Development of Muscarinic Cholinergic Inhibition of Adenylate Cyclase in Embryonic Chick Heart

ITS RELATIONSHIP TO CHANGES IN THE INHIBITORY GUANINE NUCLEOTIDE REGULATORY PROTEIN*

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Parasympathetic and sympathetic innervation of the embryonic chick heart proceed non-coordinate. β-Adrenergic agonists mediate an increase in beating rate in embryonic chick heart prior to ingrowth of the vagus nerve (Culver, N. G., and Fishman, D. A. (1977) Am. J. Physiol. 232, R116-R123) while muscarinic agonists exert relatively little effect on beating rate in hearts 2-4 days in ovo (Papanicolaou, A. J. (1979) Pharmacol. Rev. 29, 3-33). Studies of developmental changes in the ability of muscarinic agonists to inhibit adenylate cyclase activity and their relationship to the development of a physiologic response of the embryonic chick heart to muscarinic stimulation have been inconclusive. In the current study the ability of isoproterenol to stimulate adenylate cyclase activity did not change during development. Maximum stimulation above basal was 760 pmol of cAMP/10 min/mg of protein with an IC₅₀ of 1.5 × 10⁻⁹ M for isoproterenol in homogenates of hearts 2%, 3%, and 10 days in ovo and 3 days posthatching. However, inhibition of isoproterenol-stimulated adenylate cyclase activity by carbamylcholine increased from 7.6% with a IC₅₀ of carbamylcholine of 16 ± 5.0 μM at day 2% in ovo to 29% with an IC₅₀ of 0.4 ± 0.1 μM at day 10 in ovo and to 43% with a IC₅₀ of 0.6 ± 0.1 μM at 3 days posthatching. Since previous data had demonstrated the presence of muscarinic receptors as early as 2% days in ovo (Galper, J. B., Klein, W., and Catterall, W. A. (1977) J. Biol. Chem. 252, 8692-8699), studies of developmental changes in guanine nucleotide-coupling proteins were carried out to determine whether early in development muscarinic receptors were uncoupled from a physiologic response. Studies of pertussis toxin-catalyzed ADP-ribosylation of homogenates of embryonic chick heart with [³²P]NAD demonstrated the presence of two ADP-ribosylated proteins at 39,000 and 41,000 kDa, respectively. Both ADP-ribosylation and immunoblotting of homogenates with an antibody to the 39-kDa guanine nucleotide-binding protein in bovine brain demonstrated that the 39-kDa α protein increased 1.8-fold between days 2% and 3% in ovo and another 1.8-fold from day 3% to 10 in ovo in parallel with the increase in the extent of muscarinic inhibition of adenylate cyclase activity. Between day 10 in ovo and 3 days posthatching the 39-kDa protein remained constant, but muscarinic receptor number measured by [³H]quinuclidinyl benzilate binding increased 2.6-fold from 200 fmol/mg of protein to 520 fmol/mg of protein in parallel with the increase in inhibition of adenylate cyclase activity by carbamylcholine. The data suggest that early in embryonic development coupling of muscarinic receptors to inhibition of adenylate cyclase activity may be limited by the availability of the 39-kDa guanine nucleotide regulatory protein but that later in development physiologic response to muscarinic stimulation might be limited by muscarinic receptor number.

The ability of the embryonic chick heart to respond to muscarinic and β-adrenergic stimuli does not develop coordinate. Prior to ingrowth of the vagus nerve (days 4-5 in ovo) both β-adrenergic receptors, as demonstrated by the binding of the β-adrenergic antagonist (—)[³H]dibhydroalpranolol (1, 2), and muscarinic receptors, as measured by the binding of the muscarinic antagonist [³H]QNB (3, 4), are present on the cell membrane of the developing embryonic chick heart. However, while a β-adrenergic agonist-mediated increase in beating rate (5) and adenylate cyclase activity (2) was demonstrated in hearts prior to ingrowth of the vagus nerve, muscarinic agonists exerted relatively little effect on beating rate in hearts 2-4 days in ovo (3, 4). These data suggest that muscarinic receptors are not coupled to a physiologic response prior to vagal innervation of the heart. Hence the embryonic chick heart might offer a good model for the study of factors necessary for coupling of muscarinic receptors to a physiologic response.

Guanine nucleotides have been shown to be necessary for coupling of β-adrenergic agonist binding to stimulation of adenylate cyclase (7) and muscarinic agonist binding to inhibition of adenylate cyclase (6, 8) via guanine nucleotide regulatory proteins. This interaction with guanine nucleotides has also been shown to decrease the affinity of the muscarinic

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The abbreviations are: QNB, quinuclidinyl benzilate; R₅, high affinity receptor; R₇, low affinity receptor; N₉, a stimulatory guanine nucleotide regulatory protein complex; N₀, an inhibitory guanine nucleotide regulatory complex; NAD, nicotinamide adenine dinucleotide; IAP, islet-activating protein.
receptor for agonists (9, 10) during conversion of the muscarinic receptor from a high affinity form (R_H) to a low affinity form (R_L) (11, 12).

Recent studies have demonstrated that hormonal stimulation and inhibition of adenylate cyclase are mediated by two distinct guanine nucleotide-binding proteins, N_i and N_j, respectively (13). Both N_i and N_j are heterotrimers composed of subunits α-βγ. The α subunits of N_i and N_j are structurally and functionally different while the β and γ subunits are very similar (14-16). The α subunit of N_i, molecular weight 41,000, is ADP-ribosylated by a toxin isolated from Bordetella pertussis with the loss of hormonal and GTP-dependent inhibition of adenylate cyclase and conversion of the muscarinic receptor to a low affinity form (14, 17, 18). Recently, bovine brain has been shown to contain two proteins, a 39- and a 41-kDa protein which are substrates for ADP-ribosylation by pertussis toxin (19, 20). The 41-kDa protein is the species found in most membranes and is felt to be the α subunit of N_i, α_i (17, 18); the role of the 39-kDa proteins in brain referred to as α_j is not well understood.

We demonstrated previously that the absence of a physiologic response to muscarinic stimulation in cultures of hearts studied prior to ingrowth of the vagus nerve was associated with the inability of guanine nucleotides to convert high affinity muscarinic receptors (R_H) to a low affinity form (R_L). The appearance of a physiologic response during incubation of these cells with specific lots of horse serum was associated with the development of guanine nucleotide sensitivity of R_L (21). In the current studies of embryonic chick heart homogenates, we demonstrate that prior to ingrowth of the vagus nerve, another GTP-dependent function, muscarinic inhibition of adenylate cyclase activity, is also markedly depressed. One interpretation of these data is that prior to vagal innervation of the heart, a guanine nucleotide-coupling protein might be absent or present in an inactive form. Data are presented which support the hypothesis that the appearance of a physiologic response to muscarinic stimulation during maturation of the embryonic chick heart is associated with an increase in the levels of an α 39-kDa guanine nucleotide-binding protein in the heart.

**MATERIALS AND METHODS AND RESULTS**

Developmental Changes in Isoproterenol Stimulation of Adenylate Cyclase Activity—In order to study developmental changes in muscarinic inhibition of isoproterenol-stimulated adenylate cyclase activity at various embryonic ages, it was first necessary to establish conditions for the assay of muscarinic inhibition of adenylate cyclase in chick heart homogenates. The experiments described below were carried out in 3 types: 1) activity in the absence of added GTP (referred to as basal activity); 2) activity in the presence of 10^-4 M GTP; 3) activity in the presence of 10^-4 M GTP plus isoproterenol. In homogenates of chick hearts 10 days in ovo 10 μM isoproterenol caused a 1.6-fold increase (220 pmol/mg of protein/10 min) in adenylate cyclase activity over basal levels (Table I, column 2). Addition of 10^-3 M carbamylcholine decreased isoproterenol-stimulated adenylate cyclase activity by 115 pmol/mg/10 min or 52% (Table I, column 2). Inhibition was blocked by 10^-5 M atropine consistent with a specific muscarinic effect.

The dependence of muscarinic inhibition of adenylate cyclase activity on the concentration of carbamylcholine in homogenates of chick hearts 10 days in ovo is depicted in the experiment summarized in Fig. 2. A maximal inhibition of 52%, 3%, and 10 days in ovo and 3 days posthatching. In the experiments summarized in Fig. 1, the dose-response curve for isoproterenol-stimulated adenylate cyclase activity is compared for hearts of various embryonic ages. There is no statistically significant difference between the extent of stimulation by isoproterenol (750 pmol of cyclic AMP/mg/10 min), the concentration of isoproterenol required to elicit a maximal stimulation (10^-4 M), and the concentration of isoproterenol at which stimulation is half-maximal (1.5 x 10^-4 M) for all embryonic ages studied. Although data for hatched chick hearts appear to fall below the data for embryonic hearts, the scatter in the hatched chick data is more marked and hence this difference is not statistically significant. Hence between days 2% in ovo and hatching, no developmental change in the ability of β-adrenergic agonists to stimulate adenylate cyclase activity can be detected by these methods.

Inhibition of Adenylate Cyclase Activity by Muscarinic Agonists in Homogenates of Embryonic Chick Hearts—In order to study muscarinic cholinergic inhibition of adenylate cyclase in embryonic chick heart, it was first necessary to establish conditions for the assay of muscarinic inhibition of adenylate cyclase in chick heart homogenates. In these studies adenylate cyclase activity is separated into 3 types: 1) activity in the absence of added GTP (referred to as basal activity); 2) activity in the presence of 10^-4 M GTP; 3) activity in the presence of 10^-4 M GTP plus isoproterenol. In homogenates of chick hearts 10 days in ovo 10 μM isoproterenol caused a 1.6-fold increase (220 pmol/mg of protein/10 min) in adenylate cyclase activity over basal levels (Table I, column 2). Addition of 10^-3 M carbamylcholine decreased isoproterenol-stimulated adenylate cyclase activity by 115 pmol/mg/10 min or 52% (Table I, column 2). Inhibition was blocked by 10^-5 M atropine consistent with a specific muscarinic effect.

The dependence of muscarinic inhibition of adenylate cyclase activity on the concentration of carbamylcholine in homogenates of chick hearts 10 days in ovo is depicted in the experiment summarized in Fig. 2. A maximal inhibition of

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**Fig. 1. Effect of varying concentrations of isoproterenol on adenylate cyclase activity at various developmental stages of embryonic chick heart.** Assays of adenylate cyclase activity were performed at 37°C with homogenates of chick hearts of the indicated embryonic ages as described under "Materials and Methods." Final concentrations of GTP and NaCl were 100 μM and 80 mM, respectively. Ordinate is the increase in adenylate cyclase activity above levels in the presence of GTP alone. Each point is the mean of four determinations carried out in triplicate.
Muscarinic Inhibition of Adenylate Cyclase

TABLE I

<table>
<thead>
<tr>
<th>Adenylate cyclase activity*</th>
<th>pmol/mg/10 min ± S.E.</th>
</tr>
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<tbody>
<tr>
<td>(1)</td>
<td>(2)</td>
</tr>
<tr>
<td>—Na</td>
<td>+Na (80 mM)</td>
</tr>
</tbody>
</table>

GTP (10^{-4} M)                              340 ± 15  388 ± 16  
GTP plus isoproterenol (10^{-5} M)           590 ± 10  609 ± 11  
GTP plus isoproterenol plus carbachol (10^{-5} M)  550 ± 12  493 ± 15  
plus atropine (10^{-5} M)                     585 ± 10  602 ± 12  
Inhibition by carbachol                      40        115

* The differences between successive pairs of numbers in column 2 were significant at the p = 0.003 level.

In order to determine whether muscarinic cholinergic inhibition of isoproterenol-stimulated adenylate cyclase activity increased with embryonic age in a manner similar to that for the development of inhibition of adenylate cyclase in the absence of GTP (Table II, compare columns 2 and 4). Inhibition increased from 20 ± 6 (n = 8) pmol/mg/10 min or 7% at 2%-days in ovo to 33 ± 2 (n = 25) pmol/mg/10 min or 11% at 3%-days in ovo to 66 ± 16 (n = 20) pmol/mg/10 min or 20% in hearts 10 days in ovo to 1000 ± 16 (n = 10) pmol/mg/10 min or 45% in hearts 3 days posthatching.

Developmental Changes in Muscarinic Inhibition of Isoproterenol-stimulated Adenylate Cyclase Activity—In order to determine whether muscarinic cholinergic inhibition of isoproterenol-stimulated adenylate cyclase activity also increased with embryonic age, we compared the ability of various concentrations of carbachol to inhibit isoproterenol-stimulated adenylate cyclase activity in homogenates of hearts from embryos 2%-3%, 3%-days, and 10 days in ovo and 3 days posthatching. The data summarized in Table II, columns 5 and 6, and plotted in Fig. 4 indicate that prior to ingrowth of the vagus nerve (days 2%-3%-days in ovo) adenylate cyclase activity in embryonic chick hearts is relatively insensitive to inhibition by carbachol. However, maximal inhibition of isoproterenol-stimulated adenylate cyclase activity and sensitivity to carbachol increased continuously even after ingrowth of the vagus nerve. Hence, maximal inhibition increased from 60 ± 16 (n = 7, S.E.) pmol/10 min/mg of protein or 7.6% of the isoproterenol-stimulated level at 2%-days in ovo to 120 ± 12 (n = 7) pmol/10 min/mg or 11% of the isoproterenol-stimulated level at day 3%-days to 320 ± 24 (n = 8) pmol/10 min/mg or 29% of the isoproterenol-stimulated level at day 10 in ovo to 1040 ± 28 pmol/10 min/mg (n = 5) or 46% of the isoproterenol-stimulated level at 3 days posthatching. The total increase in the extent of inhibition during development was 13-fold. Furthermore, the sensitivity to carbachol increased with embryonic age (Fig. 4). The concentration required to mediate a half-maximal inhibition of adenylate cyclase activity (IC_{50}) in the presence of 16 μM isoproterenol in homogenates of hearts 2%-days in ovo was 16.5 ± 5 μM (S.E., n = 7) carbachol compared to 0.6 ± 0.1 μM (n = 7) carbachol in homogenates of hearts 3 days posthatching (Table III). This represents a 26-fold increase in sensitivity of adenylate cyclase activity to inhibition...
by carbamylcholine during development. The inhibition of adenylate cyclase by muscarinic agonist was totally abolished by $10^{-4}$ M atropine at all ages studied.

One explanation of these data is that prior to ingrowth of the vagus nerve the affinity of the inhibitory system for GTP, Na+, or Mg2+ is decreased. However, we demonstrated that increasing GTP levels up to $10^{-3}$ M and/or increasing Na+ concentration to 100 or 150 mM or increasing Mg2+ levels from 10 to 24 mM had no effect on the extent of inhibition of adenylate cyclase by carbamylcholine in hearts from embryos 3½ days in ovo. Hence, while $\beta$-adrenergic stimulation of adenylate cyclase is relatively stable during embryonic development (Fig. 1), muscarinic inhibition of basal GTP-stimulated and $\beta$-adrenergic-stimulated adenylate cyclase activity develops continuously throughout embryonic life.

**Developmental Changes in Guanine Nucleotide-coupling Proteins Demonstrated by ADP-ribosylation by Pertussis Toxin of a 39- and 41-kDa Protein in Chick Heart Homogenates**—Data presented here and in previous studies (21) suggest that prior to ingrowth of the vagus nerve, two processes which require the interaction of guanine nucleotides with Nt, 1) inhibition of adenylate cyclase and 2) guanine nucleotide-mediated conversion of high affinity muscarinic receptors to a low affinity form (21), either cannot be demonstrated at all or are markedly attenuated. One explanation of these findings is that early in embryonic development, Nt is either absent or present in an inactive form. In order to study developmental changes in Nt, crude membrane preparations from embryonic chick hearts of various embryonic ages were incubated with islet-activating protein in the presence of $^{32}$P NAD+ and high levels of protease inhibitors. Following polyacrylamide gel electrophoresis of membranes solubilized in SDS, autoradiography of the dried gels demonstrated two specifically labeled bands, 41 and 39 kDa (Fig. 5) at each embryonic age. Densitometry scanning of the autoradiographs demonstrated that the intensity of the 39-kDa band increased 1.7-fold from day 2½ to 3½ in ovo, 1.5-fold from day 3½ to 10 in ovo, and was unchanged from day 10 until hatching (Figs. 5b and 6). The 41-kDa band increased 1.5-fold from day 2½ to 3½ in ovo, was unchanged from day 3½ to 10 in ovo, and may have decreased slightly from day 10 until hatching (Figs. 5b).

One explanation for these differences in ADP-ribosylation might be that at various embryonic ages, differences in ribosylation reflect differences in accessibility of pertussis toxin and/or NAD+ to the substrate. To rule out this possibility, experiments similar to those shown in Fig. 5 were carried out with membranes incubated for 5 min with low concentrations of cholate (0.1-0.01%) prior to incubation with IAP. These experiments demonstrated no effect of detergent on the relative labeling of the 39,000- and 41,000-kDa proteins seen in Fig. 5 and suggested that accessibility of the substrate is not responsible for differences in ADP-ribosylation.

A second possible explanation is that the differences between $^{32}$P labeling of the two peaks might be due to differences in the relative rates of ribosylation of these two proteins. To rule out this possibility the time course for the ADP-ribosylation of the 39- and 41-kDa bands from hearts 3½ days in ovo and in hatched chick heart membranes was determined. Ribosylation was maximal for both bands and at both ages by 15 min and did not change for as long as 90 min.

Although these data strongly support developmental increases in the 39-kDa IAP substrate, ADP-ribosylation of this protein by IAP is an indirect method of quantitation and may be limited as a tool for quantitation because of incomplete ribosylation. For example, accessibility may be limited even in the presence of detergent, or protease activity may interfere

### Table II

**Age dependence for carbamylcholine inhibition of basal, GTP-stimulated, and isoproterenol-stimulated adenylate cyclase activity**

<table>
<thead>
<tr>
<th>Age</th>
<th>Basal* Activity</th>
<th>Inhibition</th>
<th>%</th>
<th>GTP* (1) Activity</th>
<th>Inhibition</th>
<th>%</th>
<th>Isoproterenol* Activity</th>
<th>Inhibition</th>
<th>%</th>
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<tbody>
<tr>
<td>days in ovo</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>2½</td>
<td>295</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>299</td>
<td>20</td>
<td>(7)</td>
</tr>
<tr>
<td>3½</td>
<td>281</td>
<td>6</td>
<td>(2)</td>
<td></td>
<td>296</td>
<td>33</td>
<td></td>
<td>(11)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>317</td>
<td>20</td>
<td>(6)</td>
<td></td>
<td>338</td>
<td>66</td>
<td></td>
<td>(20)</td>
<td></td>
</tr>
<tr>
<td>Hatched</td>
<td>1690</td>
<td>376</td>
<td>(22)</td>
<td></td>
<td>1700</td>
<td>1000</td>
<td></td>
<td>(56)</td>
<td></td>
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</table>

* Data for basal adenylate cyclase activity and carbamylcholine inhibition of adenylate cyclase represent the mean of 5 determinations carried out in triplicate. Basal adenylate cyclase activity for 2½, 3½, and 10 days in ovo was not significantly different while the difference between inhibition of basal adenylate cyclase was significantly different between any two ages by Student's t test.

* Minus carbamylcholine.

* Plus carbamylcholine.

Activity is expressed as pmol of cyclic AMP formed/10 min/mg of protein. Inhibition is calculated as the ratio of inhibition to total activity × 100. For all embryonic ages, standard deviation did not exceed 10%.

For hatched chick hearts, standard deviations did not exceed 15%.

* Data derived from experiments similar to those for determination of basal adenylate cyclase activity with the addition of $10^{-4}$ M GTP.

* Data derived from experiments shown in Fig. 4.

* Data derived from maximal adenylate cyclase activity at each age (basal plus the isoproterenol stimulated values in Fig. 1).
with the pertussis toxin substrate activity of α39. Furthermore, ADP-ribosylation of these proteins has been shown to be dependent on the presence of the β subunit of N1 (19). Hence differences in ADP-ribosylation with embryonic age may reflect developmental differences in β levels. For these reasons determination of levels of β and the 39-kDa IAP substrate by immunoblotting constitute a more accurate method for quantitating the levels of these proteins in the developing heart.

Immunoblotting of the β and α39 Subunits of N1 at Various Embryonic Ages—Antibodies with a high degree of specificity for β and α39 from bovine brain have been generated and carefully characterized in our laboratory (36). No antibody to α41 is presently available. In order to determine developmental changes in β and α39, membranes from hearts 2½, 3½, and 10 days in ovo and 3 days posthatching were subjected to polyacrylamide gel electrophoresis, electrotransferred from the gel to a nitrocellulose filter, incubated either with anti-β or anti-α39 antibody followed by ¹²⁵I protein A as described under “Materials and Methods” and the specific binding of ¹²⁵I protein A determined by autoradiography. Coomassie Blue staining of nitrocellulose filters demonstrated that the electrotransfer was complete (data not shown). The antibody to bovine β cross-reacts with the chick heart proteins as demonstrated by the comigration of bands from chick heart homogenates with β protein purified from bovine brain (Fig. 7a).

Comparison of the relative intensity of β bands in autoradiographs of immunoblots of proteins from chick hearts of various embryonic ages reveals that levels of β were unchanged between day 2½ to 3½ in ovo, increased 2-fold between day 3½ and 10 in ovo, but were unchanged between day 10 and 3 days posthatching (Fig. 7, a and b). The absence of a change in β levels between days 2½ and 3½ in ovo when the extent of ADP-ribosylation of α39 and α41 increased suggested that a developmental increase in β could not account for the increase in ADP-ribosylation of either α39 or α41 between day 2½ to 3½ in ovo. Furthermore, if levels of β were limiting during development, ADP-ribosylation of α41 might be greater or increase more rapidly during development than α39 since α41 from bovine brain has a somewhat higher affinity for β than α39 (19). However, these data do not rule out the possibility that the increase in β between days 3½ and 10 in ovo could contribute at least in part to the increased ADP-ribosylation of α39 between these ages.

Measurement of levels of α39 in embryonic chick heart with antibody to bovine brain α39 demonstrated cross-reactivity between α39 from these species (Fig. 8, a and b). Studies of developmental changes in α39 levels measured by immunoblotting with anti-α39 demonstrated that α39 increased 2-fold between day 2½ and 3½ in ovo, increased 2-fold further between day 3½ and day 10 in ovo, but was unchanged between day 10 in ovo and 3 days posthatching.

Comparison of the quantitation of relative levels of α39 by ADP-ribosylation and immunoblotting (Fig. 9) demonstrated that both methods gave remarkably similar results. By both methods, α39 increased a total of 2.4–2.9-fold between day 2½ in ovo and day 10 in ovo with a half-maximal increase at day 5 in ovo. Differences between α39 determined by the two methods are significant only at day 2½ in ovo. These data support the conclusion that relative levels of α39 measured by ADP-ribosylation accurately reflect developmental changes in α39. Furthermore, since the availability of β and the relative accessibility of α41 and α39 to IAP for ADP-ribosylation appear not to play a role in the extent of ADP-ribosylation, the ADP-ribosylation of α41 may give a good estimation of the relative level of α41.

Developmental Changes in the Number of Muscarinic Receptors—In order to determine whether the increase in muscarinic inhibition of adenylate cyclase activity seen during embryonic development reflected changes in the number of muscarinic receptors, we studied levels of muscarinic receptors as a function of embryonic age. Although we had previously demonstrated that muscarinic receptor number as measured by the binding of [3H]QNB was unchanged between day 2½ to day 18 in ovo (4), studies of receptor number in embryos older than 18 days in ovo had not been carried out. Furthermore, over the last several years, several factors, including...
Fig. 5. a, autoradiography following polyacrylamide gel electrophoresis of embryonic chick heart proteins ADP-ribosylated with pertussis toxin in the presence of $[^{32}P]NAD^+$. Homogenates were prepared and incubated for 30 min at 37 °C with pertussis toxin and $[^{32}P]NAD^+$, solubilized, and subjected to polyacrylamide gel electrophoresis and autoradiography as described under "Materials and Methods." Each lane contains 100 μg of protein as determined by the method of Lowry (33). Molecular weights were determined by plotting the distance migrated in millimeters for standard proteins stained with Coomassie Blue versus the logarithm of their molecular weights. The standards included trypsinogen, carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, egg albumin, bovine albumin, molecular weights 24,000, 29,000, 36,000, 45,000, and 66,000, respectively. Calculated molecular weights for the two bands were 39 ± 0.4 kDa and 41 ± 0.3 kDa (S.E., n = 9). The first lane contains α₃₉ kDa standard purified from bovine brain; lanes 1, 3, 5, and 7 contain homogenates of hearts 2½ days in ovo, 3½ days in ovo, 10 days in ovo, and 3 days posthatching, respectively. Even-numbered lanes contain the same homogenates as in the preceding lane incubated in the absence of islet-activating protein. b, densitometry tracings of autoradiographs of α₃₉ kDa and α₄₁ kDa from a. Autoradiographs were scanned on an LKB laser densitometer and the baseline for scans at each embryonic age superimposed. Data is typical of scans of 10 different determinations. ---, control base line; -- ---, 2½ days in ovo; --- ---, 3½ days in ovo; --- ---, 10 days in ovo; --- --- 3 days posthatched. kD, kilodaltons.
Muscarinic Inhibition of Adenylate Cyclase

Agonists to Inhibit Adenylate Cyclase Activity and Changes in the relative weight at each age determined. Data were normalized approximated by symmetry with the free edge. Peaks were cut out and the overlapping edge of each peak were traced individually and the overlapping edge of each peak.

These data suggest that early in embryonic development muscarinic inhibition of adenylate cyclase activity increases in parallel with the levels of $\alpha_{9}$, while between day 10 in ovo and 3 days posthatching inhibition of adenylate cyclase activity increases in parallel with receptor number. One explanation of these data is that early in embryonic development, muscarinic receptors are present but uncoupled from the muscarinic response because levels of $\alpha_{9}$ in the cell are limiting. Subsequently $\alpha_{9}$ increases to levels sufficient to couple existing receptors to a physiologic response, and the number of muscarinic receptors appears to limit the physiologic response. Since $\alpha_{41}$ increases only 1.5-fold between day 2½ and 3½ in ovo and remains unchanged or even decreases slightly from day 3½ in ovo until 3 days posthatching (see X on Fig. 10), it appears not to parallel the change in physiologic responsiveness. Hence these data suggest that $\alpha_{9}$ may be the form of a which couples the muscarinic receptor to inhibition of adenylate cyclase in the embryonic chick heart.

DISCUSSION

Adenylate cyclase in hearts 2½-3½ days in ovo demonstrates a markedly decreased response to muscarinic inhibition compared to hearts 10 days in ovo or 3 days posthatching. This relative unresponsiveness could be due to 1) the absence of muscarinic receptors in these hearts, 2) the absence of a specific subtype of muscarinic receptors necessary for physiologic activity, 3) the absence of an essential subunit of the muscarinic receptor or post-translational modification of the muscarinic receptor necessary for interaction with N, 4) the absence or abnormality of a subunit of N, either (a) $\alpha_{1}$, the guanine nucleotide-binding protein which couples formation of an agonist-receptor complex to the inhibition of adenylate cyclase activity or (b) $\beta$, which appears to dissociate from N, to mediate inhibition of adenylate cyclase, or 5) decreased sensitivity of the inhibitory process to GTP, Na$^+$, or Mg$^{2+}$. Several of these possible explanations can be easily eliminated. Although muscarinic receptors are present in the embryonic chick heart as early as 2½ days in ovo, the number of receptors does not increase between day 2½ and 10 in ovo when adenylate cyclase inhibition by muscarinic agonists increases 2.6-fold. Hence at least early in embryonic development, a change in the number of muscarinic receptors in the heart does not explain the increased responsiveness of adenylate cyclase in these cells to muscarinic agonists. Data presented in this study also demonstrated that increasing GTP levels up to $10^{-3} \text{ M}$ and/or increasing Na$^+$ concentration to 100 or 150 mM or increasing Mg$^{2+}$ levels from 10 to 24 mM had no effect on the extent of inhibition of adenylate cyclase by carbachol in hearts from embryos 3½ days in ovo. These findings rule out a decreased sensitivity of the inhibitory process to GTP, Na$^+$, or Mg$^{2+}$ as the cause of the decreased inhibitory response of adenylate cyclase activity to muscarinic agonists.

Our previously reported data demonstrate that cells cultured from embryonic chick hearts prior to ingrowth of the vagus nerve remained unresponsive to muscarinic stimulation for up to 9 days in culture (21). In these cultures $10^{-3} \text{ M}$ carbachol had no effect on beating rate and only a small effect on $K^+$ permeability as measured by the rate of $\text{K}^+$ efflux. Guanine nucleotides did not mediate the conversion of high affinity receptors ($R_H$) to a low affinity form ($R_L$) in homogenates of these cultures (21). Following growth for 3
Fig. 7. a, immunoblotting assay of β at various embryonic ages. Proteins were prepared, solubilized, and polyacrylamide gel electrophoresis, immunoblotting, and autoradiography carried out as described under “Materials and Methods.” Equal amounts of protein, 110 μg, were loaded into each lane. Lane 1, immunoblot of purified β from bovine brain; lane 2, immunoblot of samples from hearts 2½ days in ovo; lane 3, 3½ days in ovo; lane 4, 10 days in ovo; and lane 5, 3 days posthatching. This experiment is typical of 5 similar determinations. b, histogram of relative levels of binding of 125I protein A to immunoblots of β. The area of the nitrocellulose which corresponded to the bands seen in the autoradiographs in a was cut out and 125I determined in a γ-counter by the method of Shreftoff. All values were normalized to the value at 2½ days in ovo and plotted as a percentage of the value in the hatched chick taken as 100%. Values are the mean of n determinations; errors are S.E.

Fig. 8. a, immunoblot assay of α38. Proteins were prepared, solubilized, and polyacrylamide gel electrophoresis and immunoblotting carried out as described under “Materials and Methods.” Equal amounts of protein (100 μg) were loaded onto each lane. Lane 1, immunoblot of purified α38 from bovine brain; lane 2, immunoblot of samples from hearts 2½ days in ovo; lane 3, 3½ days in ovo; lane 4, 10 days in ovo; and lane 5, 3 days posthatched. Data are typical of 10 experiments. b, histogram of relative levels of 125I protein A binding to immunoblots of α38. The areas of the nitrocellulose blot which corresponded to the bands on the autoradiographs in a were cut out and 125I determined in a γ-counter by the Shreftoff method. All values were normalized to the value at day 2½ in ovo and the value in the hatched chick taken as 100%. Data represent the mean of n determinations. Errors are the S.E.

days in media supplemented by specific lots of horse serum, these cells developed a K+ permeability response to muscarinic agonists. This development of a physiologic response was associated with a 1½-fold increase in the number of high affinity receptors and the appearance of the ability of guanine nucleotide to convert Rh to Rg. These data suggested that high affinity receptors are necessary for a physiologic response to muscarinic stimulation and that inadequate numbers of high affinity receptors may be present in hearts 2½ to 3½ days in ovo (21).
MUSCARINIC INHIBITION OF ADENYLATED CYCLASE

The finding that \( \alpha \) might be limiting early in embryonic development is intriguing especially in view of the finding that inhibitory guanine nucleotide regulatory proteins appear to be present in high concentrations in tissues such as brain (26). Determination of the relative numbers of \( \alpha_{39} \) and muscarinic receptors would allow direct demonstration of whether the number of receptors present actually exceeds the number of \( \alpha_{39} \) molecules between days 2½ and 10 in ovo. However, such a quantitation is not possible because the extent of cross-reactivity of the antibody to bovine brain \( \alpha_{39} \) with the chick heart \( \alpha_{39} \) is not known. Furthermore, although ADP-ribosylation of \( \alpha_{39} \) gave relative levels of \( \alpha_{39} \) in close agreement with those found by immunoblotting, ADP-ribosylation may not accurately quantitate \( \alpha_{39} \) since the reaction may not go to completion; hence both methods could underestimate \( \alpha_{39} \).

Recently Halvorsen and Nathanson (33) and Malbun et al. (34) have presented data which suggest that \( \alpha_{39} \) and \( \alpha_{41} \) can both be detected in embryonic chick heart and rat heart, respectively. Unlike the data presented here, Halvorsen and Nathanson demonstrated that \( \alpha_{39} \) appears to be absent as late as day 4-5 in ovo and appears at day 6 in ovo. Data on changes in \( \alpha_{39} \) after day 8 in ovo are not presented. Furthermore, these authors were unable to demonstrate an increase in muscarinic inhibition of adenylate cyclase activity during that phase of embryonic development which they studied.

The close parallel between the increase in the number of muscarinic receptors and the increase in inhibition of adenylylate cyclase between day 10 in ovo and 3 days posthatching suggests that there are few "spare" receptors at this point in development. This finding is in agreement with our prior observation (35) that the decrease in muscarinic receptors as measured by the binding of [3H]methylscopolamine following prolonged exposure to agonist closely paralleled the decrease in the ability of agonists to inhibit beating and to increase K+ permeability, suggesting that the number of receptors was critical for the mediation of a physiologic response.

A review of data in Fig. 4 and Table III reveals that although the extent of muscarinic inhibition of adenylylate cyclase activity increases continuously throughout development, the IC50 for carbacholcholine which decreases 26-fold between day 2½ and day 10 in ovo is constant between day 10 in ovo and hatching. One possible explanation for these findings is that increased levels of \( \alpha_{39} \) cause both an increase in coupling of the receptor to inhibition of adenylate cyclase and an increase in the affinity of the receptor for agonist. This might increase both extent of inhibition and IC50 for inhibition. An increase in total receptor number after day 10 in ovo in the presence of levels of \( \alpha_{39} \) adequate to couple these new receptors to inhibition of adenylylate cyclase might increase only the extent of inhibition.

Recently, Hosey et al. (36) presented data which suggested that between day 18 in ovo and hatching, [3H]QNB binding increased. Although these authors saw an increase in the extent of oxotremorine inhibition of basal adenylate cyclase activity from 17 to 26% between day 18 in ovo and hatching, they saw no increase in inhibition of isoproteorehlor-stimulated adenylate cyclase activity in the presence of low levels of \( \beta \) as the cause of a decreased inhibitory effect of muscarinic agonists on adenylylate cyclase early in embryonic development. The level of \( \alpha_{31} \) does not increase during embryonic development (Fig. 5, a and b), and the data presented here do not rule out a role of increased levels of \( \alpha_{31} \) in coupling muscarinic receptors to inhibition of adenylylate cyclase. However, of all these parameters, changes in the level of \( \alpha_{39} \) appear to correlate best with the increase in muscarinic inhibition of adenylylate cyclase seen between days 2½ and 10 in ovo.
Muscarinic Inhibition of Adenylate Cyclase

Bruce T. Liang, Mark R. Hellmich,* Eva J. Neer and Jonas B. Galper

Muscarnic inhibition of adenylate cycle activity between day 13 in ovo and hatching. The studies presented here demonstrate a gradual increase of muscarinic receptor number from day 12 in ovo until 3 days after hatching and a parallel increase in muscarinic inhibition of both basal and isoproterenol-stimulated adenylate cyclase activity.

The finding that muscarinic inhibition of β-adrenergic stimulated adenylate cyclase activity is a non-competitive process rules out the possibility that the ligands compete directly at the receptor or that the two guanine nucleotide regulatory proteins compete for the same site on the catalytic unit of adenylate cyclase. Hence they exert their effects on adenylate cyclase activity either by independent actions directly on the catalytic subunit of adenylate cyclase or by an interaction between Na₃ and Na₄ or both. These observations are in close agreement with the model presented by Katada et al. (37) which suggests that agonist binding to an inhibitory receptor in the presence of GTP results in the release of β from Na₃ which can then associate with Na₄ to deactivate it.

The functions of α₃ₙ and α₄ₙ have not been well established. However, in many systems, α₃ₙ has been the predominant ADP-ribosylated protein detected when pertussis toxin blocks hormonal inhibition of adenylate cyclase (17, 19). Several laboratories have reported the presence of both α₃ₙ and α₄ₙ in the heart (33, 34). The data presented here do not establish which of these α proteins couple muscarinic stimulation to inhibition of adenylate cyclase. Data have been presented by other groups suggesting that purified α₃ₙ and α₄ₙ, reconstituted with solubilized receptors both couple muscarinic receptors to effects of GTP on affinity for agonist (32). However, the close correlation of an increase of α₃ₙ with increased adenylate cyclase inhibition suggests that in the chick heart, α₃ₙ might be responsible for this interaction with adenylate cyclase.

Recent evidence suggests that guanine nucleotide coupling proteins may be involved in a number of functions unrelated to cyclic AMP production. These proteins may be involved in stimulation of phosphoinositide turnover (38), Ca²⁺ movements in guinea pig neutrophils, and release of arachadonic acid (39). Hence, α₃ₙ and α₄ₙ may interact not only with adenylate cyclase but may also serve several independent functions in the embryonic chick heart. The decreased level of muscarinic responsiveness of the embryonic chick heart and its association with decreased levels of α suggest that the developing chick heart could offer an interesting model for the study of the interaction of α proteins with the adenylate cyclase system and other regulatory processes in the heart.

### References


### Supplementary Material

To: Development of Muscarinic Cholinergic Inhibition of Adenylate Cyclase

**Materials**

ATP, GTP, creatine phosphate, creatine kinase, lima bean trypsin inhibitor, soybean trypsin inhibitor, 1,10-phenanthroline, L-isoproterenol, acetylcholine sulfate, carbamylcholine, N,N-bis(methylamido)ethanol, all obtained from Sigma. α₃ₙ protein A was from Amerham. [³H]ATP was from Amerham/Searle. Cyclic [³P]AMP was from New England Nuclear. [³H]GTP was from ICN and islet activating protein (IAP) was from List Biochemicals, Campbell, CA. Bovine albumin was from Miles Scientific Naperville, IL. Leupatin was generously supplied by Dr. A. L. Goldberg, (Harvard Medical School). Embryonated chicken eggs were purchased from SPFavs, Inc.

**METHODS**

Development of Muscarinic Cholinergic Inhibition of Adenylate Cyclase

Its Relationship to Changes in the Inhibitory Guanine Nucleotide Regulatory Protein

Bruce T. Liang, Mark R. Hellmich,* Eva J. Neer and Jonas B. Galper**
atropine were added in the order and the concentrations indicated in the text. Following incubation for 10 min at 37°C diluting solution containing unlabeled cAMP, ATP and \(^{32}P\)-cAMP was added and the reaction mixture boiled for two min., loaded onto Dowex AG50X2 (Biorad, Inc.) columns and the cAMP peak collected. The inhibition of remaining adenosine and guanine nucleotides was accomplished by Ba(OH)\(_2\) and ZnSO\(_4\) precipitation. Following Ba(OH)\(_2\) and ZnSO\(_4\), precipitation recovery of \(^{32}P\)-cAMP was determined by measurement of the ratio of \(^{32}P\) to \(^{14}C\) in an aliquot of the supernatant in a Beckman liquid scintillation counter. Recoveries of \(^{32}P\)-cAMP were usually 40%.

Preparation of tissue: Hearts of chick embryos of the indicated gestational age were suspended in TMSD buffer (50 mM Tris-HCl, 1 mM EDTA, 0.2 M sucrose and 0.6 mM dithiothreitol, and 75 mM NaCl), Dounce homogenized and filtered through gauze at 4°C. The filtrate was adjusted approximately 0.5 mg protein/cc and 50 uL samples assayed in each tube. In some experiments the filtrate was centrifuged at 30,000g for 15 min and resuspended in the same buffer at the concentration indicated.

Effect of protease inhibitors and alamethicin on muscarinic inhibition of adenylate cyclase activity.

Inhibition of adenylate cyclase activity by carbamylcholine varied significantly from preparation to preparation. The addition of a mixture of protease inhibitors [10 mM iodoacetate, 10 mM iodoacetamide, 0.1 M leupeptin, 0.18 mg/ml, 1.10 phenanthroline and 1.4 mM EDTA markedly improved reproducibility. Several groups have indicated that hormone mediated inhibition of adenylate cyclase activity was sensitive to trypsin and other proteases (24). The extent of inhibition of isoproterenol-stimulated adenylate cyclase activity was more reproducible (40-50%), especially in older age embryos, in the presence of the surface active antibiotic alamethicin (14 uM). All experiments were carried out in the presence of protease inhibitors and alamethicin unless otherwise indicated.

ADP-ribosylation with Pertussis Toxin.

ADP-ribosylation was carried out for the times indicated at 37°C. The assay mixture contained 10 uM NAD+, 0.3-0.5 uCi \(^{32}P\)-NAD, 2.5 mM ATP, 2 mM GTP, 10 uM isonitrazid, 10 mM thymidine, 10 mM MgCl\(_2\) and 25 mM pertussis toxin, 10 mM phosphocreatine, 64u, creatine phosphokinase in a total volume of 25 uL. Tissue homogenized in TMSD buffer was added to the reaction mixture at 50-100 ug/sample. Protease inhibitors, soybean trypsin inhibitor, leupeptin and leupeptin were present at the concentrations described previously from the time of initial homogenization of the tissue. The reaction was stopped by the addition of 25% SDS in Laemmli sample buffer (25) followed by boiling for 1 min. Analysis of peptides following ADP-ribosylation was performed on 9% acrylamide gels prepared according to Laemmli (26). The gels were exposed to Kodak XAR film with or without enhancing screens for 1-2 days at -70°C.

Immunoblotting: Antibodies were prepared and characterized as described previously (26). Immunoblotting was carried out by a modification of the method of Towle et al. (27). Heart proteins were solubilized in 2% SDS in Laemmli sample buffer as described under ADP-ribosylation and separated by SDS-polyacrylamide gel electrophoresis on 9% acrylamide gels. The gels were equilibrated in transfer buffer 20 mM Tris-\(\text{HCl}\), 150 mM glycine, 0.015% SDS and 20% methanol for 30 min. The proteins were then electrophoretically transferred to nitrocellulose at 30V overnight. The dried filters were incubated with 3% BSA. In 10 mM Tris, pH 7.6, 150 mM NaCl for 60 min at 22°C to decrease nonspecific binding, followed by incubation overnight at 4°C with antiserum diluted 1:200 with the same buffer. Nitrocellulose was then washed twice with 10 mM Tris, 150 mM NaCl for 10 min, once with 10 mM Tris, 150 mM NaCl and 0.05% Tween 80 for 20 min, and again with 10 mM Tris, 150 mM NaCl for 10 min. Protein A (100,000-150,000 cpm/ml) in 10 mM Tris, 150 mM NaCl, 3% BSA was incubated with blocks for 1 hr and the wash procedure repeated. After drying, blots were exposed to Kodak XAR film with enhancing screens for 1-5 days at -70°C.

Protein Assay: Proteins were determined according to the method of Lowry et al. using bovine serum albumin as the standard (28).

RESULTS

Further characterization of muscarinic inhibition of adenylate cyclase activity

Effects of Na+ and GTP on muscarinic inhibition of \(\beta\)-adrenergic stimulated adenylate cyclase in the embryonic chick heart.

Hormonal inhibition of adenylate cyclase activity mediated via opiate receptors in brain, \(\beta\)-adrenergic receptors in the platelet (29) and muscarinic receptors in the heart (6) has been shown to be dependent on Na+ ions. Although Na+ has little effect on GTP-stimulated adenylate cyclase activity in chick heart homogenates (Table 1), inhibition of carbamylcholine was barely significant in Na+-free medium and increased nearly 3-fold in 80 mM Na+. The concentration of GTP required for half-maximal inhibition of adenylate cyclase activity has been shown to be 5-fold greater than that for stimulation of adenylate cyclase (8). Studies of GTP dependence of inhibition of adenylate cyclase activity in homogenates of embryonic chick heart has been complicated by the presence of high levels of endogenous guanine nucleotides in the crude samples studied here. Efforts to wash membrane pellets and to fractionate the membrane preparations led to large losses in responsiveness of adenylate cyclase to inhibition by muscarinic agonists. However, studies carried out on crude homogenates of embryonic chick heart demonstrated a 3-fold increase in carbamylcholine mediated inhibition of adenylate cyclase as GTP concentration was varied from 0 to \(10^{-6}\)M with a half-maximal effect at 7 uM GTP.

Kinetics of inhibition of isoproterenol stimulated adenylate cyclase activity.

Although the finding that muscarinic inhibition of adenylate cyclase is only partial even at high concentrations of muscarinic agonist suggests that inhibition is non-competitive, the mechanism of inhibition has not been rigorously established by classical analysis of competition data by Lineweaver-Burke plots.

The effect of various doses of carbamylcholine on the concentration effect curve for isoproterenol stimulation of adenylate cyclase activity is presented in the double reciprocal plot shown in Fig. 3. The finding that these curves intersect at a single point on the x-axis is consistent with the interpretation that carbamylcholine decreased the extent of stimulation by isoproterenol, without affecting the \(K_I\) for the binding of isoproterenol at the \(\beta\)-adrenergic receptor. However, even at the highest concentrations studied, carbamylcholine was not capable of totally inhibiting isoproterenol stimulated adenylate cyclase activity (Figs. 2 and 3). Hence carbamylcholine is a partial non-competitive inhibitor of isoproterenol stimulated adenylate cyclase activity. The effectiveness of an inhibitor is given by the equation

\[
\frac{V}{V-V'} = \frac{1}{1 + \frac{[I]}{K_I}}
\]

where \(V\) is the velocity in the absence of inhibition, \(V'\) is the velocity in the presence of inhibitor at concentration \([I]\), \(V'\) is the velocity in the presence of a large excess of inhibitor (30). The values of \(1/V, 1/V'\) and \(1/\sqrt{V-V'}\) are given by the intersection with the vertical axis of a plot of \(1/\text{velocity}~\text{versus}~1/\text{isoproterenol concentration}\) in the presence of the appropriate concentration of inhibitor. The value for \(K_I\) derived form the data in Fig. 3 was \(1\times10^{-8}\)M for \(I = 10^{-7}\)M carbamylcholine, and \(V'\) the velocity in the presence of \(10^{-7}\)M carbamylcholine.

Fig. 3. Stimulation of adenylate cyclase by various concentrations of isoproterenol in the presence of several concentrations of carbamylcholine. Hearts from embryos 10 days in age were homogenized and adenylate cyclase activity assayed at the indicated concentrations of isoproterenol as described in Methods. GTP and Na+ were 100 uM and 80 mM respectively. Carbamylcholine was either absent, \(\bullet\), or present at 1 uM, \(\triangle\); or 10 uM, \(\bigtriangleup\). Data were plotted as the inverse of the concentration of isoproterenol (M) \(10^{-10}\) versus the inverse of the stimulation of adenylate cyclase activity above GTP-stimulated levels in pmol/10 min/mg protein x 10^5.