene product of interest and for EF-1α, an ubiquitously expressed gene (Krieg et al., 1989) employed as an internal control for mRNA loading, was reliably linear for all three gene products assayed in this study over an input cDNA range of 0.025 to 0.15 µg when EF-1α was amplified for 20 cycles and the other gene product amplified for 30 cycles (these numbers of cycles were determined to be non-saturating). We routinely employed 0.05-0.1 µg input cDNA, and amplified for 10 cycles in the presence of goosecoid or Xbra primers before adding primers for EF-1α and amplifying for an additional 20 cycles.

Controls run with each PCR assay included samples containing primers only (no template) and template only (no primers). Additionally, a reaction was run employing extracted RNA that had not been reverse transcribed, to confirm that amplified bands were not due to contamination of RNA extracts with genomic DNA. No products were generated in any of these control reactions. Product identities were confirmed both by their sizes and by restriction analysis; the amplified regions of goosecoid and Xbra contained single restriction sites for HpaII or AluI, respectively.

PCR primers

**EF-1α:** Sense primer 5′-CCTGAATCCACCGCCAGATTGGTG-3′ (positions 1088-1113, Krieg et al., 1989; GenBank/EMBL accession number M25504); antisense primer 5′-GAGGTAGTCTC-GAGAAGCTCTCCACG-3′ (positions 1283-1308).

**Xbra:** Sense primer 5′-GGTGGAGCCAGATTATGTTAACT-3′ (positions 591-615, Smith et al., 1991; GenBank/EMBL accession number M77243); antisense primer 5′-CCCGTGCTGTGACT-CATTGCAACA-3′ (positions 1126-1150).

**goosecoid:** Sense primer 5′-CAGAGTATCCTGCTTTCT-GAGGGTAGTCT-3′ (positions 41-65, Blumberg et al., 1991; GenBank/EMBL accession number M63872); antisense primer 5′-AGTAGTATGTGATCCACGGC-3′ (positions 345-370).

**RESULTS**

Exogenous 5-HT receptors are functionally expressed near the onset of mesoderm induction and activate the PI cycle on the dorsal and ventral sides

In order to employ exogenous receptors to alter PI cycle activity in a regionally specific manner during mesoderm induction, it was necessary to achieve functional expression by the 32- to 64-cell stage (Jones and Woodland, 1987) in both the dorsal and ventral marginal zone. To test this, we measured intracellular free [Ca²⁺] ([Ca²⁺]i) responses to added 5-HT in embryos that had been injected with both the fluorescent dye, fura-dextran (at the 1-cell stage, to permit diffusion of the dye throughout the embryo) and 5-HT1cR mRNA (at the 4-cell stage, to restrict receptor expression to the dorsal or ventral sites of mRNA injection). Because PI cycle activity stimulates intracellular Ca²⁺ mobilization (via the second messenger, IP₃), functional expression is revealed by ligand-dependent [Ca²⁺]i increases at the sites of mRNA injection, and the relative magnitudes of these on the dorsal and ventral sides of the embryo reflect whether these receptors are equally competent to stimulate this signaling pathway dorsally and ventrally.

[Ca²⁺]i increases were observed when embryos injected with 5-HT1cR mRNA at the dorsal and/ or ventral midlines were treated with 5-HT at the 64-cell stage (approximately 100 minutes after injection of mRNA) (Fig. 1A). These responses were strictly dependent upon ligand, and were of similar magnitude and timing at both midlines (Fig. 1B). These observations established that the exogenous receptors are functionally expressed by the onset of mesoderm induction, are accessible to external ligand, and function equally well on both sides of the embryo, but they do not reveal the precise location of the ligand-accessible receptors—whether in the old membrane at the embryo’s surface or in new membrane beneath the superficial cell-cell junctions.

Routinely, we observed that functional expression did not occur in all the progeny of a blastomere injected at the 4-cell stage, but rather only in those arising inside a roughly circular region within about 15°-25° of the injection site (Fig. 1A). This was observed whether the injection site was located in the animal hemisphere, at the equator, or in the vegetal hemisphere, and was true for both dorsal and ventral injections. We
These tadpoles represent the range of phenotypes typically observed. tions (not shown). At high concentrations (1
but their temporal forms differed at high and low concentra-
treatment with 5-HT per se. microinjection with the exogenous mRNA or
assuming that it was the result of receptor activation and not
microinjection of the PI cycle inhibitor, Li⁺ (Kao et al., 1986; Busa and Gimlich, 1989), in keeping with the hypothesis that locally elevated activity of the PI cycle signal transduction pathway blocks dorsoanterior determination whereas low activity in this pathway is permissive for dorsalization. However, the dorsal-deficient phenotype observed here might result from effects on processes other than axis determination and mesoderm induction. For example, roughly similar phenotypes are observed when gastrulation movements are inhibited (Gerhart et al., 1989). To better determine where and how the signaling events stimulated by these exogenous receptors act to yield dorsoanterior deficiency, we further studied the effects of 5-HT₁cR stimulation on excised animal caps induced with activin A.

5-HT₁cR stimulation blocks convergent extension movements induced in vitro by activin A and in vivo by endogenous mesoderm-inducing signals

Animal caps cultured in simple salt solutions differentiate only as atypical epidermis and remain spherical, whereas explants induced to form mesoderm by treatment with appropriate growth factors undergo a pronounced elongation that is an early marker of mesoderm induction and is thought to be due to convergence extension movements (Symes and Smith, 1987). These morphogenetic movements are specific to the dorsal marginal zone and play a central role in establishing the dorsal-ventral body axis (see Keller et al., 1991 for review).

To test the effect of PI cycle stimulation on this early marker of dorsal mesoderm induction, we prepared animal caps from embryos injected with 5-HT₁cR mRNA in the animal hemisphere region of each blastomere at the 4-cell stage. Following excision, the caps were treated with activin A in combination with either 5-HT or mianserin. Whereas caps not treated with activin (and thus, not induced to form mesoderm) displayed no elongation, caps treated with activin plus the antagonist, mianserin, elongated significantly (Fig. 3A). Activation of 5-HT₁cR simultaneously with activin treatment completely blocked this elongation response. This was not a non-specific toxic effect, since a subsequent treatment with activin alone

| Table 1. Summary of phenotypic responses to dorsal or ventral expression and stimulation of 5-HT₁cR |
|-------------------------------------------------|--------|--------|--------|
| mRNA injection site | 5-HT   | Mianserin | DAI    |
| mRNA injection site |        |          |        |
| D      | V      | D      | D      |
| 5-HT   | +      | +      | −      | +      |
| Mianserin | −      | −      | +      | +      |
| DAI    | 1.8    | 5.0    | 4.9    | 5.0    |
| s.e.m. | +0.1   | +0.0   | +0.1   | +0.0   |
| n      | 24     | 30     | 29     | 8      |

4-cell stage embryos were injected in either two dorsal (D) or ventral (V) cells with 38 pg 5-HT₁cR mRNA.

Ligand concentrations were 10 nM 5-HT, 100 nM mianserin.

Phenotypes were scored employing the dorsoanterior index (DAI) of Kao and Elinson (1988), in which a DAI 5 tadpole is normal, DAI 0 is completely microcephalic, and intermediate values reflect intermediate degrees of dorsoanterior deficiency.

**Dorsal (but not ventral) 5-HT₁cR stimulation disrupts dorsal-ventral axis formation in whole embryos**

Ligand-dependent stimulation of 5-HT₁cR at the ventral equatorial region had no obvious effect on subsequent embryonic phenotype (Fig. 2A; Table 1), except for slight and occasional edema. In contrast, stimulation of receptors expressed in the dorsal equatorial region yielded grossly dorsoanterior-deficient tadpoles (Fig. 2B). Depending upon the clutch of eggs employed, the phenotypic response ranged from microcephaly to the complete absence of recognizable dorsoanterior structures; the average response over several clutches was extreme microcephaly (Table 1). This response was ligand-dependent and was blocked by the 5-HT₁cR antagonist, mianserin, indicating that it was the result of receptor activation and not merely of microinjection with the exogenous mRNA or treatment with 5-HT per se.
activin (Slack et al., 1988; Cooke et al., 1989). Potentiates elongation in animal caps induced with FGF or previously observed for the PI cycle inhibitor, Li+, which stimulating receptor. This effect, too, is the opposite of that stage with 5-HT. Thus, an early marker of mesoderm induction prepared from embryos expressing ventral 5-HT1c R and at the 64-cell stage with mianserin alone) elongated, as did caps (prepared from slightly larger masses of tissue from older embryos; Fig. 3B). In these studies positive controls (caps expressing dorsal 5-HT1c R and treated at the 64-cell stage with 5-HT, whereas little or no elongation was observed in either negative controls (prepared from UV-irradiated embryos) or in caps prepared from embryos expressing ventral 5-HT1c R and treated at the 64-cell stage with 5-HT, whereas little or no elongation was observed in either negative controls (prepared from UV-irradiated embryos) or in caps prepared from embryos expressing dorsal 5-HT1c R and treated at the 64-cell stage with 5-HT. Thus, an early marker of mesoderm induction that is not dependent upon the involutional movements of gastrulation is inhibited by activation of this exogenous PI cycle-stimulating receptor. This effect, too, is the opposite of that previously observed for the PI cycle inhibitor, Li+, which potentiates elongation in animal caps induced with FGF or activin (Slack et al., 1988; Cooke et al., 1989).

**Stimulation of 5-HT1cR specifically inhibits expression of an early response gene of dorsal mesoderm induction**

To determine whether the results described above reflect a general suppression of mesoderm induction by the signaling pathway activated by 5-HT1cR or, instead, the specific inhibition of dorsal mesoderm induction, we assayed via RT-PCR the expression of two early response genes of mesoderm induction. Goosecoid is a homeobox gene expressed in the organizer region of intact embryos in cells fated to become dorsal-most mesoderm, and in animal caps is rapidly but transiently expressed in response to activin, but not FGF (Blumberg et al., 1991; Cho et al., 1991). It thus serves as a useful marker of dorsal-most mesoderm induction. Xbra, another early response gene of mesoderm induction, is expressed throughout the whole of the early prospective mesoderm, and in animal caps its expression is induced by both activin and FGF (Smith et al., 1991; Cunliffe and Smith, 1992); in our studies it serves as a marker of generic (as opposed to dorsal-most) mesoderm induction by activin.

As previously reported, we observed that both goosecoid and Xbra are transiently expressed in animal caps following activin treatment, with peak goosecoid expression typically preceding that of Xbra (Fig. 4A,B). In caps expressing 5-HT1cR, simultaneous treatment with activin and 5-HT (filled squares in Fig. 4A) consistently depressed peak goosecoid expression relative to treatment with activin plus mianserin (open squares). In the five experiments, the results of which are shown in Fig. 4A, peak goosecoid expression was depressed by 5-HT1cR stimulation to a level indistinguishable from that observed in control uninduced explants (circles). In contrast, activin-induced Xbra expression in the same clutches of animal caps was not significantly affected by 5-HT1cR stimulation (Fig. 4B). These distinct responses of goosecoid and Xbra indicate that 5-HT1cR stimulation selectively inhibits dorsal-most (but not generic) mesoderm induction by activin. Here, too, the effect of a PI cycle-stimulating receptor is the opposite of that of the inhibitor, Li+, which enhances activin-induced goosecoid expression without affecting that of Xbra (Tadano et al., 1993).

**DISCUSSION**

Here, we have shown that exogenous receptors can be employed to alter, in a spatially and temporally precise manner, the activity of the PI cycle during early mesoderm induction. We have employed this approach to investigate whether the PI cycle signaling activity previously demonstrated to accompany the onset of mesoderm induction could modulate the induction process by restricting the extent of dorsal-most mesoderm specification, and to begin to identify the molecular targets of this modulation. A striking result of this study is that stimulation of the exogenous receptors has effects on development that are precisely complementary to those of Li+, hypodorsalizing whole embryos when the receptors are expressed and stimulated dorsally, and inhibiting both the convergent extension movements and goosecoid expression induced by activin in animal caps. It is possible that neither Li+ nor 5-HT1cR have direct effects on only PI cycle activity (we are unaware, however, of any other reported signaling activities of the 5-
However, the rather precise complementarity of the developmental effects of these two manipulations – which, to our knowledge, have in common only the ability to alter PI cycle activity, but in opposite directions and by unrelated mechanisms – argues strongly for the interpretation that the developmental effects reported here are consequences of PI cycle activation by 5-HT1c R and, conversely, that lithium’s developmental effects are the result of its inhibition of endogenous PI cycle-mediated signaling. The present work thus suggests that PI cycle-mediated signaling is sufficient to modulate the dorsal-ventral character of mesoderm induction in *Xenopus*. Whether it is also necessary is currently under investigation. As is apparent in Fig. 1A, the PI cycle stimulation we have imposed in the present studies achieved larger [Ca2+]i differences than any apparent dorsal/ventral [Ca2+]i differences in unstimulated embryos. However, it is important to note, first, that the dose of mRNA employed in the experiments shown in Fig. 1 was, for purposes of illustration, twice that employed in the experiments reported in subsequent figures (the lower doses of receptor mRNA yield much smaller [Ca2+]i transients, though still larger than any apparent dorsal/ventral differences in unmanipulated embryos). Second, the highly scattering, opaque nature of *Xenopus* embryo cytoplasm prevents us from observing Ca2+ signaling events in all but the most superficial cells of the intact embryo.
The observation that PI cycle activation specifically depresses goosecoid expression provides a potential starting point for a molecular description of how this signal transduction pathway may function in mesodermal patterning. goosecoid is expressed in a dorsolateral gradient in the early gastrula, and experiments involving microinjection of goosecoid mRNA into ventral blastomeres have revealed that relatively small differences in mRNA content (in the order of two-fold) profoundly influence the range of mesodermal types elicited by endogenous inducers (Niehrs et al., 1994), suggesting that goosecoid gene expression plays an active role in mesodermal patterning. Our results raise the possibility that the graded expression of goosecoid in the intact embryo is regulated by spatially restricted PI cycle activation during mesoderm induction, and that this underlies the PI cycle’s effects on patterning.

Although it is not possible, at present, to determine directly whether the PI cycle activation accompanying the onset of mesoderm induction is graded along the dorsal-ventral axis, the restriction of lithium’s teratogenic action (in whole embryos) to the ventral hemisphere (Kao et al., 1986) and the 5-HT1cR’s to the dorsal hemisphere (Figs 2, 3B) argue in favor of this since, as an uncompetitive inhibitor, lithium’s effect on PI cycle activity will be greatest in those cells with the highest levels of activity (Nahorski et al., 1991), and as a PI cycle activator, the 5- HT1cR’s effects will be greatest in cells not already experiencing high levels of PI cycle-mediated signaling. Three possible models of how dorsoventrally graded PI cycle activity might be established in the embryo are presented in Fig. 5. In the first two, PI cycle activity might be stimulated specifically by one (Fig. 5A) or by two or more (Fig. 5B) intercellular signal(s), one of which must be restricted to the ventrolateral region to yield graded signaling activity. Alternatively, PI cycle activity could be stimulated along the entire extent of the dorsal-ventral axis by a single intercellular signal, and be inhibited solely at the dorsal midline by another signal (Fig. 5C). Among possible alternatives to these models, those in which graded activity arises not from the graded distribution of ligands, but rather from a graded distribution of components of the PI cycle signaling pathway such asPIP2 or endoplasmic reticulum IP3 receptors, are ruled out by our observation that stimulated 5-HT1cR yield equivalent [Ca2+]i signals in both the ventral and dorsal halves of the embryo.

Models A and B of Fig. 5 predict that the putative modulator responsible for achieving graded PI cycle activity is an activator of the cycle, acting ventrally, whereas Model C predicts it to be an inhibitor, acting dorsally. Additionally, model B permits, and model C requires, a second and more homogeneously acting agent to activate the pathway. Based on immunoprecipitation and western blot analyses of association between tyrosine-phosphorylated FGF receptors (FGFR) and phospholipase C-γ1 (PLC-γ1), Ryan and Gillespie (1994) have suggested that FGFR-mediated PLC-γ1 activation is involved in signaling during mesoderm induction. It remains to be established whether the degree of activation observed in that study is sufficient to alone account for the degree of PI cycle activation accompanying the onset of mesoderm induction. If so, FGF itself could be the PI cycle activator of any of the models in Fig. 5. However, in animal cap assays the action of activin is dominant over that of FGF (Cooke, 1989), whereas we have shown that, at least under the conditions employed here, PI cycle activation is dominant over activin, raising the possibility that FGF may not be a sufficiently potent PI cycle activator in Xenopus embryos to function as either of the activating signals of models A and C. However, if two or more intercellular signals sum to achieve the required level of activity, as in model B, then no one of them need be a potent activator of this pathway.

In another recent study exploring the possible role of FGF-1 mediated PLC-γ1 activation, a chimeric receptor possessing the intracellular domains of the FGF receptor induced mesoderm formation in animal caps (as reflected by explant elongation and muscle actin gene expression) even when mutated to prevent its binding to and activation of PLC-γ (Muslin et al., 1994). Whereas these data support the hypothesis that PLC-γ activation is not required for induction of muscle via the type-1 FGF receptor, they do not directly address the hypothesis, presented here, that PI cycle activity modulates dorsal-most mesoderm induction by dorsal inducers such as activin. Receptor-linked tyrosine kinases and G protein-coupled receptors activate different isozymes of PLC (Rhee and Choi, 1992). The present work and those of Muslin et al. (1994) and Ryan and Gillespie (1994) indicate that both forms of this enzyme are functionally present in the Xenopus embryo; which (if either) is responsible for the PI cycle activity accompanying mesoderm induction is currently under study.

Model C in Fig. 5 is perhaps the least parsimonious, in that no intercellular signaling molecule has been demonstrated to inhibit PI cycle activity under physiological conditions in any cellular system. However, potential examples of receptor-mediated inhibition of both G protein-coupled and tyrosine kinase-activated PLC isozymes have been reported (Bizzarri et al., 1990; Shi et al., 1993), and more conventional heterologous desensitization could also yield a mechanism like that of model C. It is intriguing to note that certain mesoderm induction modulators such as Xwnt-8 and noggin have effects on mesoderm induction that are largely indistinguishable from those of Li+ (and therefore might function as PI cycle inhibitors). We are currently investigating whether any of the known mesoderm induction modulators such as Wnts, noggin or BMP-4 act as inhibitors or activators of PI cycle activity in Xenopus embryos.

REFERENCES

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