Development of muscarinic-cholinergic stimulation of inositol phosphate production in cultured embryonic chick atrial cells

Evidence for a switch in guanine-nucleotide-binding protein coupling

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We have demonstrated that muscarinic stimulation of inositol phosphate production in cultured atrial cells from chicks at 14 days *in ovo* is partially sensitive to inhibition by pertussis toxin. In these cells, muscarinic agonist binding is coupled to phospholipase C activity via at least two guanine-nucleotide-binding proteins (G-proteins), one sensitive to pertussis toxin and the other (G_p) insensitive to pertussis toxin [Barnett, Shamah, Lassegue, Griendling & Galper (1990) *Biochem. J.* 271, 437–442]. In the current study we demonstrate that during embryonic development of the chick heart, muscarinic stimulation of inositol phosphate production decreases by 50% between days 5 and 14 *in ovo* in cells cultured from both atrium and ventricle. In atrial cells, however, pertussis toxin-sensitive muscarinic stimulation of inositol phosphate production increased from undetectable levels at day 5 *in ovo* to 40% of total stimulation at day 12 *in ovo*. Muscarinic stimulation of inositol phosphate production in the ventricle did not become sensitive to pertussis toxin at any age studied. In permeabilized atrial cells from embryonic chicks at 5 days *in ovo*, guanosine 5'-[γ -thio]triphosphate (GTP[S]) stimulated $InsP_1$ levels by $40 \pm 10\%$ (mean $\pm$ S.E.M., $n = 3$), $InsP_2$ levels by $117 \pm 18\%$ and $InsP_3$ levels by $51 \pm 8\%$, suggesting that at day 5 *in ovo* all of the muscarinic-stimulated inositol phosphate production was coupled to phospholipase C via G_p . H.p.l.c. analysis demonstrated that, in spite of these changes in coupling of phospholipase C to different G-proteins, no changes could be demonstrated in the isomers of $InsP_3$ produced in response to carbamylcholine at both days 5 and 14 *in ovo*. These data demonstrate that embryonic development of the chick atrium is associated with a switch in coupling of muscarinic receptors to phospholipase C from G_p to a pertussis toxin substrate. This developmental switch in coupling of G-proteins may be related to possible developmental switches in levels of muscarinic receptor isoforms or switches in the subtype of phospholipase C.

INTRODUCTION

Parasympathetic responsiveness in the embryonic chick heart earlier than day 4 *in ovo* is either absent or markedly decreased compared with the response in hearts after 12 days *in ovo* [1–3]. Thus the negative chronotropic response to muscarinic stimulation which is associated with activation of muscarinic-gated K^+ current [4–6] and the muscarinic-mediated inhibition of adenylate cyclase activity are markedly decreased at days 2.5–4 *in ovo* compared with responses in hearts at 12 days *in ovo* [7]. Both the negative chronotropic response [5,6] and the inhibition of adenylate cyclase activity are coupled to muscarinic receptors via a pertussis toxin-sensitive guanine-nucleotide-binding protein (G-protein) [8]. G-proteins are heterotrimers composed of α , β and γ subunits [8–10]. The α subunits of these heterotrimers consist of a family of proteins, many of which have been shown to be substrates for ADP-ribosylation by NAD^+ in the presence of pertussis toxin. At least two of these G-proteins, with molecular masses of 39 kDa (α_0) and 41 kDa (α_1) have been identified in homogenates of embryonic chick atrial cells [8]. Pertussis toxin substrates which migrate on a one-dimensional gel with an electrophoretic mobility of 40–41 kDa have been shown to be composed of a family of at least three G-proteins, designated α_{1-1} , α_{1-2} and α_{1-3} [11].

We have previously demonstrated that muscarinic receptor number does not change in embryonic chick hearts between days

2.5 and 10 *in ovo* [3]. In contrast, at days 3.5–4 *in ovo*, guanosine 5'-[$\beta\gamma$ -imido]triphosphate (Gpp[NH]p), a non-hydrolysable GTP analogue, had no significant effect on the affinity of muscarinic receptors for agonist binding [12], suggesting that at this stage of development these receptors were not coupled to a G-protein. However, by day 10 *in ovo*, the affinity of muscarinic receptors for carbamylcholine had decreased by 100-fold in the presence of Gpp[NH]p [12]. These data suggest that between days 2.5 and 10 *in ovo*, an interaction of muscarinic receptors with G-proteins develops. Whether this interaction is a result of an increase in expression of the pertinent G-proteins, or rather of an enhanced association of the receptor with existing G-proteins, is unclear. It has been shown that levels of α_0 in chick heart increased 1.7-fold between days 2.5 and 3.5 *in ovo*, and 1.6-fold between days 3.5 and 10 *in ovo* [7]. Levels of α_1 also increased over this time course [7]. These increases in levels of G-proteins were accompanied by an increase in the coupling of muscarinic receptor stimulation both to a negative chronotropic effect and to inhibition of adenylate cyclase activity [3,8]. These data suggest that the development of muscarinic responsiveness might be associated with an increase in the levels of either α_0 or an isoform of α_1 , or with the appearance of an isoform of α_1 not previously expressed. Muscarinic stimulation of the chick heart also results in an increase in phospholipase C activity, with a corresponding accumulation of inositol phosphates [13,14]. In cultures of

Abbreviations used: G-protein, guanine-nucleotide-binding protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; Gpp[NH]p, guanosine 5'-[$\beta\gamma$ -imido]triphosphate.

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embryonic chick ventricular cells, inositol phosphate production is mediated by a pertussis toxin-insensitive G-protein [15]. In contrast, in the embryonic chick atrial cell, muscarinic receptor-stimulated inositol phosphate production appears to be mediated by at least two G-proteins, one sensitive and the other (G_p) insensitive to pertussis toxin [13]. The finding that other muscarinic functions coupled to the receptor via a pertussis toxin substrate appear during embryonic development suggest that the coupling of phospholipase C to the muscarinic receptor may also be developmentally regulated. In the experiments outlined here, we determined the time course of development of pertussis toxin-insensitive and pertussis toxin-insensitive muscarinic-stimulated phospholipase C activity and analysed its relationship to changes in levels of G-proteins and the appearance of other muscarinic functions.

MATERIALS AND METHODS

Sources of materials were as in the preceding paper [13].

Culture of embryonic chick atrial cells

Heart cell cultures were prepared by a modification of the method of DeHaan [16]. Embryonic age was determined by the method of Hamberger & Hamilton [17]. Embryos were removed from embryonated Leghorn chicken eggs at various embryonic ages, hearts were removed, and atria were separated from ventricles. Atria and ventricles were then incubated separately with 0.025% (w/v) trypsin in Ca^{2+}/Mg^{2+} -free Hanks' salt solution at 37 °C for 8 min. The trypsin solution was removed and diluted into Hanks' buffered saline solution containing 50% heat-inactivated horse serum at room temperature. After successive incubation of the tissue with trypsin, cells were sedimented at 650 g and resuspended in growth medium M199 containing 6% fetal calf serum at a density of 1.6×10^5 cells/cm² in 35 mm Petri dishes (Falcon, Oxnard, CA, U.S.A.), and incubated at 37 °C in a humidified atmosphere of 5% CO₂. Cell cultures from hearts at each embryonic age studied were determined to contain equal numbers of myocytes.

Assay of inositol phosphates

Inositol phosphates were determined by a modification of the method of Berridge *et al.* [18], as described previously [13]. Briefly, cultured embryonic chick atrial and ventricular cells from hearts at various embryonic ages were incubated for 3 days with 2.5 μ Ci of *myo*-[³H]inositol/ml. On day 3 of culture, cells were washed with a buffer containing 25 mM-Hepes, pH 7.4, 20% (w/v) Medium M199, 0.9 mM-NaH₂PO₄, 5 mM-dextrose, 0.8 mM-MgSO₄, 1.8 mM-CaCl₂, 4.4 mM-KCl, 117 mM-NaCl and 15 mM-LiCl. Cells were equilibrated with buffer for 4 min, followed by incubation for 5 min in fresh medium with or without 0.1 mM-carbamylcholine. The buffer was removed, 1 ml of chloroform/methanol/HCl (1:2:0.05, by vol.) (CMH) was added, and the sample was transferred to glass centrifuge tubes and combined with a second 0.5 ml CMH rinse. Organic and aqueous phases were separated by the addition of 0.4 ml of chloroform and 0.7 ml of water to each centrifuge tube, and the aqueous layer was rinsed further with 0.8 ml of chloroform, transferred to a polystyrene tube and evaporated for 1 h under N₂. The sample was then diluted with 2 ml of water and the pH was adjusted to 7.0 with 10 M-KOH. Chromatography was carried out using anion-exchange columns (AG1X8 resin, formate form) and $InsP_1$, $InsP_2$ and $InsP_3$ were eluted sequentially into glass scintillation vials as described previously [13].

Assay of inositol phosphates in permeabilized cells

Cultured chick atrial and ventricular cells were incubated for

3 days with 2.5 μ Ci of *myo*-[³H]inositol/ml and permeabilized on day 3 of culture with 10 ng of saponin/ml, as described previously [13]. Morphological examination of myocytes cultured from each embryonic age studied demonstrated that incubation with 10 ng of saponin/ml had no deleterious effect.

Determination of $InsP_3$ isomers and $InsP_4$

Cultures of chick atrial and ventricular cells were cultured on 60 mm culture dishes and incubated for 3 days with 25 μ Ci of *myo*-[³H]inositol/ml. Total inositol phosphates were extracted as described above and the aqueous samples were lyophilized. $InsP_3$ and $InsP_4$ were separated by h.p.l.c. on a Partisil 10 SAX column (Whatman, Clifton, NJ, U.S.A.) equipped with an AX guard column and a silica precolumn and equilibrated with 0.585 M-ammonium formate silica precolumn (pH 3.7 using H₃PO₄). Isomers were eluted as a flow rate of 1 ml/min with a gradient from 0.585 M- to 1.275 M-ammonium formate containing the following steps: 0.585 M, 5 min; 0.585–0.795 M, 16 min; 0.795–1.050 M, 5 min; 1.050 M, 5 min; 1.050–1.275 M, 16 min; 1.275 M, 5 min. Fractions were collected every 0.5 min, and the radioactivity in each sample was quantified by liquid scintillation counting. Isomers were identified by co-elution with known standards. $Ins(1,3,4)P_3$ eluted at 0.74 M-ammonium formate, $Ins(1,4,5)P_3$ at 0.79 M-ammonium formate and $Ins(1,3,4,5)P_4$ at 1.26 M-ammonium formate.

Statistical analysis

Data for dose-response and time course experiments were analysed by analysis of variance. Student's *t*-tests were used as indicated in the text or Figure legends.

RESULTS

Development of carbamylcholine-stimulated inositol phosphate production in atria and ventricles

In order to determine the time course of development of muscarinic stimulation of inositol phosphate production, we measured the ability of 0.1 mM-carbamylcholine to stimulate $InsP_2$ and $InsP_3$ production in cultures of atria and ventricles of hearts from embryonic chicks at 5, 7, 10, 12 and 14 days *in ovo*. In both atria and ventricles the ability of muscarinic agonists to stimulate inositol phosphate production decreased from 275% above basal at day 5 *in ovo* to 100% above basal at day 14 *in ovo* for $InsP_2$, and from 60–75% above basal at day 5 *in ovo* to 30–40% above basal at day 14 *in ovo* for $InsP_3$ (Fig. 1). Basal levels of both $InsP_2$ and $InsP_3$ in atria and ventricles did not change significantly during development. Furthermore, basal levels of $InsP_2$ and $InsP_3$ in ventricles at days 5, 7, 10, 12 and 14 *in ovo* were not significantly different from those in atria. The time course for the developmental decrease in carbamylcholine-stimulated inositol phosphate formation was different in atria and ventricles. The decrease was maximal at day 6 *in ovo* in the ventricle, whereas in the atrium the decrease was more gradual, with a half-maximal loss of muscarinic-stimulated phospholipase C activity by day 7 *in ovo*. Similar studies of developmental changes in $InsP_1$ demonstrated that $InsP_1$ levels also decreased to 50% of control levels between days 5 and 14 *in ovo* (results not shown).

Development of pertussis toxin sensitivity of carbamylcholine-stimulated inositol phosphate production in cultured chick atrial cells

In order to determine the time course of development of pertussis toxin-sensitive inositol phosphate production in cultured chick atrial cells, cells from cultures of atria from chicks at 5, 7, 10, 12 and 14 days *in ovo* were incubated for 15 h under

Fig. 1. Development of muscarinic receptor stimulation of inositol phosphate production in cultured embryonic chick atrial and ventricular cells

Cells cultured from atria (●) and ventricles (○) of embryonic chick hearts of various ages were incubated with 0.1 mM-carbamylcholine for 5 min, and InsP_2 (a) and InsP_3 (b) production was determined as described in the Materials and methods section. Each data point represents the mean percentage increase above basal (bars = S.E.M.) for at least six experiments performed in triplicate. Basal levels in atrium for InsP_2 were (c.p.m.): 979 ± 128 ($n = 3$), 831 ± 95 ($n = 9$), 836 ± 70 ($n = 6$), 916 ± 75 ($n = 8$), and 848 ± 50 ($n = 13$), and for InsP_3 basal levels were 1039 ± 80 , 1069 ± 125 , 1153 ± 103 , 1278 ± 115 and 1110 ± 77 at days 5, 7, 10, 12 and 14 *in ovo* respectively. Data for ventricles were not significantly different from those for atria.

control conditions or with 0.5 ng of pertussis toxin/ml, and the ability of 0.1 mM-carbamylcholine to stimulate production of inositol phosphates was determined. Incubation with pertussis toxin had no effect on basal levels of inositol phosphates in atrial cultures at any embryonic age studied. The time course of appearance of a pertussis toxin-sensitive muscarinic stimulation of inositol phosphate production is shown in Fig. 2. In this analysis, inositol phosphate production (defined as percentage increase above basal levels) was normalized to inositol phosphate production at day 5 *in ovo* taken as 100%. Data summarized in Fig. 2 demonstrate a developmental decrease both in total muscarinic-stimulated inositol phosphate production (●) and in the pertussis toxin-insensitive fraction of inositol phosphate production (■). The pertussis toxin-sensitive fraction of inositol phosphate production is the difference between the total muscarinic-stimulated inositol phosphate production and the pertussis toxin-insensitive inositol phosphate production. The pertussis toxin-sensitive fraction of inositol phosphate production at each embryonic age was plotted as a percentage of total inositol phosphate production at that embryonic age (▲). Total InsP_3 production decreased by 50% between days 5 and 14 *in ovo*, and InsP_2 production decreased by 60% over the same time course. Pertussis toxin-insensitive production of both InsP_2 and InsP_3 decreased by 64%. The fraction of muscarinic-stimulated inositol phosphate production sensitive to pertussis toxin increased from undetectable levels at day 5 *in ovo* to 40% of the total remaining inositol phosphate production at day 12 *in*

Fig. 2. Developmental changes in pertussis toxin-sensitive muscarinic-stimulated inositol phosphate production in embryonic chick atrial cells

Embryonic chick atrial cells were cultured as described in the Materials and methods section. On the second day in culture, pertussis toxin was added to the culture medium to give a final concentration of 0.5 ng/ml. Following incubation with pertussis toxin for 15 h, cells were washed, and exposed 0.1 mM-carbamylcholine for 5 min, and the levels of inositol phosphates were determined (a, InsP_2 ; b, InsP_3). Data for inositol phosphate stimulation in control (●) and pertussis toxin-treated cells (■) are expressed as percentage increase above basal at each embryonic age, normalized to the value at days 5 *in ovo* taken as 100%. Pertussis toxin-sensitive inositol phosphate production (▲) was calculated by dividing the difference between pertussis toxin-insensitive inositol phosphate production and total inositol phosphate production at each embryonic age by total inositol phosphate production at that age. Basal levels were as reported in the legend to Fig. 1 and were unaffected by pertussis toxin. Each data point represents the mean percentage increase above basal $\pm$ S.E.M. for six experiments performed in triplicate.

ovo, with a half-maximal increase observed at day 8 *in ovo*. A pertussis toxin-sensitive muscarinic stimulation of InsP_1 production also increased with embryonic age (results not shown).

Insensitivity of carbamylcholine-stimulated inositol phosphate production in ventricles to pertussis toxin at all embryonic ages

Inositol phosphate production in embryonic chick ventricle cells at day 13 *in ovo* has been shown to be insensitive to pertussis toxin [14,15]. We have demonstrated that at day 14 *in ovo* muscarinic stimulation of inositol phosphate production in cultures of chick atria is partially sensitive to pertussis toxin [13]. In order to determine whether muscarinic stimulation of phospholipase C activity in ventricular cells might be partially sensitive to pertussis toxin early in embryonic development but then becomes insensitive to pertussis toxin, the sensitivity of muscarinic stimulation of phospholipase C activity to pertussis toxin at various embryonic ages was studied. Ventricles were cultured from chicks at days 5, 7, 10, 12 and 14 *in ovo*. Replicate

Table 1. Analysis of the accumulation of isomers of InsP_3 and InsP_4 in cultured embryonic chick heart cells

Cells cultured from atria or ventricles from hearts of chicks at 5 or 14 days *in ovo* were labelled for 3 days with 25 μCi of $\text{myo-}[^3\text{H}]\text{inositol}$ and incubated for 10–12 min with a Hepes-buffered growth medium containing 15 mM-LiCl followed by a 5 min incubation with 0.1 mM-carbamylcholine. Total inositol phosphates were extracted and separated by h.p.l.c. as described in the Materials and methods section. Data are presented as d.p.m. of $\text{myo-}[^3\text{H}]\text{inositol}$ incorporated into the indicated isomers. Data are the means $\pm$ S.E.M. of six separate determinations from replicate cultures and are typical of two separate experiments. Carbamylcholine had no statistically significant effect on the levels of $\text{Ins}(1,4,5)\text{-P}_3$ or InsP_4 at either day 5 or 14 *in ovo*, as determined by Student's *t* test.

Isomer	[^3H]Inositol phosphate (d.p.m.)			
	5 days <i>in ovo</i>		14 days <i>in ovo</i>	
	Control	Carbamylcholine	Control	Carbamylcholine
(a) Atrial cells				
$\text{Ins}(1,3,4)\text{P}_3$	1042 $\pm$ 104	4810 $\pm$ 226	187 $\pm$ 28	743 $\pm$ 46
$\text{Ins}(1,4,5)\text{P}_3$	4422 $\pm$ 53	3878 $\pm$ 543	2216 $\pm$ 144	2435 $\pm$ 102
InsP_4	2309 $\pm$ 250	3719 $\pm$ 475	756 $\pm$ 198	538 $\pm$ 183
(b) Ventricular cells				
$\text{Ins}(1,3,4)\text{P}_3$	1688 $\pm$ 12	5111 $\pm$ 647	220 $\pm$ 23	1028 $\pm$ 109
$\text{Ins}(1,4,5)\text{P}_3$	7946 $\pm$ 352	6992 $\pm$ 253	2026 $\pm$ 162	2326 $\pm$ 154
InsP_4	3213 $\pm$ 399	2827 $\pm$ 142	548 $\pm$ 44	631 $\pm$ 76

plates were incubated with or without 0.5 ng of pertussis toxin/ml for 15 h and the ability of 0.1 mM-carbamylcholine to stimulate inositol phosphate production was determined at each age. Incubation with pertussis toxin had no effect on either basal or carbamylcholine-stimulated InsP_1 , InsP_2 or InsP_3 levels in ventricles at any age studied (results not shown).

Developmental changes in the isomers of InsP_3 formed in response to carbamylcholine

One possible consequence of the coupling of muscarinic receptors to phospholipase C via a pertussis toxin-insensitive G-protein at day 5 *in ovo* and the coupling of the muscarinic response by both pertussis toxin-sensitive and pertussis toxin-insensitive pathways with increased embryonic age is that these two pathways might result in the production of different isomers of InsP_3 at each age. To determine whether such developmental changes occur, the products of muscarinic stimulation of inositol phosphate production were analysed by h.p.l.c. A 5 min incubation of a culture of chick atrial cells from embryos at 5 days *in ovo* with 0.1 mM-carbamylcholine resulted in a 4-fold increase in the $\text{Ins}(1,3,4)\text{P}_3$ isomer from a basal level of 1042 $\pm$ 104 d.p.m. to 4810 $\pm$ 226 d.p.m. (Table 1). No significant increase in either $\text{Ins}(1,4,5)\text{P}_3$ or InsP_4 could be detected. At day 14 *in ovo*, both basal and stimulated levels of all isomers were decreased compared with day 5 *in ovo* (Table 1). However, muscarinic stimulation increased the $\text{Ins}(1,3,4)\text{P}_3$ isomer by 4-fold from 187 $\pm$ 28 d.p.m. in control cells to 743 $\pm$ 46 d.p.m. in cells incubated with 0.1 mM-carbamylcholine (Table 1). As was demonstrated for cultures of atria from chicks at 5 days *in ovo*, carbamylcholine had no effect on the levels of either $\text{Ins}(1,4,5)\text{P}_3$ or InsP_4 (Table 1).

Similarly, in ventricles of hearts from chicks at 5 days *in ovo*, $\text{Ins}(1,3,4)\text{P}_3$ was the major product of muscarinic stimulation of phospholipase C activity, increasing 3-fold from 1688 $\pm$ 120 d.p.m. to 5111 $\pm$ 647 d.p.m. at 5 min (Table 1). By day 14 *in ovo*, although basal and muscarinic-stimulated levels of both InsP_3 isomers were lower than in hearts from chicks at 5 days *in ovo*, $\text{Ins}(1,3,4)\text{P}_3$ was increased by 4-fold (Table 1), whereas the $\text{Ins}(1,4,5)\text{P}_3$ and InsP_4 levels were unaffected by carbamylcholine. Because of low levels of incorporation of label into both $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4 at day 14 *in ovo*, it was not possible to obtain data at earlier time points. However, at the times studied

Table 2. Stimulation by GTP[S] of inositol phosphate production in permeabilized chick atrial and ventricular cells

Permeabilized cells from chicks at 5 days *in ovo* were exposed to 10 μg of saponin/ml and assayed for inositol phosphates as described in the Materials and methods section. Data are expressed as percentage stimulation (mean $\pm$ S.E.M.). This was obtained by subtracting the mean c.p.m. for cells exposed to saponin alone ($n = 3$) from the mean c.p.m. for cells exposed to saponin plus 0.1 mM-GTP[S] ($n = 3$) and dividing by the mean c.p.m. for cells exposed to saponin alone for individual experiments.

Inositol phosphate	Cells...	Stimulation (%)	
		Ventricular	Atrial
InsP_1		30 $\pm$ 7	40 $\pm$ 10
InsP_2		166 $\pm$ 5	117 $\pm$ 18
InsP_3		74 $\pm$ 8	51 $\pm$ 8

no developmental changes in the cumulative levels of muscarinic-receptor-stimulated InsP_3 isomers could be detected.

Effect of GTP[S] on inositol phosphate production in saponin-treated cultured atrial and ventricular cells from chicks at 5 days *in ovo*

In order to determine if the pertussis toxin-insensitive production of inositol phosphates in atrial cells from chicks at 5 days *in ovo* was mediated by a G-protein, we examined the ability of GTP[S] to stimulate inositol phosphate production in saponin-permeabilized cells from cultures of atria and ventricle of hearts from chicks at 5 days *in ovo*. In atrial cells, in the presence of 10 ng of saponin/ml, 0.1 mM-GTP[S] increased InsP_3 formation from a basal level of 583 $\pm$ 23 c.p.m. to 875 $\pm$ 31 c.p.m., InsP_2 formation from a basal level of 683 $\pm$ 7 c.p.m. to 1478 $\pm$ 112 c.p.m. and InsP_1 formation from a basal level of 2381 $\pm$ 76 c.p.m. to 3331 $\pm$ 151 c.p.m. In ventricular cultures, InsP_3 levels increased from 719 $\pm$ 100 c.p.m. to 1214 $\pm$ 131 c.p.m., InsP_2 from 813 $\pm$ 94 c.p.m. to 2252 $\pm$ 300 c.p.m. and InsP_1 from 2392 $\pm$ 166 c.p.m. to 3107 $\pm$ 221 c.p.m. The responses of InsP_1 , InsP_2 and InsP_3 to GTP[S] in saponin-permeabilized cells as a percentage of basal levels are summarized in Table 2. Hence, at

day 5 *in ovo*, an embryonic age at which muscarinic receptor stimulation of inositol phosphate production is not sensitive to pertussis toxin, phospholipase C activity is coupled to a G-protein in both atria and ventricles.

DISCUSSION

These experiments demonstrate that in chick atria the identity of the G-protein which couples muscarinic receptors to phospholipase C activity is developmentally regulated. During embryonic development, total muscarinic-stimulated inositol phosphate production decreases in both chick atrial and ventricular cells. In atria between days 5 and 12 *in ovo* a pertussis toxin-insensitive fraction of muscarinic-stimulated phospholipase C activity decreased by 64%. Concomitantly, a fraction of pertussis toxin-sensitive muscarinic-stimulated phospholipase C activity increased from undetectable levels at day 5 *in ovo* to 40% of the remaining muscarinic-stimulated inositol phosphate production at day 12 *in ovo*. In contrast, muscarinic stimulation of phospholipase C activity in ventricles decreased with development, but sensitivity to pertussis toxin was never apparent.

These data further demonstrate that muscarinic receptor activation of phospholipase C is coupled differently in atria and ventricles, depending on embryonic age. In chicks at day 5 *in ovo*, muscarinic stimulation of phospholipase C activity appears to be coupled to the muscarinic receptor via a pertussis toxin-insensitive G-protein (G_p) in both atria and ventricles. This is supported by the observation that, in atrial and ventricular cells cultured from hearts of chicks at 5 days *in ovo*, GTP[S] induced accumulation of inositol phosphates in permeabilized cells. We have previously shown that this pertussis toxin-insensitive pathway persists in atrial cells at day 14 *in ovo* [13], but that at day 14 there is an additional component to the response, which is pertussis toxin-sensitive. Masters *et al.* [14] have demonstrated that G_p appears to be responsible for all of the GTP[S]-stimulated phospholipase C activity in ventricular cells from hearts of chicks at 13 days *in ovo*. Orellana & Brown [19] also demonstrated that in the ventricle, the stimulation of phospholipase C activity decreased during embryonic development. Our data indicate that in both atria and ventricles it is coupling via the pertussis toxin-insensitive pathway that is decreasing. These data support the conclusion that the G-protein(s) coupling the muscarinic receptor to phospholipase C activity in atrial cells changes from G_p at day 5 *in ovo* to both G_p and a pertussis toxin substrate by day 12 *in ovo*. In ventricular cells, G_p couples the muscarinic receptor to phospholipase C throughout development. The decrease in muscarinic-stimulated inositol phosphate production during development appears to be more rapid in the ventricle than in the atrium (Fig. 1). This could be explained, at least in part, by the gradual increase in a pertussis toxin-sensitive fraction of inositol phosphate production in the atrium, but not in the ventricle, such that the decrease in total inositol phosphate production would appear to be attenuated in the atria.

Several studies have suggested that the relative levels of G-proteins might be critical for the regulation of the level of a specific physiological response. During embryonic development of the chick heart, levels of α_o and α_i increase in parallel with the development of the ability of muscarinic agonists to inhibit adenylate cyclase activity [7] and the appearance of a negative chronotropic response to muscarinic stimulation [3]. Watkins *et al.* [20] demonstrated that during differentiation of 3T3-L1 cells, levels of α_o , α_o and α_i increased significantly in parallel with changes in the coupling of receptors to a number of biochemical processes. Developmental increases in muscarinic-stimulated pertussis toxin-sensitive inositol phosphate production reported

here could be associated with the availability of increased levels of α_o or of a specific isoform of α_i which, before day 5 *in ovo*, was either present in insufficient levels to couple the receptor to phospholipase C or was not expressed.

These data also suggest that muscarinic responses in chick heart may be separated into two types: those present earlier in embryonic development, such as activation of phospholipase C, which are coupled via G_p , and those which develop after day 3.5 *in ovo*, such as the negative chronotropic response to muscarinic stimulation [1–3], inhibition of adenylate cyclase activity [11] and the pertussis toxin-sensitive stimulation of phospholipase C activity, all of which are coupled via a pertussis toxin substrate. The developmental regulation of the appearance of these latter responses, which are critical for parasympathetic modulation of cardiac function, suggests that increased coupling of the muscarinic receptor to these responses via a pertussis toxin-insensitive pathway plays a critical role in their appearance.

The developmental decrease in the coupling of the muscarinic receptor to phospholipase C activity via G_p could represent either a decrease in G_p below some critical level with a resultant uncoupling from the receptor and phospholipase C, or some alteration in the receptor or phospholipase C which results in decreased affinity of the receptor or phospholipase C for G_p . One interpretation of the studies of GTP[S]-stimulated phospholipase C activity presented here is that levels of G_p do not decrease during development, but that a decrease in the interaction of the muscarinic receptor with G_p may be responsible for the 64% decrease in pertussis toxin-insensitive muscarinic stimulation of phospholipase C activity. This conclusion is supported by comparison of the data presented here for GTP[S]-stimulated inositol phosphate production at day 5 *in ovo* (Table 2) with our data for GTP[S] stimulation of inositol phosphate production at day 14 *in ovo* [13]. In atria from chicks at day 5 *in ovo*, GTP[S] stimulated InsP_3 production by 0.5-fold, compared with 2.2-fold at day 14 *in ovo*. GTP[S] stimulated InsP_2 production in atrial cells from chicks at day 5 *in ovo* by 1.2-fold, compared with 2.2-fold in atria of chicks at day 14 *in ovo*. Although during development pertussis toxin-insensitive muscarinic stimulation of phospholipase C activity decreased by 64%, GTP[S] stimulation of phospholipase C activity appeared to increase by almost 4-fold. Variations in cell number and sensitivity to saponin between cultures of atria from chicks at day 5 and day 14 *in ovo* appear not to account for these differences in GTP[S] stimulation (see the Materials and methods section). One interpretation of these data is that although G_p -coupled muscarinic receptor stimulation of inositol phosphate production decreases during embryonic development, the coupling of G_p to phospholipase C activity actually increases during embryonic development. Hence, during development, the level of G_p does not decrease, but the ability of G_p to couple the muscarinic receptor to phospholipase C is impaired.

Previous studies have demonstrated that both the muscarinic receptor and phospholipase C exist in at least five isoforms [21–23]. Transfection studies with cDNAs coding for the M_1 and M_4 receptors have shown that these receptors are coupled to phospholipase C activation and to the stimulation of adenylate cyclase, whereas transfection studies with M_2 and M_3 demonstrate a modest coupling to phospholipase C and a tight coupling to inhibition of adenylate cyclase [24]. Data presented here and in our previous studies demonstrated that during early embryonic development of the chick heart, muscarinic stimulation of the atrium results in a high level of inositol phosphate production and a low level of inhibition of adenylate cyclase activity [7], a response characteristic of the M_1 -mediated pathway. By day 14 *in ovo*, muscarinic receptors are coupled to a lower level of phospholipase C stimulation (Fig. 2) and to a 10-fold

higher inhibition of adenylate cyclase activity [7], a response more characteristic of an M_2 receptor-mediated pathway. One possible explanation for these changes is that during embryonic development the predominant form of the muscarinic receptor is switched from an M_1 form coupled to phospholipase C via a pertussis toxin-insensitive G-protein to an M_2 form coupled to phospholipase C via a pertussis toxin-sensitive G-protein. Alternatively, an isoform shift to a subtype of phospholipase C which preferentially couples to a pertussis toxin substrate is also consistent with data presented here.

These data clearly demonstrate a switch in G-protein coupling to phospholipase C during embryonic development of the chick heart, and are consistent with a model in which these changes in coupling reflect isoform switches in receptors and phospholipase C. Taken together, the data raise the intriguing possibility that regulation of expression of isoforms of G-proteins, muscarinic receptors and effectors during embryonic development may play a major role not only in the process of differentiation but also in the regulation of the level of a given physiological response in the cell.

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