Transforming Growth Factor β (TGFβ) Signaling via Differential Activation of Activin Receptor-like Kinases 2 and 5 during Cardiac Development

ROLE IN REGULATING PARASYMPATHETIC RESPONSIVENESS*

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Little is known regarding factors that induce parasympathetic responsiveness during cardiac development. We demonstrated previously that in atrial cells cultured from chicks 14 days in ovo, transforming growth factor β (TGFβ) decreased parasympathetic inhibition of heart rate by the muscarinic agonist, carbachol, by 5-fold and decreased expression of Goα2. Here in atrial cells 5 days in ovo, TGFβ increased carbachol inhibition of heart rate 2.5-fold and increased expression of Goα2. TGFβ also stimulated Goα2 mRNA expression and promoter activity at day 5 while inhibiting them at day 14 in ovo. Over the same time course expression of type I TGFβ receptors, chick activin receptor-like kinase 2 and 5 increased with a 2.3-fold higher increase in activin receptor-like kinase 2. Constitutively active activin receptor-like kinase 2 inhibited Goα2 promoter activity, whereas constitutively active activin receptor-like kinase 5 stimulated Goα2 promoter activity independent of embryonic age. In 5-day atrial cells, TGFβ stimulated the p3TP-lux reporter, which is downstream of activin receptor-like kinase 5 and had no effect on the activity of the pVent reporter, which is downstream of activin receptor-like kinase 2. In 14-day cells, TGFβ stimulated both pVent and p3TP-lux. Thus TGFβ exerts opposing effects on parasympathetic response and Goα2 expression by activating different type I TGFβ receptors at distinct stages during cardiac development.

A decrease in heart rate in response to parasympathetic stimulation (negative chronotropic response) involves the binding of acetylcholine to M₁ muscarinic receptors and the disassociation of the heterotrimeric G-protein, Gαi₂, into αi₂ and βγ subunits. The latter activates the inward rectifying K⁺ channel, GIRK1, increasing diastolic depolarization and decreasing heart rate (1). A decrease in the force of contraction in response to muscarinic stimulation (negative inotropic effect) involves the binding of the α₂ subunit to adenylate cyclase followed by a decrease in cAMP production. Several studies support the conclusion that control of Goα₂ expression regulates the response of the heart to parasympathetic stimulation. The development of parasympathetic responsiveness in the embryonic chick heart is associated with an increase in Goα₂ expression (2). Furthermore, growth of chick atrial cells in the absence of lipoproteins, which has been shown to result in an increased response to parasympathetic stimulation, is associated with an increase in the expression of Goα₂ (3, 4). Finally, expression of Goα₂ in the porcine atrioventricular node resulted in an increase in parasympathetic tone (5).

A role for TGFβ in the development of the parasympathetic response of the heart was suggested by studies in which medium conditioned by co-culture of chick heart cells and ciliary ganglia induced a negative chronotropic response to carbachol in chick heart cells 3.5 days in ovo (dio). This induction of a parasympathetic response was accompanied by an increase in Goα₂ expression (6) and was reversed by addition of a neutralizing antibody to TGFβ₁ to the medium.² In contrast, we recently demonstrated that in atrial cells from hearts 14 dio, TGFβ₁ decreased the expression of Goα₂ and decreased the negative chronotropic response to carbachol (7). These data suggest that TGFβ exerts opposing effects on parasympathetic responsiveness at different stages of cardiac development.

The TGFβ family is composed of at least three 25-kDa homodimeric proteins, TGFβ₁, TGFβ₂, and TGFβ₃. TGFβ signaling involves the binding of TGFβ ligand to two transmembrane serine threonine kinases, the type I TGFβ receptor I (TBRI) and the type II TGFβ receptor (TBRII). TBRII has a constitutively active cytoplasmic kinase domain and an extracellular domain that binds TGFβ₁ and TGFβ₂. TGFβ₁ binding results in the phosphorylation of TBRI by TBRII. TBRII then activates a signaling cascade, which may include a series of transcription factors known as Smads (8). Other TGFβ family members such as the activins and bone morphogenic proteins (BMPs) also signal through a type I receptor by binding to specific type II TGFβ receptors.

²The abbreviations used are: TGFβ, transforming growth factor β; TBRI, type I TGFβ receptor; TBRII, type II TGFβ receptor; ActRII, type II activin receptor; BMP, bone morphogenic protein; BMPR, type II BMP receptor; GAPDH, glyceraldehyde phosphate dehydrogenase; ALK, activin receptor-like kinase; chALK, chick ALK, chALK⁺, constitutively active chALK; PAP, plasmogen activator inhibitor; pBS, β-galactosidase.

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receptors for activin (ActRII and ActRIIB) and BMP (BMPRII) (9). To date, seven type I receptors have been identified and their activin receptor-like kinases (ALKs) 1–7. The ligand specificity of these ALKs has been determined by their ability to bind to a given ligand and to activate downstream signals in the presence of a specific type II receptor subtype. ALK1 and ALK5 are activated by TGFβ via TBRIs (9, 10). ALK5 in association with TBRIs specifically stimulates the phosphorylation of activin receptor inhibitor (PAI-1) promoter. ALK5 mediates growth arrest in mink lung epithelial cells following the formation of the ALK5/TBRII complex and the phosphorylation of ALK5 (11). ALK2 interacts with TBRIs as well as ActRII and BMPRII type II receptors (12). ALK2 does not mediate TGFβ signaling in mink lung epithelial cells but has been implicated in the TGFβ-stimulated epithelial-mesenchymal transformation in the mammary gland of the mouse (13). The regulation of TGFβ receptor signaling by selective interactions with different type I receptors is an intriguing mechanism that might help explain the pleiotropic effects of TGFβ. Here we demonstrate that TGFβ mediates opposing effects on Gαq expression and the response of the heart to parasympathetic stimulation at different stages of chick heart development and that these pleiotropic effects are due to differential activation of ALK2 and ALK5 by TGFβ.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Embryonic chick atrial myocyte cultures were prepared by a modification of the method of DeHaas (14) as described previously (15). Eggs were staged according to stage 27, and the 14-day embryo corresponded to stage 40.

**RNase Protection Analysis**—A Gαq RNase protection probe was generated from a Psfl fragment derived from the chick Gαq cDNA subcloned into a Psfl vector and linearized with T7 RNA polymerase (Roche Molecular Biochemicals) in the presence of [32P]UTP (800 Ci/mmol, PerkinElmer Life Sciences), this template gave a 307-nucleotide antisense riboprobe. The glyceraldehyde phosphate dehydrogenase (GAPDH) RNase protection probe, used as a control, was generated from a cdNA template (gift of R. Runyan), which was linearized with HindIII. Using T3 RNA polymerase, this template gave a 290-nucleotide antisense riboprobe. Probes were purified by PAGE on a 6% gel, and the major band corresponding to the predicted molecular weight for the riboprobe was excised and eluted overnight. Total RNA was isolated from cultures of embryonic chick atrial cells 14 dio using guanidinium CsCl centrifugation as described (18). RNase protection was carried out as described previously (15). Riboprobes were hybridized to 15 μg of total RNA prepared from cells treated with either vehicle or 5 ng/ml TGFβ1. The samples were treated with RNase and analyzed by PAGE on 6% gels containing urea followed by autoradiography. Radioactive exposure was 6 h for Gαq and 2 h for GAPDH. The relative intensity of the bands was determined by densitometry scanning using NIH Image Pro.

**Measurement of Changes in Beat Rate**—Embryonic chick atrial cells from hearts of embryos 5 dio cultured on coverslips at 5 × 10^5 cells/cm^2 were treated either with vehicle (4 mM HCl and 0.5 mg/ml bovine serum albumin) or with 5 ng/ml TGFβ1, and placed in a perfusion chamber as described (15), on the stage of a Zeiss inverted phase contrast microscope (17). Using a Picoscope (Model 1010, Western Blotting, Polyclonal (rabbit) antisera to the carboxyl-terminal decapeptide from rat Gαq was a gift of David Manning. TBRIs, ALK2, and ALK5 antibodies were prepared as described (18, 19). Cultured chick atrial cells 5 and 14 dio were grown for 3 days in fetal calf serum, homogenates were prepared and Western blot analysis was carried out as described (15). Equal amounts of protein were loaded as determined by a DC protein assay (Bio-Rad). Equal loading was determined by Coomassie staining.

**Luciferase and Alkaline Phosphatase Assays**—Embryonic chick atrial cells 5 and 14 dio were cultured in medium supplemented with fetal calf serum. On the second culture day, 1 μg of Gαq-Luc consisting of 1.5 kb of the 5′ upstream region of the chick Gαq promoter ligated to a luciferase reporter (7) and 0.2 μg of a human placental alkaline phosphatase under the control of an SV40 promoter (pSV2Apap, a gift of L. Erolanni) were transfected into heart cells cultured on 35-mm plates by the use of FuGENE 6 transfection reagent (Roche Molecular Biochemicals) as recommended by the manufacturer. Total DNA was maintained at 2.1 μg by addition of pBluescript (pBS) DNA. At 16 h posttransfection, cells were washed in phosphate-buffered saline and solubilized in lysis buffer at 425 μl/plate (24 mM glycyglycyglycine, 15 mM MgSO4, 4 mM EGTA, 1% Triton X-100, and 1 mM dithiothreitol). The extract was sonicated three times for 10 s and then centrifuged at 13,000 × g for 3 min at 4 °C, and the supernatant was assayed for luciferase and alkaline phosphatase activity as described (20). In other experiments, cells were transfected with the pVENT promoter luciferase reporter construct, Xvent2-luc, containing ~250 bp of Xvent2 promoter sequences, which was a gift from Christof Niehrs, or p3TP-lux containing the putative TGFβ-responsive region of the human PAI-1 (plasminogen activator inhibitor) promoter, which was a gift of Joan Massague. In some experiments, cells were co-transfected with pVENT-Luc, p3TP-lux, or GUS2-Luc and contuitively active TBRIs (constitutively active chicken ALK2 (chALK2+) and chicken ALK5 (chALK5+)). Constitutively active TBRIs were generated as described (21, 22). Briefly, chALK2 and chALK5 (19) cDNAs were altered in the GS box (chALK2 Q202D, chALK2G204D) (23, 24). The specificity of these constitutively active mutants of chALK2 and chALK5 was determined by cotransfection of chick atrial cells with p3TP-lux, which is specifically activated by chALK5 or cotransfection with pVENT, which is specifically activated by chALK2. chALK5−stimulated p3TP-lux 2.8 ± 0.4-fold (± S.E., n = 6, p < 0.01) while having no significant effect on pVENT promoter activity. chALK2−stimulated pVENT promoter activity 2.0 ± 0.3-fold (± S.E., n = 6, p < 0.01) while having only a minimal effect on p3TP-lux promoter activity.

**Statistics**—Statistical analysis was by Student’s t test.

**RESULTS**

**TGFβ1 Enhances the Negative Chronotropic Response to Muscarinic Stimulation in Atrial Cells from Hearts 5 dio**—During embryonic development, the negative chronotropic response of the chick heart to muscarinic stimulation developed between 2 and 7 dio (25). To determine the effect of TGFβ on the development of the parasympathetic response, embryonic chick atrial cells from 5 dio hearts were incubated for 16 h with either 5 ng/ml TGFβ, or vehicle, and beat rate was determined in the presence of carbamylcholine. In the absence of TGFβ1, 0.1 mM carbamylcholine decreased beat rate by 30 ± 1% (± S.E., n = 21, p < 0.001, Fig. 1). However, after incubation with TGFβ1, carbamylcholine decreased beat rate by 76 ± 1% (± S.E., n = 21, p < 0.01, Table I). These effects on beat rate were reversible within 5 min after reperefusion of cells with carbamylcholine-free medium. Thus TGFβ1 increases the chronotropic response to carbamylcholine by more than 2.5-fold in atrial myocytes from hearts 5 dio. This result is opposite to the effect of TGFβ1 in cells from atri of hearts 14 dio in which we demonstrated that TGFβ1 decreased the chronotropic response to carbamylcholine by more than 5-fold (Table I) (7).

**Developmental Changes in TGFβ1 Regulation of Gαq Expression**—The expression of Gαq increases in parallel with the development of parasympathetic responsiveness in the embryonic chick heart (2). Hence the opposing effects of TGFβ on the negative chronotropic response of the heart to muscarinic stimulation might be associated with alterations in Gαq expression. To test this hypothesis, we determined whether TGFβ1 altered Gαq expression in atrial myocytes cultured from hearts between 5 and 14 dio. Incubation of cells from hearts 5 dio with TGFβ1 increased the level of Gαq mRNA, whereas in cells derived from hearts 7, 9, and 14 dio, TGFβ1 decreased levels of...
Fig. 1. Effect of TGFβ on the negative chronotropic response to carbamylcholine (Carb) in embryonic chick atrial cells from hearts 5 dio. The data illustrate the effect of 0.1 mM carbamylcholine on the spontaneous beat rate of cells grown for 3 days in medium supplemented with fetal calf serum in the presence of either 5 ng/ml TGFβ, or an equal volume of vehicle (4 mM HCl and 0.5 mg/ml bovine serum albumin) as described under “Experimental Procedures.” The left side of each panel represents the basal beat rate response stable for 5 min prior to perfusion with buffer containing carbamylcholine. The right side of each panel represents the beat rate following a 2-min perfusion with 0.1 mM carbamylcholine.

TABLE I

| Developmental changes in TGFβ, regulation of the parasympathetic response in embryonic chick atrial cells |
|---------------------------------------------------------------|
| Percent inhibition of beat rate by carbamylcholine in atrial cells 5 and 14 dio treated with vehicle or 5 ng/ml TGFβ. Data are the means ± S.E. |
| 5 dio<br>Veh | TGFβ | 14 dio<br>Veh | TGFβ |
| Vehicle | 5 dio | 14 dio | Vehicle | TGFβ |
| 31.1 ± 1.4% | 75.4 ± 1.5% | 94.8 ± 2.1% | 18.3 ± 1.8% |

Data derived from Ref. 7.

Gαi2 mRNA (Fig. 2A). The mean of five experiments similar to that in Fig. 2A demonstrated that when compared with vehicle, TGFβ, stimulated Gαi2 mRNA 2.10 ± 0.16-fold (± S.E., n = 5, p < 0.002) at day 5 in ovo while decreasing Gαi2 mRNA at days 7, 9, and 14 in ovo by 0.44 ± 0.06-fold (± S.E., n = 4, p < 0.003); 0.52 ± 0.06-fold (± S.E., n = 5, p < 0.002); and 0.60 ± 0.02-fold (± S.E., n = 5, p < 0.001) respectively. Similarly, TGFβ, stimulated expression of Gαi2 protein in cells from hearts 5 dio (Fig. 2B) by 2.30 ± 0.10-fold (± S.E., n = 3, p < 0.002, Fig. 2C) but decreased Gαi2 protein in extracts of cells from hearts 14 dio (Fig. 2B) by 0.42 ± 0.04-fold (± S.E., n = 4, p < 0.001, Fig. 2C). Finally, TGFβ, stimulated Gαi2 promoter activity in chick atrial cells from hearts 5 dio by 2.40 ± 0.40-fold (± S.E., n = 5, Fig. 3A) and decreased Gαi2 promoter activity by 54 ± 6% (± S.E., n = 4) in atrial cells from hearts 14 dio (Fig. 3B). These data demonstrate that the opposing effects of TGFβ on the negative chronotropic response to muscarinic stimulation are accompanied by similar alterations in Gαi2 expression.

Developmental Changes in the Expression of TGFβ Receptors—These opposing effects in the response of chick atrial cells to TGFβ might reflect changes in the expression of TGFβ receptors involved in signaling at different stages of cardiac development. Western blot analysis demonstrated that embryonic chick atrial cells expressed TBRII, ALK2, and ALK5 (Fig. 4, A and C). ALK2 and ALK5 have been reported to mediate distinct responses to TGFβ signaling (9–11, 13). For this reason, we studied developmental changes in these two TGFβ receptors. chALK2 and chALK5 were initially expressed at low levels at day 5 in ovo but increased markedly between days 5 and 14 in ovo (Fig. 4A). Comparison of the fold increase in ALK2 and ALK5 expression between 5 and 14 dio demonstrated that chALK2 increased 2.30 ± 0.20-fold (± S.E., n = 4, p < 0.01) more than chALK5 (Fig. 4B). TBRII levels increased 4.40 ± 0.20-fold (± S.E., n = 3) between 5 and 14 dio (Fig. 4, C and D). Thus each receptor increased between 5 and 14 dio with the largest increase in chALK2.

Differential Activation of chALK2 and chALK5 by TGFβ at Days 5 and 14 in Ovo—To determine whether TGFβ signaling might preferentially activate chALK2 or chALK5 at different stages of cardiac development, we compared the effect of TGFβ on p3TP-lux and pVent reporter activity in atrial cells from hearts 5 and 14 dio. chALK5 specifically activates the p3TP-lux reporter (12). pVent is known to be activated by BMP, not by TGFβ, and is one of the best known reporters of ALK2 activation (26). To determine whether ALK2 might be mediating a TGFβ response, in our system, pVent was used as a reporter of ALK2 activation. In atrial cells from hearts 5 dio, 5 ng/ml TGFβ stimulated p3TP-lux activity 5.20 ± 0.30-fold (± S.E., n = 5, p < 0.002, Fig. 5A), whereas in atrial cells from hearts 14 dio, TGFβ stimulated p3TP-lux by 2.5 ± 0.1-fold (± S.E., n = 6, p < 0.003, Fig. 5B). In atrial cells from hearts 5 dio, TGFβ had no effect on pVent reporter activity (Fig. 5C). However, in atrial cells 14 dio, we observed an unexpected 2.2 ± 0.2-fold (± S.E., n = 7, p < 0.003, Fig. 5D) increase in pVent reporter activity in response to TGFβ. These data demonstrate that in chick atrial cells, pVent is activated by TGFβ and that this activation is specific for cells 14 dio. These data also suggest that in chick atrial cells 5 dio TGFβ signals via chALK5 and not chALK2.

chALK2 and chALK5 Differentially Regulate Gαi2 Promoter Activity—The ability of chALK2 to mediate a TGFβ response at 14 dio but not at 5 dio suggests a potential mechanism for the opposing effects of TGFβ at these ages. Specifically, chALK2 might act to decrease Gαi2 expression, whereas chALK5 might act to increase Gαi2. To test this hypothesis, atrial cells from hearts 14 dio were transfected with chALK5Δ, chALK5Δ stimulated Gαi2 promoter activity 2.5 ± 0.20-fold (± S.E., n = 4) (Fig. 6A, column 3). Furthermore, chALK5Δ not only reversed TGFβ inhibition of Gαi2 promoter activity but also stimulated Gαi2 promoter activity 2.4 ± 0.1-fold (± S.E., n = 4) above basal (Fig. 6A, column 4). In contrast, transfection of cells from chick atria 14 dio with chALK2Δ not only mimicked the effect of TGFβ but completely inhibited Gαi2 promoter activity (Fig. 6B, column 3).

If chALK2 mediates the inhibition of the Gαi2 promoter by TGFβ signaling, then overexpression of chALK2 in atrial cells from chicks 5 dio should inhibit TGFβ-stimulated Gαi2 pro-
FIG. 2. Developmental changes in the effect of TGFβ1 on expression of Goα2 in chick atrial cells from 5 to 14 dio. A, effect of TGFβ1 on Goα2 mRNA. Embryonic chick atrial cells 5, 7, 9, and 14 dio were cultured as described under “Experimental Procedures.” On the second culture day, either 5 ng/ml TGFβ1 or an equal volume of vehicle was added. Cells were incubated for 16 h, total cell RNA was prepared, and RNase protection was performed as described previously. Upper panel, Goα2; lower panel, GAPDH. The intensity of the bands protected by the antisense riboprobe to GAPDH was identical for cells incubated with vehicle (V) and TGFβ1 (T), indicating equal loading of RNA. These data are typical of four similar experiments. B, effect of TGFβ1 on Goα2 protein. Embryonic chick atrial cells 5 and 14 dio were cultured as described previously under “Experimental Procedures.” On the second culture day, either TGFβ1 (5 ng/ml) or vehicle was added for 16 h. Cells were harvested on the third day, and Goα2 expression was determined by Western blot analysis. Lanes 1 and 3, Goα2 expression in cells incubated with vehicle; lanes 2 and 4, Goα2 expression in cells incubated with TGFβ1. These data are typical of three similar experiments. C, densitometry scanning of three experiments similar to that in panel B. Data are normalized to the value in vehicle treated cells 5 dio taken as 1.

The data presented here provide novel insight into TGFβ signaling and the regulation of parasympathetic responsiveness in the heart. TGFβ stimulates the negative chronotropic response of chick atrial cells 5 dio to carbamylcholine, whereas it decreases the inhibition of beat rate by carbamylcholine in atrial cells 14 dio (7). These effects of TGFβ correlate with alterations in Goα2 expression. At 5 dio, TGFβ stimulates Goα2 expression, and at 14 dio, TGFβ inhibits Goα2 expression. Examination of two TBRIs reported to play a role in TGFβ signaling reveals that chALK2 inhibits Goα2 expression, whereas chALK5 stimulates Goα2 promoter activity, and that these effects are independent of the developmental stage of the atrial myocytes.

DISCUSSION

The data presented here provide novel insight into TGFβ signaling and the regulation of parasympathetic responsiveness in the heart. TGFβ stimulates the negative chronotropic response of chick atrial cells 5 dio to carbamylcholine, whereas it decreases the inhibition of beat rate by carbamylcholine in atrial cells 14 dio (7). These effects of TGFβ correlate with alterations in Goα2 expression. At 5 dio, TGFβ stimulates Goα2 expression, and at 14 dio, TGFβ inhibits Goα2 expression. Examination of two TBRIs reported to play a role in TGFβ signaling reveals that chALK2 inhibits Goα2 expression, whereas chALK5 stimulates Goα2 promoter activity, and that these effects are independent of the developmental stage of the atrial myocytes.
The induction of a parasympathetic response is a critical step in the physiological development of the mammalian heart. The regulation of the parasympathetic responsiveness of the heart not only controls the rate and force of contraction but also may play a role in the development of cardiac arrhythmias (27, 28). We have demonstrated previously that during embryonic development of the chick heart, the negative chronotropic response to carbamylcholine increased markedly between 5 and 7 dio, reaching a plateau at 7 dio (25). The development of the parasympathetic response in the embryonic chick heart was associated with an increase in Gα₁₂ expression (2). Regulation of Gα₁₂ expression has been associated with the control of parasympathetic responsiveness in the adult heart. A recent study demonstrated that overexpression of Gα₁₂ in the porcine atrioventricular node resulted in a decrease in atrioventricular conduction and a decreased response to sympathetic stimulation consistent with an increase in parasympathetic tone (5). Here we demonstrate a striking parallel between developmental changes in TGFβ regulation of the response of the heart to parasympathetic stimulation and TGFβ regulation of Gα₁₂ ex-
expression. These data emphasize the importance of the regulation of Gαq expression on parasympathetic responsiveness and cardiac function.

Our data support the notion that the transition of TGFβ signaling in atrial cells from a stimulatory effect on Gαq expression and parasympathetic response to an inhibitory effect during embryonic development reflects differential activation of the TGFβ type I receptors, chALK2 and chALK5. Complexes of ALK5 and TBRII bind TGFβ, to mediate TGFβ effects such as growth arrest in mink lung epithelial cells (11). Although ALK2 binds TGFβ when co-expressed with TBRII, it does not mediate growth arrest in mink lung epithelial cells. A role for ALK2 has been described during the TGFβ-dependent epithelial-mesenchymal transformation of mouse mammary epithelial cells (13). A similar TGFβ-stimulated epithelial-mesenchymal transformation occurs in the atrioventricular cushion during valvulogenesis. Studies using an in vitro culture system demonstrated that anti-chALK2 antisera blocked transformation, whereas anti-chALK5 antisera was without effect (19). The finding that specific TGFβ effects may be attributed to ALK2 or ALK5 suggested that the specificity of the downstream response to TGFβ signaling is dependent on the identity of the TBRI activated in a given cell type. In support of this conclusion, chALK2 and chALK5 were shown to exert opposing effects on Gαq promoter activity. Constitutively active chALK2 inhibited Gαq promoter activity, and constitutively active chALK5 stimulated Gαq promoter activity independent of the embryonic age of the cell in which they were expressed.

Hence differential activation of chALK2 and chALK5 by TGFβ at 5 and 14 dio might result in opposing effects of TGFβ on Gαq expression during cardiac development. To test this hypothesis, we compared the effect of TGFβ on p3TP-lux reporter activity in atrial cells from atria 5 and 14 dio. The pVent reporter is activated by BMP signaling via ALK2 (22, 26, 29), whereas the p3TP-lux reporter is activated by ALK5 signaling (11). TGFβ stimulated p3TP-lux reporter activity in atrial cells from hearts 5 dio but had no effect on pVent reporter activity in these cells. Furthermore, although TGFβ stimulated both p3TP-lux and the pVent reporter in cells 14 dio, the stimulation of pVent was significantly higher than p3TP-lux in these cells.

These data support the conclusion that TGFβ signaling at 5 dio occurs via chALK5 and that signaling at 14 dio occurs via both chALK2 and chALK5, with chALK2 predominating. Although it is not possible to directly compare the level of expression of chALK2 and chALK5 at 5 or 14 dio, we noted a larger increase in ALK2 expression than ALK5 expression, consistent with the conclusion that the increase in ALK2 signaling at 14 dio was due at least in part to an increase in expression levels. Taken together with the data which demonstrate that ALK5 stimulates Gαq promoter activity and ALK2 inhibits Gαq promoter activity, the finding of differential activation of ALK2 and ALK5 would account for the opposing effects of TGFβ on Gαq expression at 5 and 14 dio.

The unexpected observation that TGFβ stimulates pVent expression in chick atrial cells 14 dio is the first report of activation of a BMP-like signal by TGFβ. TGFβ signaling via ALK5 has been shown to involve Smads 2/3 (30). We demonstrated that constitutively active chALK5 did not stimulate pVent promoter activity, which indicates that Smads 2/3 cannot activate pVent in these cells. Furthermore, studies of pVent have demonstrated stimulation by the BMP-specific Smads 1/5/8 (26). This would suggest that TGFβ stimulation of pVent might be mediated by a BMP-specific pathway in these cells.

The significance of these developmental changes in TGFβ signaling may be related to a dual role of TGFβ signaling in cardiac physiology and development. In an in vitro model for parasympathetic innervation of the heart, we have demonstrated that induction of a negative chronotropic response to carbamylcholine and the expression of Gaq2 were dependent on the release of a soluble factor (6) whose effect was inhibited by a neutralizing antibody to TGFβ (2). These findings implicate TGFβ in the development of the parasympathetic response. Studies in explanted, intact chick heart have previously demonstrated a marked increase in the response of the heart to parasympathetic stimulation between days 2 and 7 in ovo (31). Here TGFβ stimulates a significant increase in both Gaq2 expression and parasympathetic response in atrial cells 5 dio. These data support the conclusion that TGFβ plays a role in the development of a parasympathetic response in the heart. At 14 dio, functional parasympathetic innervation of the chick heart is complete (31). The significance of TGFβ inhibition of Gaq2 expression and parasympathetic responsiveness at this developmental stage is unclear. However, TGFβ has been shown to play a role in a number of processes important to cardiac function such as angiogenesis, cardiac hypertrophy, inflammation, and the response of the heart to myocardial infarction (32, 33). The relationship between TGFβ inhibition of parasympathetic responsiveness and Gaq2 expression to these processes remains to be determined.
These data suggest that TGFβ is an important regulator of parasympathetic responsiveness during cardiac development and may regulate the parasympathetic response at least in part by modulating Gαq expression. Further, we suggest that TGFβ signaling may involve the activation of both ALK5 and ALK2 in atrial cells and that the relative contribution of each of these receptors determines the level of Gαq expression and parasympathetic responsiveness. Our observations suggesting differential activation of two different type I receptors are an attractive mechanism to explain the pleiotropic effects of TGFβ.

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