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Signal transduction of a tissue interaction during embryonic heart development

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During early cardiac development, progenitors of the valves and septa of the heart are formed by an epithelial-mesenchymal cell transformation of endothelial cells of the atrioventricular (AV) canal. We have previously shown that this event is due to an interaction between the endothelium and products of the myocardium found within the extracellular matrix. The present study examines signal transduction mechanisms governing this differentiation of AV canal endothelium. Activators of protein kinase C (PKC), phorbol myristate acetate (PMA) and mezerein, both produced an incomplete phenotypic transformation of endothelial cells in an in vitro bioassay for transformation. On the other hand, inhibitors of PKC (H-7 and staurosporine) and tyrosine kinase (genistein) blocked cellular transformation in response to the native myocardium or a myocardially-conditioned medium. Intracellular free calcium concentration ($[Ca^{2+}]_i$) was measured in single endothelial cells by microscopic digital analysis of fura 2 fluorescence. Addition of a myocardial conditioned medium containing the transforming stimulus produced a specific increase in $[Ca^{2+}]_i$ in "competent" AV canal, but not ventricular, endothelial cells. Epithelial-mesenchymal cell transformation was inhibited by pertussis toxin but not cholera toxin. These data lead to the hypothesis that signal transduction of this tissue interaction is mediated by a G protein and one or more kinase activities. In response to receptor activation, competent AV canal endothelial cells demonstrate an increase in $[Ca^{2+}]_i$. Together, the data provide direct evidence for a regional and temporal regulation of signal transduction processes which mediate a specific extracellular matrix-mediated tissue interaction in the embryo.

Introduction

During the embryonic development of the heart, a population of mesenchymal cells arises within the primordial valves. These cells are produced by an epithelial-mesenchymal cell transformation of endothelial cells lining the atrioventricular canal (Bolender and Markwald, 1979). Once formed, mesenchymal cells rapidly invade the underlying extracellular matrix and become progenitors of the valves and septa of the adult heart (see Markwald *et al.,* 1984 for review).

Previous studies of this process demonstrated that endothelial transformation is produced by a stimulus that is derived from the adjacent myocardium and found in the extracellular matrix (ECM) (Runyan and Markwald, 1983). In subsequent studies, it was shown that both extracts of the ECM (Krug *et al.,* 1985, 1987) and conditioned media from AV canal myocardial cultures (Sinning *et al.,* 1988) could substitute for the myocardium in stimulating the formation of mesenchyme. As in vivo, this tissue interaction is regionally confined and both the myocardial source of the stimulus and the target endothelium are localized to the AV canal of the embryonic heart (Runyan *et al.,* 1982; Mjaatvedt *et al.,* 1987). These studies suggest that specific factor(s) can be identified that produce this cellular differentiation.

Recently, it was shown that one component of the transformation process is a member of the transforming growth factor beta (TGFβ) family of proteins found within the ECM (Potts and Runyan, 1989). Although unable to induce mesenchymal cell formation by itself, TGFβ appears to provide the functional basis for regional specificity of the tissue interaction in the heart. It is clear, however, that one or more additional molecules found in the ECM are required for cellular transformation in the AV canal.

The present study probes the basis of signal transduction of the myocardial stimulus to the endothelium. Through examination of specific stimulus-response mechanisms in the heart, we can gain an understanding of both the nature of the stimulus and the manner in which cellular

differentiation is initiated. Due to limitations on material, we have focused on reagents which act upon well-characterized components of second messenger and signal transduction pathways. This approach is further supported by direct measurement of the intracellular calcium concentration ($[Ca^{2+}]_i$) within single cells using microscopic digital analysis of fura 2 fluorescence.

Our results suggest that ECM-mediated tissue interaction in the AV canal acts through pathways mediated by activation of PKC and an increased $[Ca^{2+}]_i$. It is also shown that signal transduction in this example of "secondary induction" in the chick embryo is sensitive to an inhibitor of a class of G proteins. Additional regulation may occur through the activity of a tyrosine kinase. These data provide new insights into basic mechanisms which mediate embryonic development.

Results

Activation of protein kinase C produces a non-invasive change in phenotype

A previous report (Montesano and Orci, 1985) had shown that angiogenic invasion of collagen gels was produced by the addition of phorbol esters to adult endothelial cells. Since phorbol esters are putative activators of PKC, we sought to determine whether epithelial-mesenchymal cell transformation by embryonic AV canal endothelial cells could be mediated by similar mechanisms. Competent AV monolayers were prepared and treated with the phorbol ester, phorbol 12-myristate 13-acetate (PMA) in a range from 5 to 40 ng/ml. Similar effects were seen at all dosages tested. When treated at 12 h after explantation, cells of the endothelial monolayer showed cell-cell separation and an elongated fibroblastic phenotype. By comparison with the extent of outgrowth with untreated cultures, it appeared that the cells had an increased range of migration over the surface of the gel (Figure 1). PMA did not produce complete epithelial-mesenchymal transformation as no evidence of cell invasion could be found. This change in cellular phenotype was similar to that previously observed with a partially active fraction of cardiac ECM extract in earlier studies of cell transformation (Krug *et al.*, 1985). Unlike the cardiac extract however, PMA was not regionally specific. Ventricular endothelium showed an identical change in phenotype in response to the phorbol ester (data not shown). To test the specificity of this effect, the non-phorbol activator of PKC, mezerein, was also tested in a range of 5 to 20 mM (Figure 1C). All dosages tested were equally effective and both AV canal and ventricular endothelia responded identically to this reagent as they had to PMA.

Extended treatment with phorbol esters has been shown to downregulate the activity of PKC (McArdle and Conn, 1989). To investigate the effects of downregulation, additional explant cultures were incubated on collagen gels containing PMA (10 ng/ml) at the start of incubation. As shown in Figure 1D, pretreatment reduced the outgrowth of endothelium from the explant. These data suggest that PKC may mediate aspects of epithelial-mesenchymal transformation associated with a change in cellular phenotype or cell migration.

Phosphorylation inhibitors block epithelial-mesenchymal transformation

To further explore the role of kinases in epithelial-mesenchymal transformation, we examined the effects of a variety of kinase inhibitors in the transformation bioassay. First, the ATP analog, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was added to AV canal explants after 12 h in culture (50 ng/ml). This inhibitor has effects on a variety of cellular kinases, including PKC and protein kinase A (Kawamoto and Hidaka, 1983). Despite the presence of the AV myocardium as a source of the transducing stimulus, mesenchymal cell formation was completely inhibited (Figure 2). This inhibitor was also able to block the shape-transforming effects of PMA (Figure 2B). To further examine the role of PKC, an additional inhibitor, staurosporine (Tamaoki *et al.*, 1986), with specificity towards this enzyme was also employed. As shown in Table 1, this inhibitor was also effective in blocking epithelial-mesenchymal transformation when added to AV canal explants. Cells on the surface of the gel retained an endothelial appearance when exposed to above threshold levels of each of the inhibitors. A typical endothelial outgrowth is shown in Figure 2C. Further, when the AV canal explants were placed upon gels already containing either of these inhibitors, little if any outgrowth could be observed from the explant. These explants appeared identical to those pretreated with PMA to downregulate PKC (Figure 2D). In single experiments, two additional inhibitors, sphingosine (Merril and Stevens, 1989) and cremophor EL (Zhao *et al.*, 1989) produced identical effects (data not shown). These data

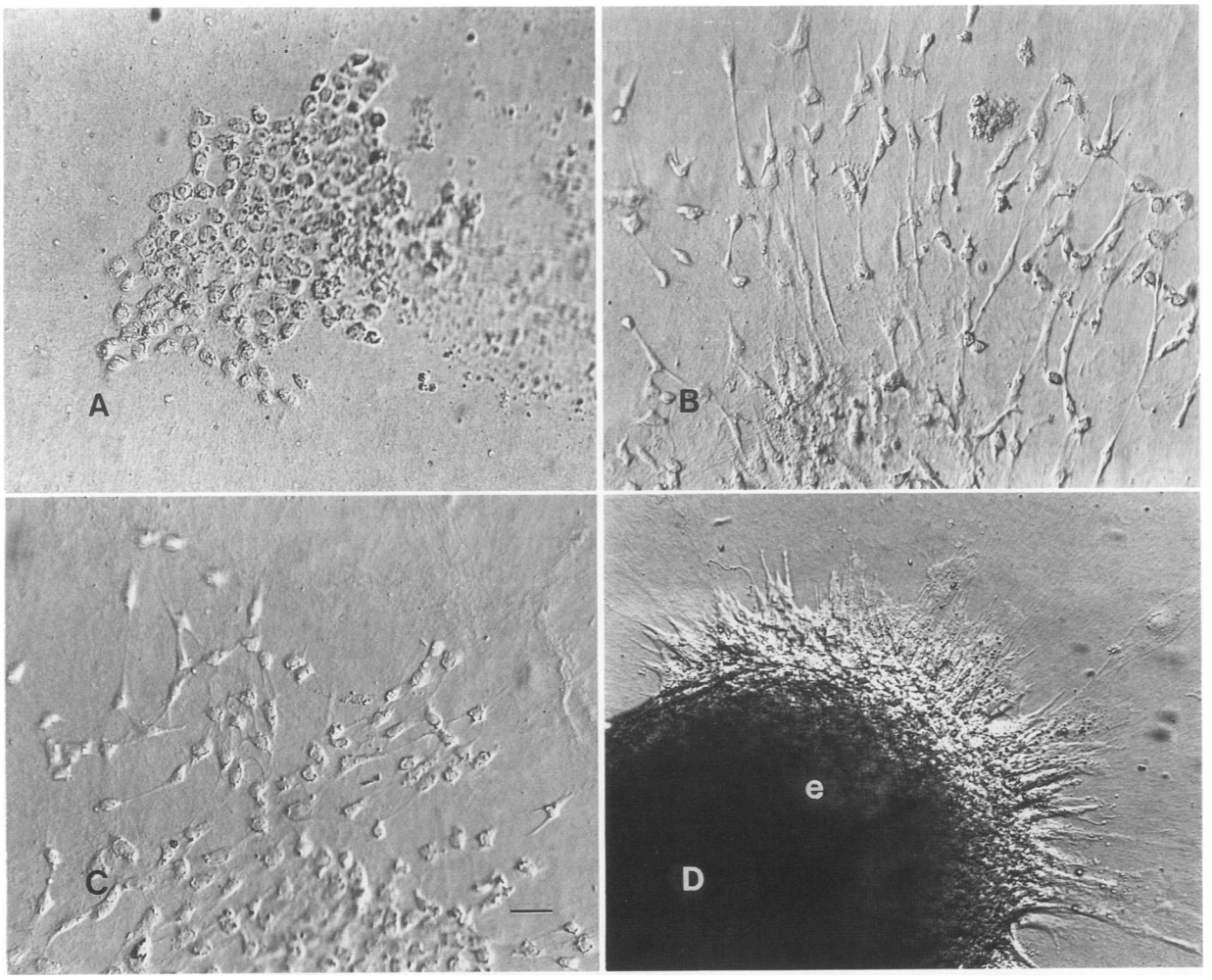

Figure 1. **Effects of PMA and Mezerein on endothelial cell phenotype.** All cultures were cultured for 24 h before photography. The myocardial portion of each explant was removed after 12 h to prevent normal mesenchymal cell formation. (A) Control AV canal endothelial monolayer on collagen gel. The majority of cells are closely apposed to one another. (B) AV monolayer ~12 h after the addition of 20 nM PMA. Cells show an extended morphology with less cell-cell contact. Despite change in shape, no cells have invaded the matrix of the underlying gel. (C) Similar change in cell morphology in the presence of 20 mM Mezerein. (D) Appearance of an AV canal explant when placed upon a gel containing 20 nM PMA. Endothelial outgrowth is prevented by exposure to this reagent. All figures are at same magnification. e, myocardial portion of explant. Bar = 50 μm.

suggest that the process of endothelial cell migration, as well as invasion, is mediated by PKC.

As a comparison with inhibitors of PKC activity, cultures were also incubated with genistein. Genistein is a recently reported kinase inhibitor which has been shown to be specific for tyrosine kinase. It is reported to have little activity towards PKC or protein kinase A (Akiyama *et al.*, 1987). This inhibitor permitted endothelial outgrowth from the explant but completely prevented epithelial-mesenchymal cell transformation (Figure 2E). Though staurosporine has also been reported to inhibit tyrosine kinase (Nakano *et al.*, 1987; Smith *et al.*, 1989), the differences between the effects of genistein and staurosporine appear to be due to their different activities towards PKC. In considering the effects of these inhibitors, it should be noted that the myocardial portion of each explant retained its beating activity at the active concentration used with each of these inhibitors (Table 1).

Subsequent experiments were undertaken to demonstrate that the effects of the various kinase inhibitors are due to their activities on the target endothelium. Competent AV canal

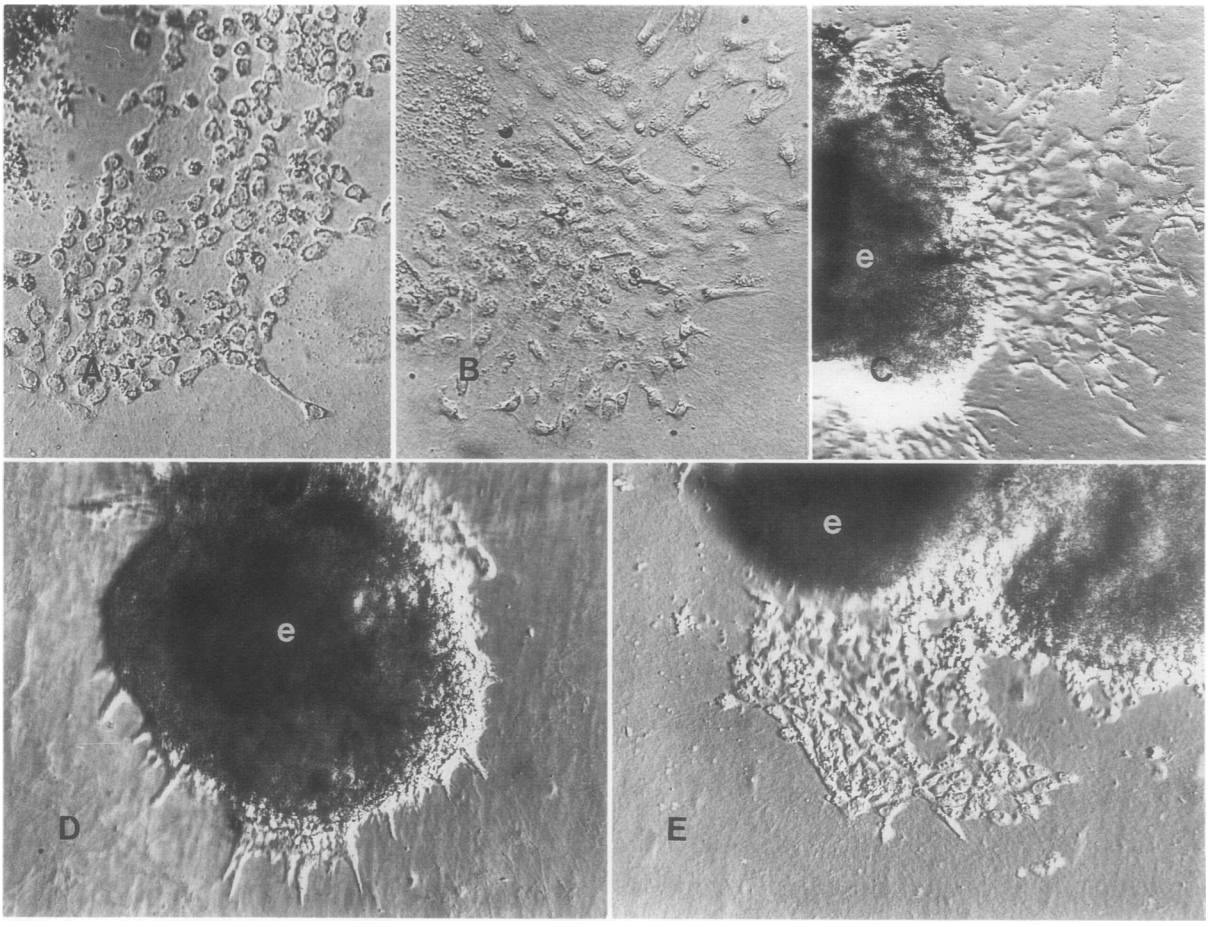

Figure 2. **Epithelial-mesenchymal cell transformation is blocked by kinase inhibitors.** Explants were left in place to provide the native stimulus for epithelial-mesenchymal cell transformation. (A) All cells remain endothelial in the presence of H-7 (20 nM) when it is added after endothelial outgrowth over the collagen gel surface has taken place. No transformed cells are found within the gel matrix. (B) Explant in the presence of 20 nM H-7 and 20 nM PMA. Outgrowth, but not invasion, had occurred before the addition of the inhibitors. H-7 blocks the effects of PMA seen in Figure 1B. (C) Explant treated with 100 pM staurosporine after endothelial outgrowth has occurred. This reagent also blocks normal mesenchymal cell formation. (D) Explants placed upon a gel containing 100 pM staurosporine are unable to produce an endothelial outgrowth. (E) Transformation, but not endothelial outgrowth, is blocked when explants are cultured on collagen gels containing genistein (19 μM).

monolayers were prepared by removal of the myocardial portion of the explants and 200 μl of conditioned medium from AV canal myocardial cells was added as a source of the stimulus. The inhibitors, staurosporine and genistein, were both effective in blocking cellular transformation when added concurrently with the conditioned medium (Table 2).

Endothelial cells respond to myocardial-conditioned medium with a specific increase in intracellular [Ca^{2+}]

The previously described experiments suggest that two or more protein kinase activities may be involved in the response of AV canal endothelial cells to the transforming stimulus. However, as each cardiac explant produces only a few hundred mesenchymal cells in a nonsynchronous manner, confirmatory biochemical analysis of phosphorylated proteins is difficult. PKC is a protein kinase which can be activated by both phospholipids and calcium (Nishizuka, 1984). Calcium activation may occur by an increase in $[Ca^{2+}]_i$ derived from either intracellular stores or calcium channel activation, but in either case, can be monitored within single cells by use of a calcium-sensitive fluoroprobe such as fura 2 (Grynkiewicz *et al.,* 1985). Thus, if signal transduction in the AV canal is mediated by

Table 1. Effects of inhibitors on cell transformation in AV canal cocultures

Inhibitor	Range tested	Minimum effective dose*	Toxic dose†
H-7	50–135 nM (20)‡	50 nM	—§
Staurosporine	10 pM–25 nM (47)	100 pM	5 nM
Genistein	2.5–190 μM (42)	19 μM	190 μM
Cholera toxin	1–100 nM (20)	—‖	—§
Pertussis toxin	0.9–45 nM (20)	9 nM	—§

* Dose required for inhibition of transformation.

† Dose required to stop myocardial cell contraction or cause release of explant from gel.

‡ Number of explants tested in parentheses.

§ Myocardial cells remained contractile at all dosages tested.

‖ Did not block cell transformation.

calcium as a second messenger, a specific change in $[Ca^{2+}]_i$ in response to the transforming stimulus should be visible.

Direct measurement of $[Ca^{2+}]_i$ was undertaken with the fluorescent probe, fura 2. As shown in Figure 3, the addition of conditioned media to competent AV canal endothelial cells caused an asynchronous increase in $[Ca^{2+}]_i$. The response shown by the two cells in Figure 3 is typical. One cell responded within 10 s to the stimulus and reached a peak by 60 s. The adjacent cell took 30–40 s to demonstrate an initial response and it appeared to plateau between 150 and 300 s. In one series of experiments, it was observed that $[Ca^{2+}]_i$ remained elevated at least 10 min after the addition of the conditioned medium. In contrast to the control experiments shown in Table 2, all AV canal cultures tested responded to the stimulus. This difference is most likely due to the concentration of the stimulus received (200 μl/1 ml gel vs. 200 μl/200 μl chamber). Further, all endothelial cells tested showed a response to the stimulus. Both the rate of response and the maximum level of $[Ca^{2+}]_i$ appeared to vary considerably from cell to cell.

A number of cells showed levels of intracellular $[Ca^{2+}]_i$ in the range of 1200–1500 nM within 30 s, whereas others took as long as 2 min before a response was evident. The maximum concentration of $[Ca^{2+}]_i$ obtained within each cell appeared to vary between 700 and 2000 nM under the conditions used for assay. The data shown in Figure 4 depict the mean increase in $[Ca^{2+}]_i$ measured in competent AV canal endothelial cells. The size of the standard errors shown reflects the asynchrony of the transformation process. When unconditioned media was added instead of conditioned media, no changes in $[Ca^{2+}]_i$ were observed (data not shown).

Since ventricular endothelia have previously been shown to be refractory to transformation by the myocardially derived stimulus (Mjaatvedt *et al.*, 1987), these cells were examined for a change in $[Ca^{2+}]_i$ when conditioned media was added. As shown in Figure 4, only a slight rise in $[Ca^{2+}]_i$ was observed during the 5-min period of analysis. A second control examined the change in $[Ca^{2+}]_i$ in AV canal endothelial cells that had previously been exposed to the myocardial stimulus. Endothelial cells left in the presence of the AV myocardium for 24 h were examined for response to the conditioned medium. As can be seen in Figures 3B and 4, these cells proved to be refractile to the addition of the transforming stimulus and demonstrated only a modest increase in $[Ca^{2+}]_i$ similar to that seen in the ventricular endothelia. The specificity of the AV canal endothelial response is consistent with the regional and temporal nature of cellular transformation previously observed both in vivo and in vitro.

Pertussis toxin, but not cholera toxin, blocks mesenchyme formation

Results obtained in the previous experiments suggest that common pathways of second messenger metabolism may be involved. One mechanism of signal transduction that is related

Table 2. Effects of inhibitors on conditioned medium stimulated cell transformation

Group*	Inhibitor conc.	n†	Percent transformed‡
Control	—	38	79
Staurosporine	1 nM	20	15§
Genistein	19 μM	19	3§
Pertussis toxin	9 nM	16	0§

* All explants received 200 μl of AV canal myocardially conditioned medium.

† Number of explants in group.

‡ Percentage of explants which responded to the addition of conditioned medium by producing invaded mesenchymal cells.

§ $P \ll 0.01$ when compared to control population by chi square analysis.

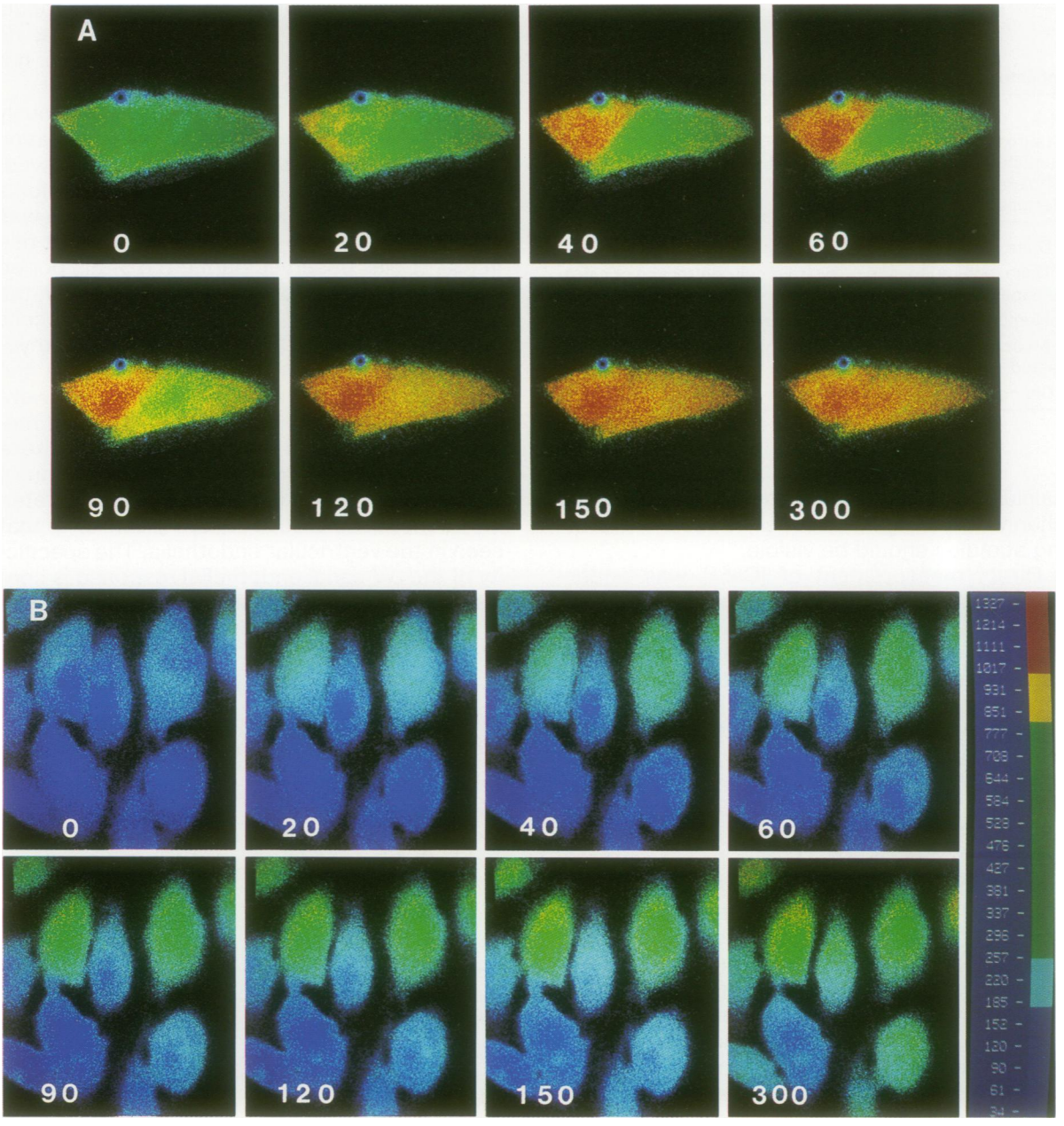

Figure 3. **Pseudocolor images of $[Ca^{2+}]_i$ as determined by fura-2 fluorescence.** (A) Series of photos taken of a pair of AV canal endothelial cells after the addition of an exogenous transforming stimulus. Cells respond asynchronously to the stimulus by an elevation of $[Ca^{2+}]_i$. (B) Elevation in $[Ca^{2+}]_i$ after the addition of stimulus to an endothelial outgrowth which was previously exposed to the myocardial stimulus overnight. A small response is seen in a subset of the cells. The cell-cell separation seen in this figure is characteristic of endothelial cells after activation. Time marked on pictures indicates seconds after the addition of the stimulus. The psuedocolor scale used in both sets of images is displayed to the right of B. Numbers on scale indicate $[Ca^{2+}]$ in nanomolars.

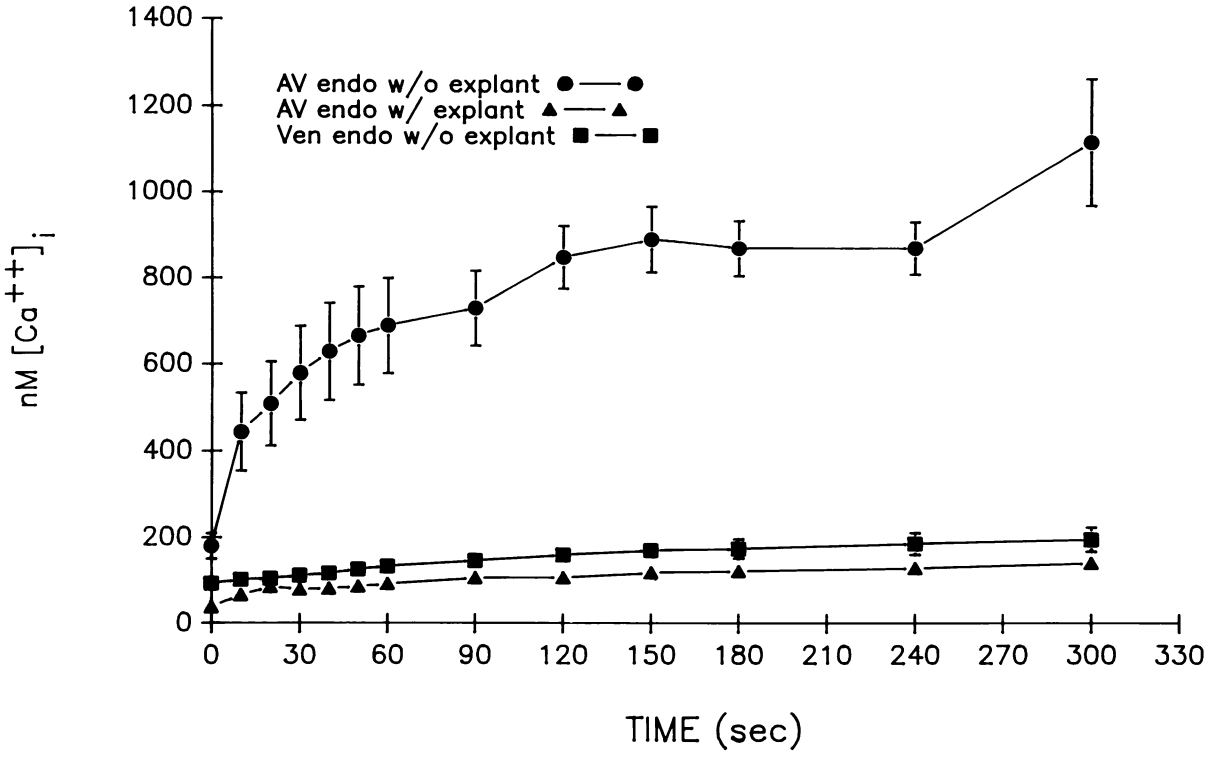

Figure 4. **Comparison of AV canal and ventricular responses to the myocardial stimulus for transformation.** Graph depicts average response of previously unstimulated AV canal cells (●, n = 27 cells), AV canal cells exposed to the myocardial stimulus during the previous night (▲, n = 41 cells), and ventricular endothelial cells (not capable of transformation and invasion) (■, n = 39 cells). Previously unstimulated (competent) AV cells respond to the conditioned medium in a clearly different manner than either the regionally or temporally incompetent cells. Error bars indicate standard error of the mean.

to both protein phosphorylation and intracellular calcium release is signal transduction by a regulatory GTP-binding protein (G protein) (Dillon *et al.,* 1988; Mitchell, 1989). The endotoxins, pertussis toxin (PT) and cholera toxin (CT), have been shown to be sensitive probes for the activity of specific members of the G protein family (Cockcroft, 1987; Okajima and Ui, 1984, Stryer and Bourne, 1986).

To determine whether signal transduction is mediated by similar G proteins in cardiac endothelia, segments of stage 14 chick embryo AV canals were explanted onto collagen gels containing various levels of PT and CT. When added to AV canal explants, PT completely prevented mesenchymal cell formation when present at concentrations > 1 ng/ml. The threshold level at which reduced mesenchymal cell formation took place was 0.2 ng/ml. Below this concentration mesenchymal cell formation was indistinguishable from controls. Figure 5 shows the complete prevention of mesenchymal cell formation seen at 1 ng/ml. As can be seen in this figure, endothelial outgrowth was unaffected by PT. As a comparison, CT was added to AV canal cultures in the same concentration ranges. Epithelial-mesenchymal cell transformation appeared identical to that seen in controls (Figure 5D). These data suggest that signal transduction of the transforming stimulus is likely to be mediated by a G protein mechanism sensitive to PT but not CT.

Discussion

The combination of experiments employed in the present study provide an outline of signal transduction by AV canal endothelia. Since direct biochemical analysis is precluded by the limited number of cells obtainable from each explant, we have relied upon the use of specific inhibitors and activators in combination with direct fluorometric analysis of $[Ca^{2+}]_i$. This approach demonstrates that the ECM-mediated tissue interaction seen in the AV canal utilizes effector mechanisms common to a variety of

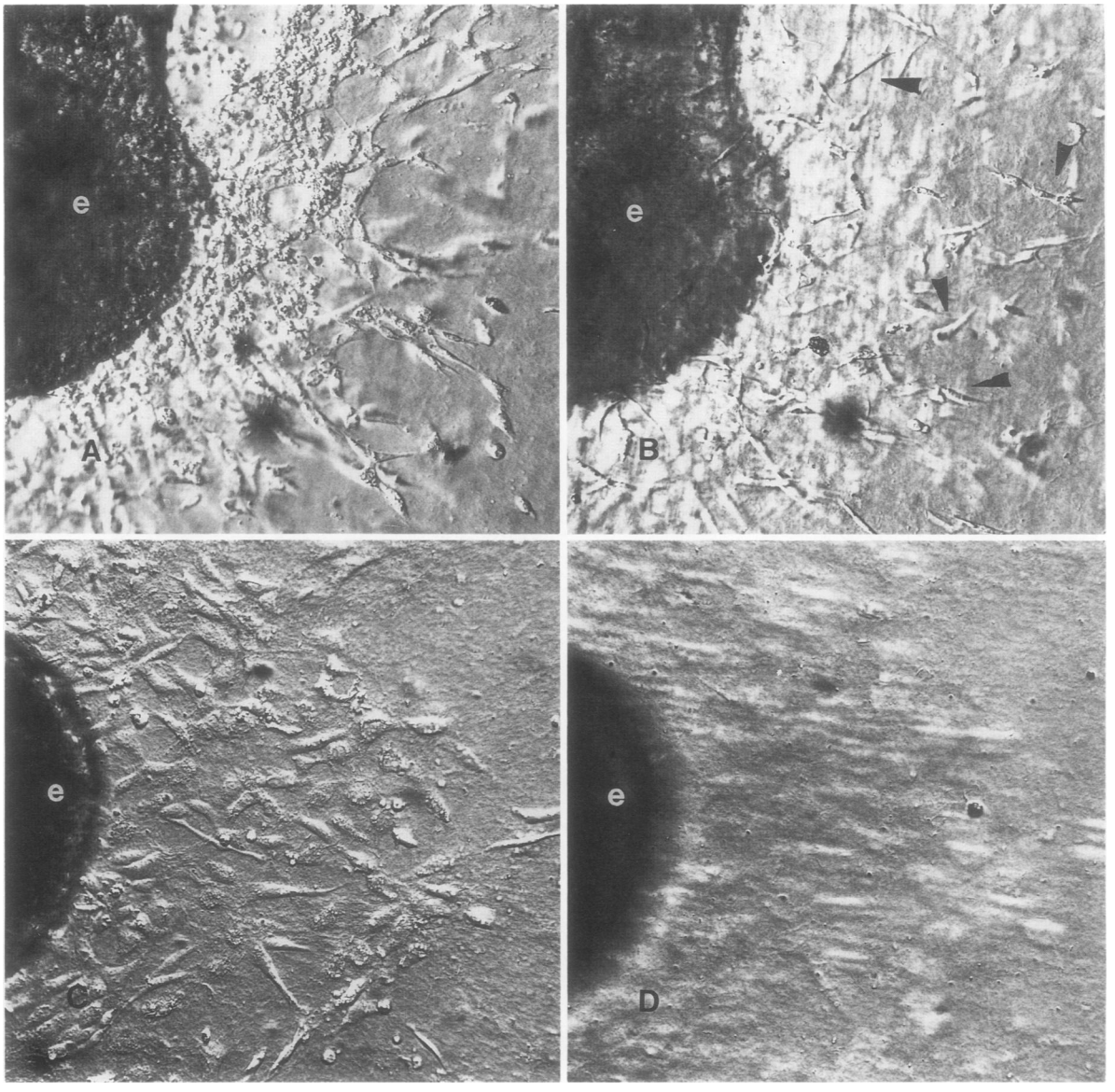

Figure 5. **Effect of bacterial endotoxins on transformation.** (A) Surface view of collagen gel culture after the addition of 10 nM cholera toxin. Normal endothelial outgrowth and cell-cell separation has occurred. (B) Same culture as in A showing mesenchymal cells which have been formed by transformation of the AV endothelium (arrowheads). Microscope is focused beneath the surface of the gel. (C) Surface view of collagen gel culture after the addition of 5 nM pertussis toxin. Endothelial outgrowth is normal. (D) Same culture as in C showing the lack of mesenchymal cells within the collagen gel. No mesenchymal cells are visible in this culture at any focal plane.

cells which respond to their environment (Dillon *et al.,* 1988; Mitchell, 1989).

Data obtained in the present study provide convincing evidence that the formation of mesenchyme in these cultures is a result, in part, of kinase activity. Although evidence points to activity of both PKC and tyrosine kinase in this process, their exact roles remain to be elucidated. PMA acts as an analogue of the intracellular metabolite diacylglycerol in activating PKC (Nishizuka, 1984). Mezerein, although not an analogue of diacylglycerol, competes with phorbol esters in binding to PKC (Jaken *et al.,* 1983; Miyake *et al.,* 1984). Since the biological effects of mezerein do not always correspond to those of phorbol esters (Jaken *et al.,* 1983), concordance in these studies suggests that it is their PKC-binding activities that are active in

the present study. The response of endothelial cells to both PMA and mezerein suggests that PKC activity is related to the shape change associated with transformation (Krug *et al.,* 1985). This is supported by the observation that the PKC inhibitor H-7 blocks the shape changing ability of PMA.

Since prior exposure to PMA will reduce the activity of PKC within cells (McArdle and Conn, 1989), PMA was added to cultures at the time of explants were added. Pretreatment with PMA prevented the normal outgrowth of endothelium from the explant. One explanation for this result is that the activity of PKC is required for endothelial migration. Data obtained by the use of kinase inhibitors was consistent with that obtained with PMA and mezerein. H-7 and staurosporine are primarily inhibitors of PKC (Kawamoto and Hidaka, 1984; Tamaoki *et al.,* 1986). Both of these reagents produced similar effects. When added at early times, they prevented endothelial outgrowth from the explant. When administered after outgrowth had taken place, they blocked the change in phenotype associated with epithelial-mesenchymal transformation. While it is possible that these responses are due to effects on molecules other than PKC, the similarity of response to H-7, staurosporine, sphingosine, and cremaphor EL suggests that PKC is the major substrate for these inhibitors.

This combination of activator and inhibitor data suggests that PKC is involved in the regulation of elements associated with both cell migration and the change of phenotype required for invasion. Other investigators have also described a role for PKC in an inductive tissue interaction (Otte *et al.,* 1988). In this case, they described a translocation of PKC activity which coincided with neural induction in the *Xenopus* embryo. The relationship between shape change and transformation seen in the present study leads to a suggestion that the role of PKC in cardiac tissue interaction is related to its effects on the cytoskeleton. This is consistent with previous studies suggesting PKC regulation of cytoskeletal organization (Shliwa *et al.,* 1984). However, since PKC has been demonstrated to exist as a family of proteins (Nishizuka, 1988), we can not rule out the possibility that different subspecies of PKC are involved in endothelial cell migration and mesenchymal transformation.

Genistein was used as a specific inhibitor of a different class of kinase activity. This reagent is reported to be a potent and specific inhibitor of tyrosine kinase activity with little activity towards PKC or protein kinase A (Akiyama *et al.,* 1987). The effect of this reagent was clearly different as it showed no inhibition of endothelial outgrowth from the AV canal explant. However, it was also effective at blocking epithelial-mesenchymal transformation by the endothelial cells. This suggests that tyrosine kinase activity may be involved in the process of transformation but not in cell migration. Since this class of kinases have been shown to mediate signal transduction by cell surface receptors (Mitchell, 1989), the data suggests that at least one component of the transformation stimulus acts through a similar mechanism.

The involvement of PKC activity in cell transformation prompted an investigation of the role of calcium in this cellular transformation. Intracellular calcium has been widely described as a second messenger and can act to regulate the activity of PKC (Nishizuka, 1988; Mitchell, 1989). In the present study, addition of conditioned medium to competent AV canal endothelial cells produced a rapid change in $[Ca^{2+}]_i$. Endothelia obtained from adjacent areas of the ventricle or from AV endothelia exposed to myocardial explants overnight, demonstrated only a small rise in $[Ca^{2+}]_i$. Thus, a regional specificity was conserved in the $[Ca^{2+}]_i$ response which corresponds to the ability of endothelia to transform in vitro and in vivo (Bernanke and Markwald, 1982; Runyan and Markwald, 1983; Mjaatvedt and Markwald, 1987). Conditioned medium was used because it provided a reliable way to synchronously introduce the invasive stimulus. Although a rise in intracellular calcium might be expected due to the presence of a growth factor or other substance found in the conditioned medium, the regional and temporal specificity of the response provides compelling evidence that stimulus for change in $[Ca^{2+}]_i$ is related to the stimulus for cell transformation. The small rise in $[Ca^{2+}]_i$ seen in the control cell populations of Figure 4 may be related to additional growth factors found in the conditioned medium. While PKC and $[Ca^{2+}]_i$ both appear to play a role in cellular transformation, the present study does not indicate how they are related. As the identity of the components of the ECM stimulus are further identified it will be possible to characterize the sequence of intracellular processes which result in transformation.

One finding of this study was that all competent AV canal cells responded to the addition of conditioned medium by an increase in $[Ca^{2+}]_i$. As shown by the cells in Figure 4 and the stan-

dard error bars on Figure 5, the response to the stimulus was variable in both timing and maximum level. A few cells responded with maximal levels of Ca^{2+} within 30 s, whereas others responded only after 2–3 min in the presence of conditioned medium. The maximal response in any one cell ranged from 700 to 2000 nM Ca^{2+}. Since, in vivo, some cells remain as luminal endothelia, it might have been expected that only a subset of cells would demonstrate a change in $[Ca^{2+}]_i$. However, data in previous studies are consistent with the idea that all AV canal endothelial cells undergo changes (hypertrophy, golgi polarization) consistent with activation (Bolender and Markwald, 1979; Krug *et al.,* 1985). It appears that only a subset of activated endothelial cells actually completes the process of cellular invasion. Further studies will address the question whether the rate or level of $[Ca^{2+}]_i$ response has any relationship to the fate of the cells.

Correspondence between kinase activity and a rise in $[Ca^{2+}]_i$ suggests a cellular pathway that involves the production of DAG and IP_3 from PIP_2. These metabolites are capable of both activation of PKC and stimulating a rise in $[Ca^{2+}]_i$ (Majerus *et al.,* 1985; Dillon *et al.,* 1988). One mechanism of signal transduction that stimulates the production these metabolites is the activation of a GTP-binding regulatory protein (Kikuchi *et al.,* 1986). The bacterial endotoxins, PT and CT, have proven useful in the analysis of signal transduction by G proteins. PT will ADP-ribosylate the catalytic a subunit of two types of G protein (Stryer and Bourne, 1986; Okajima and Ui, 1984). One, G_i, is inactivated by this toxin and becomes unable to inactivate adenylate cyclase, resulting in a rise in intracellular cAMP. The other G protein (called G_p, Cockcroft, 1987) when inactivated by PT, becomes ineffective in stimulating phosphotidylinositide dependent-phospholipase C (pi-PLC) (Cockcroft, 1987; Kelvin *et al.,* 1989). Inactivation of this pathway blocks the formation of polyphosphoinositide metabolites and subsequent changes in the level of $[Ca^{2+}]$ (Berridge and Irvine, 1984; Majerus *et al.,* 1985; Cockcroft, 1987). CT acts by ADP-ribosylation of the a subunit of G_s and produces an activation of adenylate cyclase (Stryer and Bourne, 1986). Since phenotypic transformation is blocked by PT, but not CT, it is likely that a G_p class of G protein is involved in signal transduction leading to phenotypic transformation.

Recently, we have shown that exogenous transforming growth factor beta (TGFβ), in combination with a cofactor provided by a ventricular explant, can induce transformation by AV canal endothelia (Potts and Runyan, 1989). This is consistent with other observations which suggest that members of the TGFβ family are active in morphogenetic roles during development (Weeks and Melton, 1987; Cate *et al.,* 1987; Rosa *et al.,* 1988). Preliminary evidence (Runyan, Sharma and Bhalla, unpublished observations) suggests that TGFβ does not, by itself, stimulate an increase in $[Ca^{2+}]_i$ in AV canal endothelial cells. However, TGFβ has been shown to modulate $[Ca^{2+}]_i$ and phosphotidylinositide metabolism stimulated by epidermal growth factor in Rat-1 cells (Muldoon *et al.,* 1988). We suspect that a member of the TGFβ family may have a similar facultative effect on another ECM component in the AV canal.

We hypothesize that tissue interaction in the developing heart is the product of a series of molecular interactions. The multifactorial stimulus found in the ECM may act upon several receptors on the AV canal surface. We suggest that one of these receptors is coupled to a G protein, whereas another may be, or may interact with, a tyrosine kinase. Subsequent activation of pi-PLC by the G protein produces metabolites which, in turn, activate PKC and release $[Ca^{2+}]_i$. Within a relatively short time, a subpopulation of activated cells assumes a mesenchymal cell phenotype, begins active migration, and produces characteristic membrane and ECM products such as have been described previously (Markwald *et al.,* 1984; Funderburg and Markwald, 1986; Sinning *et al.,* 1988). Our ongoing studies will specifically focus on the identification of the component (or components) of the myocardial stimulus which produce the flux in $[Ca^{2+}]_i$. Once this component has been identified, we will be in a position to further characterize the mechanisms of signal transduction outlined in the present study.

Materials and methods

Chemicals

Rat tail collagen was isolated as described (Bernanke and Markwald, 1982) from dissected rat tail tendons. Tissue culture medium M199, Tyrode's balanced salt solution, antibiotic-antimycotic solution, and chicken serum were obtained from Gibco (Grand Island, NY). ITS tissue culture supplement was obtained from Collaborative Research (Lexington, MA). All other chemicals were obtained from Sigma (St. Louis, MO) except fura 2 (Molecular Probes, Eugene, OR), genistein (ICN, Cleveland, OH), and staurosporine (Fluka, Ronkonoma, NY).

Tissue culture

Fertilized chicken eggs (Whelp Hatchery, Bancroft, IA or HyVac Laboratory Eggs, Gowrie, IA) were incubated at 38°C in a humidified incubator for 54–57 h (Stage 14) or 70–73 h (Stage 17). Upon collection, embryos were staged according to Hamburger and Hamilton (1956) and dissected from the surrounding membranes. The hearts were removed under aseptic conditions and the AV canal region was cut from the heart tube with iridectomy scissors. During collection, embryos and hearts were maintained in Tyrode's salt solution. Collected AV canal segments were then placed on the surface of collagen gels (1.6–1.8 mg of collagen/ml) that had been prepared as previously described (Bernanke and Markwald, 1982) in 35-mm tissue culture dishes (Corning, Oneonta, NY). Before placement of tissue on the gels, each gel was incubated with a medium made up of M199 with 1% chicken serum, ITS supplement (5 mg/ml insulin, 5 mg/ml transferrin, and 5 ng/ml selenium), and 1% antibiotic-antimycotic solution for at least 1 h. Excess medium was decanted just before the addition of the explants to allow them to attach to the collagen. Cardiac explant cultures were subsequently incubated at 37°C in a 5% CO_2 atmosphere until examined and photographed.

Bioassay for epithelial-mesenchymal transformation

The bioassay for epithelial-mesenchymal cell transformation has been described in detail elsewhere (Runyan and Markwald, 1983; Markwald *et al.*, 1984; Krug *et al.*, 1985, 1987; Mjaatvedt *et al.*, 1987). Briefly, explants of AV canal tissue from stage 14 chick embryo hearts are placed upon the surface of hydrated collagen gels. When desired, the myocardial portion of the explant is removed from the surface of the gel with fine forceps between 10 and 12 h after the start of incubation. This leaves an endothelial monolayer on the surface of the gel which is "competent" to respond to a transforming stimulus added at the time of myocardium removal. In the absence of the myocardium, the transforming stimulus was provided by the addition of myocardial conditioned medium (described below). Inhibitory reagents were tested in both the presence of the myocardial explant and conditioned medium. Completion of epithelial-mesenchymal transformation was assessed by "optically sectioning" the collagen gel, using Hoffman Modulation Optics, to observe invaded mesenchyme clearly within the matrix of the gel. Such observation is carried out between 20 and 24 h after the addition of a treatment (~32–36 h after the start of each stage 14 culture). For each experiment, three to five explants were observed for each treatment and each of the experiments reported here was repeated at least three times. In addition to recording the outcome of each experiment, representative explants were photographed at the gel surface and at a level within the gel that showed the maximum number of invaded mesenchymal cells.

Production of myocardially-conditioned medium

Conditioned medium from cultures of AV canal myocardial cells was recently shown to be able to induce the formation of mesenchyme from competent AV canal endothelia (Sinning *et al.*, 1988). A similar preparation of conditioned medium was prepared for this study. Briefly, 96 embryo hearts were collected from stage 16–17 embryos, and dissociated in 0.01% Trypsin and 5 mM EDTA in a calcium- and magnesium-free salt solution. After repeated trituration, the dissociated cardiac cells were centrifuged in a test tube containing 10 ml of medium 199 with 10% chicken serum. The pellet was then resuspended in medium 199 with 1% chicken serum and insulin, transferrin, and selenium as described above. The cells were then dispensed into two T25 culture flasks and enough media was added to cover the bottom of each flask (~5 ml). After 3 d the medium was replaced and at 6 d the medium was removed, rinsed with medium without serum, and then incubated for 2 h with additional medium without serum. After 2 h in serum-free medium, the medium was decanted and saved and fresh medium containing 1% chick serum was added. This procedure was repeated the next day and the conditioned serum-free medium from each of the 2 h incubations was pooled, aliquoted into 1-ml aliquots and frozen. The ability of the medium to induce mesenchymal cell formation in collagen gels was assessed by adding 1 ml of conditioned medium to each of three collagen gels containing three AV endothelial monolayers. All nine monolayers produced mesenchyme, whereas controls using unconditioned media remained endothelial.

Measurement of intracellular [Ca^{2+}]

Endothelial monolayers were prepared for $[Ca^{2+}]_i$ measurement by placing a small volume (200 μl) of gelling collagen solution on cleaned 25 mm glass cover slips. These gels were conditioned in tissue culture medium for 1 h, and then either AV canal or ventricular explants (stage 14) were placed upon the surface of the gels. Uninduced competent monolayers were produced by removing the myocardial portion of AV canal explants after 12 h. Myocardium from ventricular explants was removed at the same time. Additional medium was added to the cultures once attachment of the endothelial cells had taken place (8–12 h). Approximately 22 h after initiation of the explant cultures, the cover slips were placed in Dulbecco's modified Eagle's medium containing 1 mg/ml bovine serum albumin and 2–6 μM fura 2/AM for 2 h at 37°C, 5% CO_2. Subsequently, the coverslips were rinsed with a HEPES-buffered saline solution (140 mM NaCl, 5 mM KCl, 1.2 mM Mg, 1.2 mM $NaHPO_4$, 1.6 mM $CaCl_2$, 11 mM glucose, 1 mg/ml BSA, 25 mM HEPES, and 0.027 mM EDTA, pH 7.4) and examined in a digital image analysis apparatus. A detailed description of the apparatus and analysis procedures can be found in Sharma and Bhalla (1989). Briefly, the apparatus is designed to produce ratio images of fluorescent intensity after monochromatic illumination (340–380 nm). The dye, fura 2, shows an increase of fluorescent intensity in its ratio image in relation to the increase in the local calcium concentration. Image intensity is converted to $[Ca^{2+}]_i$ by computer and the images are displayed in a psuedocolor output to visualize the spatial distribution of $[Ca^{2+}]_i$ in the cells. A series of 13 time points (over 5 min) after the addition of myocardially conditioned medium (200 μl) were collected for one field on each cover slip. Images were obtained on a population of 2–15 cells for each experimental run and data was collected for each treatment on at least three separate occasions.

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