Co-culture of embryonic chick heart cells and ciliary ganglia induces parasympathetic responsiveness in embryonic chick heart cells

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We have developed a system for the co-culture of embryonic chick heart cells obtained from embryos at 3.5 days *in ovo* with ciliary ganglia from chick embryos at 7 days *in vivo*. After 3 days of co-culture, removal of the ciliary ganglia resulted in complete degeneration of axons within 6–8 h, leaving the post-innervated heart cell culture devoid of neurons. Embryonic chick heart cells at 3.5 days *in ovo* are unresponsive to muscarinic stimulation. However, following 3 days of co-culture with ciliary ganglia, the heart cells developed a negative chronotropic response to muscarinic stimulation (paired *t* test, $P < 0.02$) which persisted for at least 24 h after removal of the ciliary ganglion. The development of muscarinic responsiveness was associated with an increase in the levels of specific α -subunits of the guanine nucleotide binding proteins (G-proteins), with a 3-fold increase in the level of α_{39}

(39 kDa subunit) and a 2.5-fold increase in the level of α_{41} . The level of the G-protein subunit α_s remained unchanged. Culture of embryonic chick heart cells at 3.5 days *in ovo* with medium conditioned by the growth of embryonic chick heart cells and ciliary ganglia had an effect on the chronotropic response to muscarinic stimulation and on α_{39} and α_{41} levels identical to that of co-culture. These data suggest that a soluble factor released during the co-culture of embryonic chick heart cells and ciliary ganglia is capable of inducing muscarinic responsiveness. These studies suggest that innervation of the heart may induce parasympathetic responsiveness by increasing the availability of G-proteins which couple the muscarinic receptor to a physiological response.

INTRODUCTION

We have previously demonstrated that, early in the embryonic development of the chick heart, the appearance of several GTP-dependent muscarinic cholinergic functions [1–3] is associated with an increase in the levels of the G-protein α -subunits α_{39} and α_{41} (of 39 and 41 kDa respectively) [1]. Thus, while the muscarinic receptor number remained constant, muscarinic inhibition of isoprenaline-stimulated adenylate cyclase activity [1] and muscarinic stimulation of ^{42}K efflux [4] increased between days 2.5 and 10 *in ovo*, in parallel with a 1.5–3-fold increase in the levels of α_{39} and α_{41} [1]. Similarly, a pertussis toxin-sensitive muscarinic stimulation of inositol phosphate production increased from undetectable levels in cultured embryonic chick atrial cells from hearts at 5 days *in ovo* to 40% of the total carbamylcholine-stimulated inositol phosphate production at day 14 *in ovo* [2,5]. One interpretation of these data is that, early in embryonic development, muscarinic responsiveness might be limited by the availability of G-proteins.

During the embryonic development of the chick heart, vagal fibres reach the atria by the end of day 4 *in ovo* and the intra-atrial septum on day 5 *in ovo*, and enter the sinoatrial node and atrial walls at days 6–7 *in ovo* [6,7]. The finding that the time courses of vagal innervation of the heart and development of a physiological response to muscarinic stimulation were similar suggested that ingrowth of the vagus nerve might be an important signal for the development of parasympathetic responsiveness. In the present studies we tested the hypothesis that co-culture of embryonic chick heart cells with ciliary ganglia, or incubation of medium conditioned by growth of embryonic chick heart cells with ciliary ganglia, results in increased physiological responsiveness to muscarinic stimulation, mediated at least in part by the induction of increased levels of G-proteins.

MATERIALS AND METHODS

Materials

^{125}I -labelled Staphylococcal Protein A was from Amersham, Arlington Heights, IL, U.S.A. [^{32}P]NAD $^{+}$ was from New England Nuclear, Boston, MA, U.S.A. Antibody to the α_s subunit of the G-protein was a gift from A. Spiegel, National Institutes of Health, Bethesda, MD, U.S.A. Embryonated chicken eggs were from Spafas Inc. (Norwich, CT, U.S.A.).

Co-culture of ciliary ganglia with embryonic chick heart cells

Ciliary ganglia were removed from embryonic chicks at 7 days *in ovo* and 10–12 ganglia were placed directly into 35 mm culture dishes, or on to 12 mm glass coverslips in 35 mm culture dishes. Ciliary ganglia were chosen because they maintain parasympathetic character in culture [8] and few of the neurons migrate out of the explant [9], allowing the ganglion to be easily removed. Ganglia from embryos at 7 days *in ovo* were chosen to maximize the number of neurons, since between days 8 and 15 *in ovo*, 50% of the neurons in a ganglion die [10,11]. The ciliary ganglia were covered with a small volume of M199 medium supplemented with 10% chick embryo extract and placed in a 37 °C/5% CO_2 incubator for 4–6 h. Chick hearts at 3.5 days *in ovo* (Hamburger and Hamilton stages 19–21 [12]) were removed and subjected to successive trypsinization with 0.12 mg/ml trypsin by a modification of the method described previously [5]. After successive incubations with trypsin, suspensions of cells were sedimented at 650 *g* for 10 min [5]. The cells were resuspended in M199 medium supplemented with 6% fetal calf serum and adjusted to 4×10^5 cells per ml. The medium bathing the ciliary ganglia was completely removed and replaced with 2 ml of medium containing heart cells. On the third day in culture, one

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half of the medium was replaced and the ganglia were removed. Conditioned medium was prepared by the growth of embryonic chick heart cells from embryos at 3.5 days *in ovo* in the presence or absence of ciliary ganglia for 3 days. Freshly dispersed embryonic chick heart cells from embryos at 3.5 days *in ovo* were suspended in medium consisting of 50 % fresh medium and 50 % conditioned medium. After 3 days in culture, one half of the medium was replaced, and 24 hours later the cells were assayed.

Measurements of changes in beating rate

Beating was determined as described previously [13,14]. In this analysis each cell served as its own control, with the spontaneous cell beat rate determined before and after exposure to 0.1 mM carbamylcholine. Each mean value was obtained from multiple cells studied in separate cultures. Data from contractility studies were compared by paired *t* tests as indicated in the text or Figure legends.

Measurement of G-protein α -subunits

Pertussis toxin-catalysed ADP-ribosylation was carried out as described [1,2]. Western blotting was carried out by a modification of the method of Towbin et al. as described previously [14,15].

Determination of GTP levels

Embryonic chick heart cells from embryos at 3.5 days *in ovo* were cultured in 35 mm culture dishes in the presence or absence of ciliary ganglia as described. After 3 days in culture, the ciliary ganglia were removed, one half of the medium was replaced, and cells were continued in culture for another day to allow the neuronal processes to degenerate. On the fourth day in culture, GTP levels were determined as described [16].

RESULTS

Co-culture of ciliary ganglia and embryonic chick heart cells

Embryonic chick heart cells from hearts of embryos at 3.5 days *in ovo* were co-cultured with ciliary ganglia from embryos at 7 days *in ovo* as described in the Materials and methods section. A photomicrograph of a typical co-culture is shown in Figure 1(a). After 3 days in culture numerous axonal processes, many of several hundred μm in length, can be seen emanating from the ciliary ganglion. The processes grow out in close apposition to heart cells, and appear to end on the heart cell surface and to move in unison with the beating heart cells they contact. Both the heart cells and the ciliary ganglia are stable for as long as 10 days in culture provided that the medium is changed at 3 day intervals. However, if ciliary ganglia are not removed by day 7 or 8 in culture, fibroblasts from the ganglia may begin to overgrow the culture.

Figure 1(b) is a photomicrograph of the same co-culture as in Figure 1(a) immediately following removal of the ciliary ganglion. Some axonal processes may still be seen on the lawn of heart cells. These processes degenerate within 6–8 h following removal of ciliary ganglia; at 24 h following the removal of the ganglia, no axonal projections or fragments of ciliary ganglia could be detected (Figure 1c).

Effect of co-culture of ciliary ganglia and embryonic chick heart cells 3.5 days *in ovo* on the chronotropic response of heart cells to carbamylcholine

The effect of 0.1 mM carbamylcholine on the spontaneous beat rate of embryonic chick heart cells 3.5 days *in ovo* cultured in the

Figure 1 Co-culture of ciliary ganglia and embryonic chick heart cells

(a) Co-culture of heart cells from embryos at 3.5 days *in ovo* with ciliary ganglia; (b) the same culture immediately after removal of the ciliary ganglion; (c) the same cells 24 h following the removal of the ciliary ganglion.

absence (control) or presence of ciliary ganglia was determined after 3 days in culture as described in the Materials and methods section. Of the 21 control cells studied, the beat rate in 13 was either unchanged or increased, while eight decreased their beat rate (Figure 2a). Paired *t* test analysis revealed no significant effect of carbamylcholine on beat rate ($n = 21$). No significant response of the beat rate to carbamylcholine could be demonstrated in this control group even when cultures were maintained for up to 9 days. Of the 18 cells studied after culture in the presence of ciliary ganglia, 15 decreased their beat rate in response to carbamylcholine (Figure 2b). Paired *t* test analysis revealed a

Figure 2 Co-culture of embryonic chick heart cells 3.5 days *in ovo* with ciliary ganglia induces a negative chronotropic response to carbamylcholine

The response to 0.1 mM carbamylcholine of (a) control cells ($n = 21$) and (b) cells co-cultured with ciliary ganglia ($n = 18$) is shown. Paired t tests indicated a significant effect ($P < 0.02$) of carbamylcholine on the beat rate of the cells in co-culture.

significant effect of carbamylcholine on spontaneous beat rate ($P < 0.02$, $n = 18$).

In order to determine whether these effects of co-culture required the continued presence of ciliary ganglia, cells were cultured for 3 days and ganglia removed; 24 h later, the effect of 0.1 mM carbamylcholine on the beat rate was determined. Of the 10 control cells studied, paired t test analysis revealed no significant effect of carbamylcholine on beat rate ($n = 10$). Of the 11 cells studied after culture in the presence of ciliary ganglia, nine decreased their beat rate in response to carbamylcholine. Paired t test analysis revealed a significant effect of carbamylcholine on spontaneous beat rate ($P < 0.04$, $n = 11$).

Effect of co-culture of ciliary ganglia and embryonic chick heart cells 3.5 days *in ovo* on the levels of the G-protein α subunits α_{39} , α_{41} and α_s

In order to determine whether the negative chronotropic response induced by co-culture of embryonic chick heart cells 3.5 days *in ovo* with ciliary ganglia was associated with an increase in the levels of α_{39} and α_{41} , embryonic chick heart cells 3.5 days *in ovo* were cultured in the presence or absence of ciliary ganglia as described in the Materials and methods section. After 3 days in culture, the ciliary ganglia were removed and one-half of the medium was replaced. On the 4th day in culture, cells were harvested and the levels of α_{39} and α_{41} determined by ADP-ribosylation with pertussis toxin in the presence of [32 P]NAD $^{+}$ as described in the Materials and methods section. Co-culture with ciliary ganglia increased the levels of α_{39} and α_{41} in chick heart

Figure 3 Effect of co-culture of embryonic chick heart cells 3.5 days *in ovo* with ciliary ganglia on the levels of G-protein α -subunits

(a) Typical autoradiograph of a pertussis toxin-catalysed ADP-ribosylation of homogenates of control cells incubated in the presence of [32 P]NAD $^{+}$ and in the absence of pertussis toxin (lane 1) or in the presence of pertussis toxin and [32 P]NAD (lane 2). Lane 3 is a homogenate of heart cells previously co-cultured with ciliary ganglia and incubated in the presence of pertussis toxin and [32 P]NAD $^{+}$. (b) A typical immunoblot of α_s . Lane 1 is a homogenate of control heart cells; lane 2 is a homogenate of heart cells cultured in the presence of ciliary ganglia. No significant differences in the levels of the α_{52} or α_{42} bands were noted ($n = 4$).

cells by 3-fold and 2.5-fold respectively when data were normalized to values for α_{39} and α_{41} in control heart cell cultures (Figures 3a, lane 3). Data are typical of six experiments.

In order to determine whether the effects of co-culture were specific for changes in the levels of the G-protein subunits α_{39} and α_{41} , immunoblotting studies were carried out using specific antibody to the G-protein subunit α_s , as described in the Materials and methods section. A typical blot shown in Figure 3(b) demonstrates bands at 42 and 52 kDa. In contrast to our previous studies in embryonic chick heart cell cultures of atria 14 days *in ovo*, no band could be seen at 45 kDa [17]. No significant difference in band intensity was noted between cells cultured in the absence and presence of ciliary ganglia ($n = 4$).

Effect of co-culture of ciliary ganglia and embryonic chick heart cells 3.5 days *in ovo* on the levels of intracellular GTP

Another possible explanation for enhanced responsiveness of embryonic chick heart cells 3.5 days *in ovo* to muscarinic stimulation is that prior to co-culture GTP levels may be inadequate to fully activate the G-proteins present and that co-culture results in elevated GTP levels. Since the negative chronotropic response to muscarinic stimulation involves coupling of the muscarinic receptor to K $^{+}$ channels, which is a GTP-dependent process [18,19], we studied the effect of co-culture on

Figure 4 Conditioned medium induces a negative chronotropic response to carbamylcholine in embryonic chick heart cells 3.5 days *in ovo*

(a) Response to 0.1 mM carbamylcholine of embryonic chick heart cells 3.5 days *in ovo* cultured in medium conditioned by prior growth of heart cells. (b) Response to 0.1 mM carbamylcholine of embryonic chick heart cells 3.5 days *in ovo* in medium conditioned by prior growth of heart cells and ciliary ganglia. Paired *t* tests indicate a significant effect ($P < 0.02$) of carbamylcholine on the beat rate of cells in medium conditioned by prior growth of heart cells and ciliary ganglia.

Figure 5 Effect of medium conditioned by co-culture of embryonic chick heart cells 3.5 days *in ovo* with ciliary ganglia on the levels of the G-protein subunits α_{39} and α_{41}

Typical autoradiograph of [32 P]NAD $^{+}$ /pertussis toxin-catalysed ADP-ribosylation of homogenates of embryonic chick heart cells 3.5 days *in ovo* cultured for 4 days in the presence or absence of conditioned medium. Lane 1, homogenate of heart cells cultured in medium conditioned by heart cells alone; lane 2, homogenate of heart cells incubated with medium conditioned by growth of heart cells and ciliary ganglia.

GTP levels. GTP levels were measured as described in the Materials and methods section and were not significantly different (control cells 4.8 ± 0.2 nmol/mg of protein, mean $\pm$ S.E.M.,

$n = 5$; cells co-cultured with ciliary ganglia, 5.2 ± 0.3 nmol/mg of protein, $n = 5$).

Effect of incubation with medium conditioned by prior growth of ciliary ganglia and embryonic chick heart cells 3.5 days *in ovo* on the chronotropic response of heart cells to carbamylcholine

Experiments were carried out to determine whether a soluble factor released during co-culture of ciliary ganglia and embryonic chick heart cells 3.5 days *in ovo* was responsible for the induction of the negative chronotropic response to muscarinic stimulation. Of the 10 cells studied after culture in medium conditioned by heart cells alone, three decreased their beat rate while the beat rate in the remaining cells either increased or was unchanged. Paired *t* test analysis revealed no significant effect of carbamylcholine on beat rate ($n = 10$; Figure 4a). Of the six cells studied after culture in medium conditioned by both heart cells and ciliary ganglia, all six decreased their beat rate (Figure 4b). Paired *t* test analysis revealed a significant effect of carbamylcholine on beat rate ($P < 0.02$, $n = 6$).

Effect of conditioned medium on the levels of α_{39} and α_{41} in embryonic chick heart cells 3.5 days *in ovo*

Experiments similar to those in Figure 3(a) demonstrated that growth of embryonic chick heart cells 3.5 days *in ovo* in medium conditioned by co-culture of heart cells and ciliary ganglia resulted in a significant increase in both α_{39} and α_{41} when compared with cells grown in medium conditioned by heart cells alone (Figure 5). Quantification by laser densitometry of eight similar autoradiographs demonstrated that growth in conditioned medium increased the levels of α_{39} and α_{41} by 2.3-fold and 2.0-fold respectively compared with the levels in control cells.

DISCUSSION

In this model, the use of ciliary ganglia grown as explants on a lawn of embryonic chick heart cells is unique, since this allows the intact ganglia to be easily removed from the surrounding lawn of heart cells. This model further allows biochemical analyses to be performed on post-innervated cultures of heart cells in the absence of neurons and permits the study of the reversibility of both the physiological and biochemical effects of *in vitro* innervation. Other systems for the study of heart-cell-neuron interactions have used a mixed culture of dispersed cardiocytes and neurons. Steinberg et al. [20] have reported that co-culture of neonatal rat ventricular cardiocytes with sympathetic ganglia resulted in the appearance of a negative inotropic response to α -adrenergic stimulation. This group also suggested that co-culture mediated an increase in pertussis toxin substrate in the heart cells. However, individual neurons could not be removed from the cultures of the cardiocytes. Since co-culture of neurons with muscle cells also results in differentiation and maturation of neurons [21,22], it is impossible to determine whether the increases in pertussis toxin substrates reported by Steinberg [20] can be attributed to changes in cardiocytes, changes in the neurons, or both. Marvin et al. [23] demonstrated that co-culture of both sympathetic and parasympathetic ganglia with rat heart cells resulted in an increase in the chronotropic and inotropic responses of heart cells to sympathetic and parasympathetic agonists. No measurement of biochemical parameters associated with the reported changes in physiology during co-culture were made.

The development of a muscarinic response requires interaction

between the muscarinic receptor, a G-protein and effector molecules such as the catalytic subunit of adenylate cyclase, phospholipase C or an acetylcholine-sensitive K^+ channel. The absence of a physiological response to muscarinic stimulation at day 3.5 *in ovo* could be due to inadequate levels of any of these components. The lack of sufficient tissue from the co-culture system has made radioligand binding studies of muscarinic receptor number impractical. The finding that the number of muscarinic receptors in homogenates of whole hearts does not change over the time course of the embryonic development of muscarinic responsiveness (between 2.5 and 10 days *in ovo*) suggests that changes in muscarinic receptor number do not play a role in the development of muscarinic responsiveness [1]. Furthermore, whole-cell clamp studies of acetylcholine-stimulated K^+ current density following co-culture demonstrated no change in current density, suggesting that the number of K^+ channels did not change during co-culture [24]. This suggests that G-proteins are the limiting component of the system.

The finding that changes in G-protein levels occur in parallel with the embryonic development of muscarinic responsiveness suggests that changes in the level of pertussis toxin substrates play a critical role during the *in ovo* development of the chick heart [25]. The conclusion that levels of G-protein α -subunits are regulatable and may be limiting in the heart is further supported by studies which suggest that G-protein levels are critical for regulating the level of muscarinic or β -adrenergic response in the embryonic chick heart. Thus, during growth of embryonic chick atrial cells from hearts of embryos at 14 days *in ovo* in medium supplemented with lipoprotein-depleted serum, levels of α_o and α_i increased more than 2-fold while levels of α_s decreased by 3-fold. This occurred in parallel with a 10-fold increased sensitivity of beating to carbamylcholine and the disappearance of the positive inotropic response to isoprenaline [14,17]. The parallels between developmental changes in muscarinic responsiveness and G-proteins during the *in ovo* development of the heart [1], and similar changes seen following co-culture described in these studies, suggest that levels of G-proteins play a crucial role in regulating the physiological responsiveness to muscarinic stimulation and that innervation of the heart may be a signal regulating G-protein levels.

The observation that medium conditioned by prior incubation with embryonic chick heart cells and ciliary ganglia has the same effect as co-culture on chronotropic response and levels of α_{39} and α_{41} suggests that a soluble factor is responsible for the induction of muscarinic responsiveness in heart cells by inducing G-protein expression. Thus, during the normal *in ovo* development of the heart, as the vagus nerve is entering into atrial tissue at around day 4 *in ovo*, the neural fibres may be releasing a substance which induces surrounding cardiocytes to develop the phenotypic muscarinic responsiveness of atria. This occurs well

before functional vagal neurotransmission occurs in the heart [6]. Therefore our system for co-culture of parasympathetic neurons with embryonic chick heart cells cultured from hearts before ingrowth of the vagus nerve may serve as a model to study the role of innervation in the differentiation of cardiac tissues.

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