Tissue-Specific Regulation of GTP-Binding Protein and Muscarinic Acetylcholine Receptor Levels during Cardiac Development†

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ABSTRACT: A quantitative immunoblot assay was developed by using affinity-purified monospecific antibodies to quantitate levels of guanine nucleotide binding regulatory protein (G-protein) subunits in atria and ventricles during embryonic chicken cardiac development. The muscarinic acetylcholine receptor (mAChR) number was measured with [^{3}H]quinuclidinyl benzilate. On day 10 of embryonic development (day 10E) there was no difference between the atrial and ventricular membrane concentrations of β-subunit, $G_0\alpha$ subunit, or mAChR. The level of $G_i\alpha$ was found to be 44% greater in atria than in ventricles on day 10E. The atrial membrane concentration of β-subunit increased 80% between day 13E and 15E, $G_0\alpha$ increased 46% between day 10E and 15E, mAChR increased 61% between day 10E and 12E, and $G_i\alpha$ decreased 34% between day 10E and 13E. The atrial levels of β-subunit, $G_0\alpha$, $G_i\alpha$, and mAChR did not change further through day 20E. The ventricular membrane concentration of these proteins did not change between day 10E and 20E, except for that of $G_0\alpha$, which increased 47% between day 15E and 20E. The atrial specific increase in β-subunit correlated with a loss of GTP inhibition of basal adenylate cyclase activity. The difference in $G_i\alpha$ levels between atria and ventricles on day 10E correlated with a difference in carbachol sensitivity of atrial and ventricular basal adenylate cyclase activity. Thus, the levels of several components of the cholinergic neuroeffector pathway are regulated in a tissue-specific manner at a time that coincides with the onset of functional parasympathetic innervation of the embryonic chicken heart. These changes are associated with functional effects on the regulation of adenylate cyclase activity.

Guanine nucleotide binding regulatory proteins (G-proteins)[1] couple membrane receptors to a variety of effector mechanisms including adenylate cyclase (G_s and G_i), phosphodiesterase (transducin), phosphatidylinositol 4,5-bisphosphate hydrolysis, and ion channels (Gilman, 1984; Stryer et al., 1981; Cockcroft & Gomperts, 1985; Blackmore et al., 1985; Pfaffinger et al., 1985; Breitwieser & Szabo, 1985). G-proteins are heterotrimers composed of an α-, β-, and γ-subunit. The α-subunit ranges in size from 39 000 to 52 000 Da and is unique for each G-protein. The α-subunits have GTPase activity and a bacterial toxin ADP-ribosylation site (Gilman, 1984; Sternweis & Robishaw, 1984; Mumby et al., 1986). Each α-subunit appears to be associated with a common β-subunit (Gilman, 1984; Manning & Gilman, 1983). A 35 000- and 36 000-Da form of the β-subunit have been observed (Sternweis & Robishaw, 1984), although the significance of this is not known. Each G-protein also contains a γ-subunit of approximately 8000 Da, about which little is known (Hildebrandt, 1984), though the γ-subunit of transducin is immunologically distinct from the γ-subunits of other G-proteins (Gierschik et al., 1985; Roof et al., 1985).

An additional G-protein (G_0) has been observed in brain (Sternweis & Robishaw, 1984; Neer et al., 1984), heart (Halvorsen & Nathanson, 1984), and other tissues (Mumby et al., 1986; Huff et al., 1985). G_0 is also a heterotrimer with an α-subunit of 39 000 Da that, like $G_i\alpha$, can serve as an ADP-ribosylation substrate for islet-activating protein (IAP), a toxin of *Bordetella pertussis*. Both G_i and G_0 have been shown to interact with muscarinic acetylcholine receptors (mAChR) (Florio & Sternweis, 1985; Kurose et al., 1986; Haga et al., 1986). G_i couples receptors to adenylate cyclase, and a role for both the α-subunit and the $\beta\gamma$-complex has been postulated (Katada et al., 1984, 1986). An IAP substrate couples cardiac mAChR to a K^+ channel (Pfaffinger et al., 1985; Martin et al., 1985), and a G_i-like protein isolated from human erythrocytes has been shown to activate the cardiac muscarinic K^+ channel (Yatani et al., 1987). It has been

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[1] Abbreviations: G-protein, guanine nucleotide binding regulatory protein; $G_s\alpha$, α-subunit of the stimulatory G-protein of adenylate cyclase; $G_i\alpha$, α-subunit of the inhibitory G-protein of adenylate cyclase; $G_0\alpha$, 39 000-Da α-subunit of unknown function; $T\alpha$, α-subunit of transducin; mAChR, muscarinic acetylcholine receptor(s); NAD, nicotinamide adenine dinucleotide; QNB, quinuclidinyl benzilate; IAP, islet-activating protein, a toxin of *Bordetella pertussis*; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TWEEN 20, poly(oxyethylenesorbitan monolaurate); PAGE, polyacrylamide gel electrophoresis; EC_{50}, concentration producing 50% effect; BSA, bovine serum albumin.

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suggested that the $\beta\gamma$-complex and/or the α-subunit activates the cardiac muscarinic K^+ channel (Logothetis et al., 1987; Codina et al., 1987), while the α-subunit of G_0 has been implicated in the functional coupling of opiate receptors to neuronal voltage-dependent Ca^{2+} channels (Hescheler et al., 1987).

During cardiac development in embryonic chickens, the level of mAChR increases in atria but not ventricles (Kirby & Aronstam, 1983). This tissue-specific increase occurs at a time that coincides with the onset of functional parasympathetic innervation of the heart (Pappano, 1977), suggesting a role for the process of innervation in the regulation of cardiac mAChR. Since mAChR are coupled to various effector mechanisms by G_i and G_0, we sought to determine if subunits of these G-proteins are regulated similarly to mAChR and what functional effects changes in G-protein subunit levels might have. We report here the use of affinity-purified monospecific antibodies to quantitate the levels of G-protein subunits in atria and ventricles during cardiac development. The effect of changes in G-protein subunit levels on the regulation of adenylate cyclase activity is also examined.

Experimental Procedures

Materials

White leghorn chicken embryos were obtained locally from College Biologicals, Bothell, WA, and maintained at 38 °C in a humidified incubator. Embryonic ages were staged as described by Hamburger and Hamilton (1951). Phenylmethanesulfonyl fluoride, 1,10-phenanthroline, iodoacetamide, pepstatin A, TWEEN 20, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, *p*-nitro blue tetrazolium chloride, and *p*-nitrophenyl phosphate disodium salt were from Sigma, St. Louis, MO. Nitrocellulose was from Schleicher & Schuell, Inc., Keene, NH. Alkaline phosphatase conjugated goat anti-rabbit IgG was from Cappel, Cooper Biomedical, Malvern, PA. IAP was a generous gift from Jennifer Martin and was purified from the 24-h culture supernatant of *B. pertussis* (Tahoma phase I) by the method of Sekura et al. (1983). [α-^{32}P]NAD was synthesized from [α-^{32}P]ATP as described by Cassel and Pfeuffer (1978). All other chemicals were of reagent grade and were obtained from previously described sources (Halvorsen & Nathanson, 1984).

Methods

Preparation of Antisera. Rabbit antiserum RV3 was produced by immunization with partially purified G-proteins (Gierschik et al., 1986b). Rabbit antiserum AS7 was produced by immunization with the C-terminal decapeptide of the α-subunit of bovine transducin (Fallon et al., 1986).

Membrane Preparation. Embryonic chicken hearts were removed and separated into atria and ventricles in ice-cold PBS (20 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) containing protease inhibitors (0.4 mM phenylmethanesulfonyl fluoride, 1 mM 1,10-phenanthroline, 1 mM iodoacetamide, 1 μM pepstatin A), homogenized with a Brinkman Polytron at setting 6 for 20 s, and centrifuged at 80*g* for 15 min. Membranes were then pelleted from the supernatant by centrifugation at 17600*g* for 15 min, washed, and recentrifuged. The membranes were then resuspended and stored at −80 °C until use. Bovine cerebral cortex membranes were prepared as described by Hurko (1978). Partially purified G-proteins were prepared as described by Milligan and Klee (1985). Bovine transducin was purified as described (Gierschik et al., 1985).

IAP Labeling. Day 15E atrial membranes were prepared as above except that the protease inhibitors were omitted. IAP labeling of membrane proteins was carried out as previously described (Halvorsen & Nathanson, 1984) except that 0.45 μg of purified IAP was used in each reaction. Autoradiography was performed by using Kodak X-Omat film at −80 °C with a Cronex intensifying screen.

SDS Gel Electrophoresis. SDS–PAGE was performed by using a modification (Nathanson & Hall, 1979) of the discontinuous system of Laemmli (1970). The following proteins were used as molecular weight (in parentheses) standards: β-galactosidase (116 000), bovine serum albumin (68 000), pyruvate kinase (57 000), fumarase (49 000), aldolase (40 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), and carbonic anhydrase (29 000).

Quantitative Immunoblot Analysis. Proteins were electrophoretically transferred to nitrocellulose by using the method of Towbin et al. (1979). The nitrocellulose was then either stained with amido black or incubated with PBS containing 10% bovine hemoglobin for 1 h at room temperature and rinsed with distilled water. The nitrocellulose was then incubated with rabbit antisera or affinity-purified antibodies diluted in PBS and 0.5% TWEEN 20 (TPBS) overnight at room temperature. Following several rinses in TPBS, the nitrocellulose was incubated with alkaline phosphatase conjugated goat anti-rabbit IgG diluted in TPBS for 2 h at room temperature. Bound antibodies were then visualized as described by Smith and Fisher (1984) by incubating the nitrocellulose in 50 mM sodium glycinate, pH 9.6, 0.1 mg/mL *p*-nitro blue tetrazolium chloride, 0.05 mg/mL 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, 4 mM $MgCl_2$, and 0.5% TWEEN 20. The reaction was stopped after 5–10 min by rinsing with TPBS. Monospecific antibodies were affinity purified from the polyclonal antisera by using the method of Smith and Fisher (1984). Briefly, the immunostained protein band of interest was cut from the nitrocellulose, and the bound antibodies were eluted with three 30-s washes with 5 mM glycine-HCl, pH 2.3, 500 mM NaCl, 100 μg/mL BSA, and 0.5% TWEEN 20, followed by three 30-s washes with TPBS and 100 μg/mL BSA. Eluates were neutralized by addition of 0.5 M Na_2HPO_4, pH 8.6, to a final concentration of 50 mM. Quantitation of immunostained bands was performed by using affinity-purified antibodies at dilutions that were previously determined to be saturating in control experiments (data not shown). Each immunostained band was cut from the nitrocellulose, placed in a well of a 96-well, flat bottom, microtiter plate, and incubated with 100 μL of 1M Tris-HCl, pH 8.0, and 0.2 mg/mL *p*-nitrophenyl phosphate. After 10–20 min, the reaction was stopped by the addition of 100 μL of 13% K_2HPO_4 and the optical density of the solution determined at 410 nm. Comparing sample values with a standard curve generated by using known amounts of G-protein subunits allowed the concentration of G-protein subunits in the samples to be determined. Standard curves used in these experiments were prepared by using either partially purified G-protein samples, whose subunit concentrations were determined by densitometry, or bovine cerebral cortex membrane samples, whose G-protein subunit content had been previously determined by comparison with standard curves of purified G-protein subunits.

Assay of mAChR. Atrial and ventricular membranes were prepared as described above except that the buffer was 50 mM sodium phosphate, pH 7.4. The muscarinic antagonist [^{3}H]quinuclidinyl benzilate (QNB) was used in a modification of the filter-binding technique of Yamamura and Snyder (1974) as described (Halvorsen & Nathanson, 1981).

Adenylate cyclase activity in atrial and ventricular membranes was determined as previously described (Halvorsen &

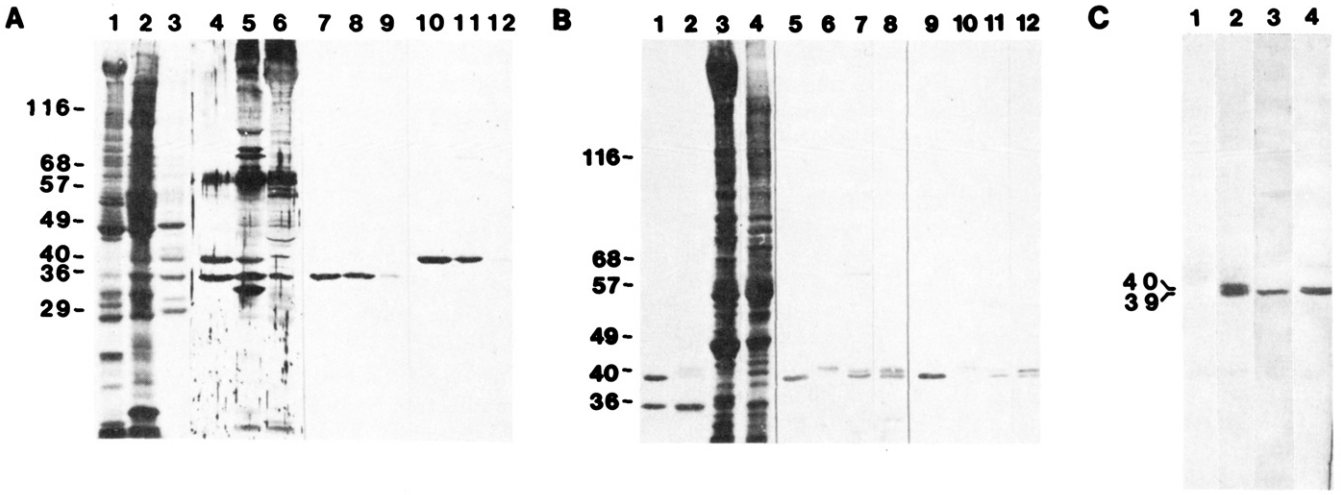

FIGURE 1: Immunoblot analysis of polypeptides recognized by antisera RV3 and AS7 and by affinity-purified antibodies. (A) Comparison of polypeptides recognized by antiserum RV3 and affinity-purified anti-β and anti-$G_0\alpha$ antibodies. Samples were subjected to SDS–PAGE on a 10% separation gel and transferred to nitrocellulose as described under Experimental Procedures. The nitrocellulose was stained with amido black (lanes 1–3) or incubated with antiserum RV3 (lanes 4–6), affinity-purified anti-β (lanes 7–9), or affinity-purified anti-$G_0\alpha$ antibodies (lanes 10–12). Bound antibody was visualized as described under Experimental Procedures. Lanes 3, 4, 7, and 10 contain 5.5 μg of partially purified G-proteins from bovine brain, and lanes 2, 5, 8, and 11 contain 75 μg of bovine cerebral cortex membranes. Lane 1 contains 75 μg, lanes 6 and 9 contain 150 μg, and lane 12 contains 300 μg of day 15E chicken atrial membranes. (B) Comparison of polypeptides recognized by antiserum AS7 and affinity-purified anti-Tα antibodies. Samples were treated as described above except that an 8% separation gel was used. The nitrocellulose was stained with amido black (lanes 1–4) or incubated with antiserum AS7 (lanes 5–8) or affinity-purified anti-Tα antibodies (lanes 9–12). Lane 1 contains 1 μg and lanes 5 and 9 contain 200 ng of bovine transducin. Lane 2 contains 2.5 μg and lanes 6 and 10 contain 300 ng of partially purified G-proteins from bovine brain. Lanes 3, 7, and 11 contain 100 μg of day 15E chicken atrial membranes. Lanes 4, 8, and 12 contain 40 μg of embryonic chicken brain membranes. (C) Comparison of IAP substrates with polypeptides recognized by affinity-purified anti-$G_0\alpha$ and anti-Tα antibodies. Membranes were incubated with [α-^{32}P]NAD in the absence (lane 1) and presence (lane 2) of IAP, subjected to SDS–PAGE, transferred to nitrocellulose, and visualized by autoradiography as described under Experimental Procedures. Lanes 3 and 4 show the results of probing lanes 1 and 2 with affinity-purified anti-$G_0\alpha$ (lane 3) or anti-Tα (lane 4) antibodies. All lanes contain 200 μg of day 15E chicken atrial membranes.

Nathanson, 1984). Protein concentration was determined by the method of Lowry et al. (1951) as modified previously (Halvorsen & Nathanson, 1981). Statistical significance was determined by using a two-sample t-test following an F-test to ensure equality of variance. For samples with unequal variance ($p > 0.05$), statistical significance was determined by using a two-sample t-test for samples with unequal variance (Cochran's method).

Results

Antiserum RV3 was raised against purified G-proteins from bovine cerebral cortex and has been previously shown to contain antibodies that recognize the 39 000-Da α-subunit of G_0 (but not the 41 000-Da α-subunit of G_i) and the β-subunit of bovine G_0, G_i, G_s, and transducin (Gierschik et al., 1986b). Antiserum RV3 clearly recognizes the 36 000-Da β-subunit; however, it is unclear whether the 35 000-Da β-subunit is also recognized (Sternweis & Robishaw, 1984; Roof et al., 1985). Antiserum AS7 was raised against the C-terminal decapeptide of the α-subunit of bovine transducin (Fallon et al., 1986) and recognizes the transducin α-subunit as well as the α-subunit of G_i.

Figure 1A (lanes 4–6) shows that antiserum RV3 recognizes the β- and $G_0\alpha$-subunits in a partially purified sample of G_i and G_0, bovine cerebral cortex membranes, and embryonic chicken atrial membranes. Figure 1B (lanes 5–8) shows that antiserum AS7 recognizes the α-subunit of bovine transducin (Tα), the α-subunit of G_i in a partially purified sample of G_i and G_0 from bovine cerebral cortex, and the α-subunit of G_i in embryonic chicken atrial membranes. Both antiserum RV3 and, to a lesser extent, antiserum AS7 recognize many polypeptides in crude membrane preparations (Figure 1A, lanes 5 and 6; Figure 1B, lanes 7 and 8). This necessitated the purification of anti-β subunit and anti-$G_0\alpha$ subunit antibodies from antiserum RV3 and antitransducin α-subunit antibodies from antiserum AS7 in order to reliably identify the β-, $G_0\alpha$-, and $G_i\alpha$-subunits in crude membrane samples. Monospecific antibodies against the β- and $G_0\alpha$-subunits were affinity purified from immunoblots of bovine cerebral cortex membranes, and antibodies against the transducin α-subunit were purified from immunoblots of bovine transducin by elution from the nitrocellulose with a series of low and neutral pH washes (Smith & Fisher, 1984). The purified antibodies exhibited a high degree of specificity for the detection of G-protein subunits even in crude membrane preparations.

The purified anti-β antibodies recognized the β-subunit in samples of partially purified G_i and G_0, cerebral cortex membranes, and embryonic chicken atrial membranes (Figure 1A, lanes 7–9). The anti-$G_0\alpha$ antibodies recognized the $G_0\alpha$-subunit in samples of partially purified G_i and G_0, cerebral cortex membranes, and embryonic chicken atrial membranes (Figure 1A, lanes 10–12). The purified anti-Tα antibodies recognized the α-subunit of bovine transducin (Figure 1B, lane 9) and the α-subunit of G_i in a partially purified sample of G_i and G_0 from bovine cerebral cortex and in embryonic chicken atrial membranes (Figure 1B, lanes 10 and 11). To ensure that the polypeptides recognized by the affinity purified antibodies in embryonic chick cardiac membranes were in fact immunologically cross-reactive with the G-protein subunits from bovine cerebral cortex, antibodies were affinity purified from immunoblots of embryonic chicken atrial membrane β, $G_0\alpha$, or $G_i\alpha$. These antibodies were then shown, in immunoblot analysis, to be able to recognize β, $G_0\alpha$, or $G_i\alpha$, respectively, in a partially purified sample of bovine cerebral cortex G-proteins (data not shown). Both antiserum AS7 and the affinity-purified anti-Tα antibodies also recognized a 39 000-Da polypeptide in embryonic chicken atrial membranes (Figure 1B, lanes 7 and 11) and in embryonic chicken brain membranes (Figure 1B, lanes 8 and 12). Antibodies eluted from this polypeptide recognized $G_i\alpha$ and not $G_0\alpha$ in a sample

of purified G-proteins from bovine cerebral cortex (data not shown). This polypeptide may be the α-subunit of G_0 or the α-subunit of an additional G_i-like protein.

The polypeptide recognized by the anti-$G_0\alpha$ antibodies comigrated with the 39 000-Da IAP substrate in embryonic chicken atrial membranes (Figure 1C). This demonstrates that the two IAP substrates in embryonic chick heart are immunologically distinct and that the 39 000-Da IAP substrate is immunologically similiar to $G_0\alpha$ from bovine brain. The 40 000- and 39 000-Da polypeptides recognized by the anti-Tα antibodies comigrated with the 40 000- and 39 000-Da IAP substrates, respectively, in embryonic chicken atrial membranes.

To determine if immunoblot analysis could be used to quantitate the levels of β-, $G_0\alpha$-, and $G_i\alpha$-subunits, we subjected increasing concentrations of partially purified G-proteins to immunoblot analysis using saturating concentrations of the affinity-purified anti-β, anti-$G_0\alpha$, or anti-Tα antibodies and then measured band density as described under Experimental Procedures. Optical density was proportional to the amount of partially purified β, $G_0\alpha$, or $G_i\alpha$ loaded onto the gel (Figure 2). The relationship was linear between 50 and 800 ng of β, between 100 and 500 ng of $G_0\alpha$, and between 20 and 150 ng of $G_i\alpha$. Optical density was also proportional to the amount of bovine cerebral cortex membranes and embryonic chicken atrial membranes loaded onto the gel (data not shown). In order to ensure the accuracy of the immunoblot assay, it was necessary to determine whether the embryonic chicken cardiac G-protein subunits were recognized by the same set of antibodies that recognized the purified bovine cerebral cortex G-protein subunits, which were to be used as quantitation standards. If, for example, the chick cardiac G-protein subunits were recognized by only a subset of the antibodies that recognize the purified bovine cerebral cortex G-protein subunits, then the level of G-protein subunits in the chick cardiac membranes would be underestimated. Embryonic chicken atrial β, $G_0\alpha$, or $G_i\alpha$ immobilized on nitrocellulose (cut from immunoblots) was incubated with the affinity-purified anti-β, anti-$G_0\alpha$, or anti-Tα antibodies, respectively. Subsequent immunoblot analysis showed that all immunoreactivity toward purified bovine cerebral cortex β, $G_0\alpha$, or $G_i\alpha$ had been removed from the affinity-purified anti-β, anti-$G_0\alpha$, or anti-Tα antibodies, respectively (data not shown). Nitrocellulose containing irrelevant proteins (cut from immunoblots) was not capable of adsorbing any immunoreactivity. This indicates that each G-protein subunit in embryonic chicken atrial membranes is recognized by the same set of antibodies, in the affinity-purified antibody samples, that recognize that G-protein subunit in bovine cerebral cortex.

Using these affinity-purified antibodies in the quantitative immunoblot assay, we determined the levels of β-, $G_0\alpha$-, and $G_i\alpha$-subunits in chicken atrial and ventricular membranes at various stages of embryonic development (Figure 3). On day 10 of embryonic development (day 10E) there was no difference between the β-subunit concentrations in atrial [55.7 ± 8.9 pmol/(mg of protein)] and ventricular [53.1 ± 10.9 pmol/(mg of protein)] membranes (Figure 3A). These levels did not change between day 10E and day 13E. Between day 13E and day 15E the amount of β-subunit in atrial membranes increased by 80%, while the amount in ventricular membranes remained constant. The atrial membrane β-subunit level remained elevated with respect to the ventricular membrane level on day 20E.

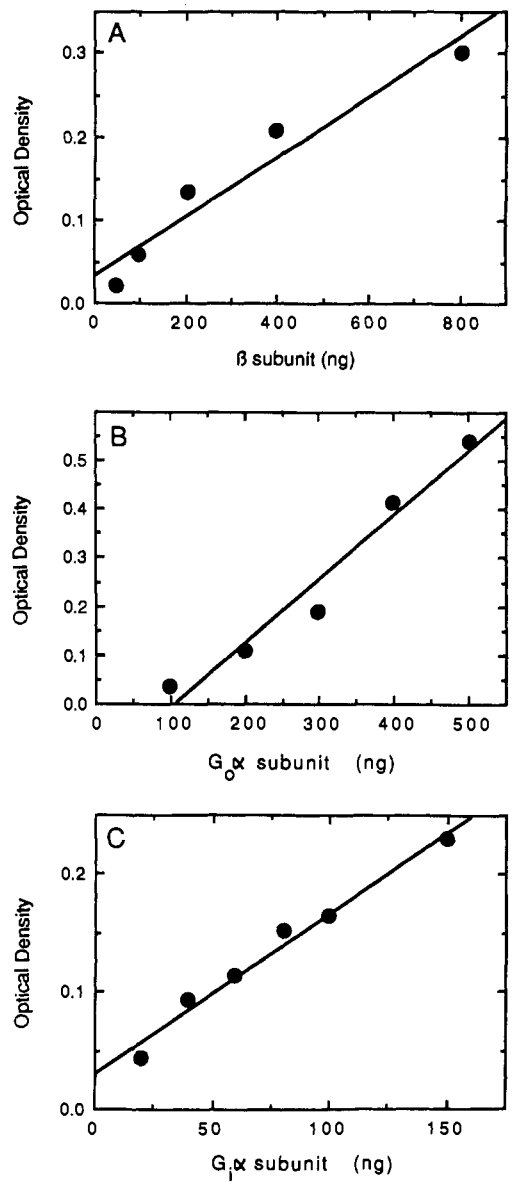

FIGURE 2: Quantitative immunoblot analysis of partially purified G-proteins. Increasing concentrations of partially purified G-proteins were subjected to immunoblot analysis as described under Experimental Procedures by using saturating concentrations of the affinity-purified anti-β (A), anti-$G_0\alpha$ (B), or anti-Tα subunit antibodies (C). The concentration of β, $G_0\alpha$, and $G_i\alpha$ in the G-protein sample was determined by densitometry. Results are the mean of duplicate determinations, which usually varied less than 10% from the mean.

On day 10E there was no difference between the $G_0\alpha$-subunit levels in atrial [8.8 ± 1.5 pmol/(mg of protein)] and ventricular [9.0 ± 0.9 pmol/(mg of protein)] membranes (Figure 3B). Between days 10E and 15E the $G_0\alpha$-subunit level in atrial membranes increased by 46%, while the level of $G_0\alpha$-subunit in ventricular membranes did not change. Between days 15E and 20E the amount of $G_0\alpha$ subunit increased by 14% in atrial membranes and by 47% in ventricular membranes. On day 20E the levels of $G_0\alpha$ were not significantly different between atrial and ventricular membranes.

Figure 3C shows that on day 10E the level of $G_i\alpha$ was 44% greater in atrial [22.1 ± 1.7 pmol/(mg of protein)] than in ventricular [15.3 ± 1.6 pmol/(mg of protein)] membranes. Between days 10E and 13E the level of $G_i\alpha$ in atrial membranes decreased by 34%, while the ventricular membrane level did not change. There was no significant difference between the atrial and ventricular membrane $G_i\alpha$ levels on day 13E, and these levels did not change between day 13E and day 20E.

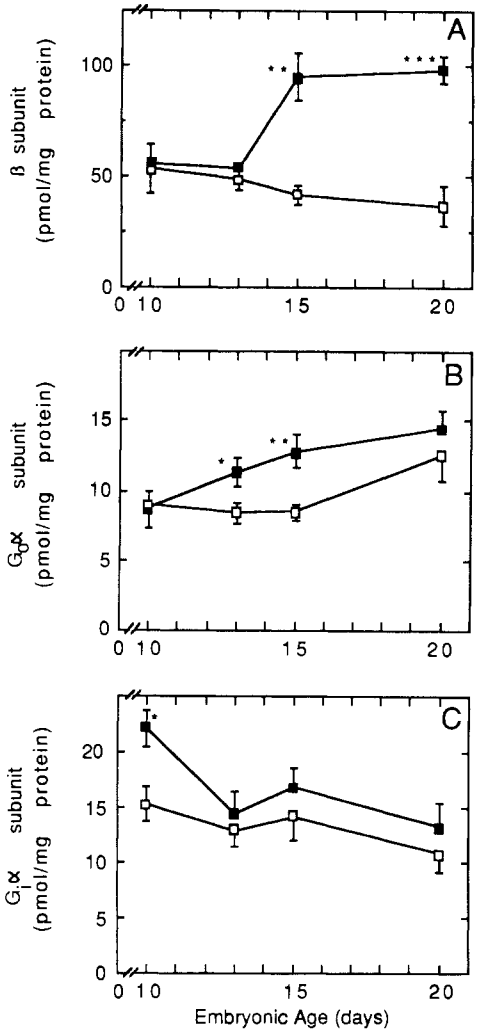

FIGURE 3: Quantitation of β-subunit, $G_0\alpha$-subunit, and $G_i\alpha$-subunit in embryonic chicken atrial and ventricular membranes. Atrial (■) and ventricular (□) membranes prepared from chicken embryos of the indicated ages were subjected to immunoblot analysis using affinity-purified anti-β subunit (A), anti-$G_0\alpha$ subunit (B), or anti-Tα subunit (C) antibodies. Each point represents the mean ± SEM of three to five separate experiments, each consisting of triplicate determinations. Statistically significant differences between atrial and ventricular samples are denoted by asterisks (two-tailed t-test: *, $p < 0.05$; **, $p < 0.02$; ***, $p < 0.01$).

In addition to $G_i\alpha$, the anti-Tα antibodies also recognize a 39 000-Da polypeptide in embryonic chicken cardiac membranes (Figure 1B). The level of this protein was determined during the quantitation of $G_i\alpha$ using the $G_i\alpha$ quantitation standards. There was no significant difference between the amount in day 15E atrial [21.9 ± 2.0 pmol/(mg of protein)] and ventricular [20.4 ± 3.0 pmol/(mg of protein)] membranes.

It has been reported that between day 12E and day 15E the atrial membrane concentration of mAChR increases by 25%–40%, while the concentration in ventricular membranes remains constant (Kirby & Aronstam, 1983). In order to compare developmental changes in mAChR concentration with those we have observed for the β-, $G_0\alpha$-, and $G_i\alpha$-subunits, we determined mAChR concentration in atrial and ventricular membranes from day 10E to day 20E using [^{3}H]QNB (Figure 4). On day 10E there was no difference between the mAChR concentration in atrial [1.23 ± 0.10 pmol/(mg of protein)] and ventricular [1.30 ± 0.18 pmol/(mg of protein)] membranes. The concentration of mAChR in atrial membranes increased by 61% between days 10E and 12E, earlier than previously reported, while the mAChR level in ventricular

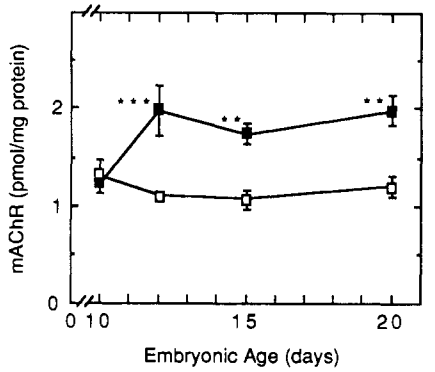

FIGURE 4: Quantitation of mAChR in embryonic chicken atrial and ventricular membranes. The mAChR concentration in atrial (■) and ventricular (□) membranes prepared from embryos of the indicated ages was determined by using [^{3}H]QNB at the saturating concentration of 750 pM. Each point represents the mean ± SEM of three to six separate experiments, each consisting of triplicate determinations. Statistically significant differences between atrial and ventricular samples are denoted by asterisks (two-tailed t-test: **, $p < 0.02$; ***, $p < 0.01$).

membranes did not change. The atrial mAChR concentration remained elevated on day 15E and day 20E with respect to the ventricular membrane concentration, which showed no change between days 10E and 20E.

In order to determine what functional effects the observed changes in G-protein and mAChR levels might have, the regulation of adenylate cyclase activity by receptor-dependent and receptor-independent mechanisms was examined in membranes prepared from atria and ventricles from chick embryos of various ages. In embryonic chick cardiac membranes GTP has an inhibitory effect upon basal adenylate cyclase activity by activating the inhibitory G-protein, G_i. This allowed examination of the effect of changes in G-protein subunit levels without interference from changes in receptor levels. Adenylate cyclase activity in the presence of 1 μM GTP, expressed as the percent of the basal value, was 78.5 ± 2.9% and 70.8 ± 5.8% in day 10E atrial and ventricular membranes, respectively, 79.1 ± 3.0% and 76.1 ± 4.5% in day 13E atrial and ventricular membranes, respectively, and 98.9 ± 3.7% and 75.5 ± 3.5% in day 15E atrial and ventricular membranes, respectively (mean ± SEM, four to five independent experiments each in triplicate). While day 10E and day 13E atrial and ventricular and day 15E ventricular membrane basal adenylate cyclase activities were inhibited to an equal extent, the basal adenylate cyclase activity of day 15E atrial membranes was unresponsive to 1 μM GTP. The atrial and ventricular membrane values at day 15E were significantly different ($p < 0.01$).

The response of basal adenylate cyclase activity to a range of GTP concentrations is shown in Figure 5. Similar concentration-effect curves were obtained in day 13E atrial and ventricular membranes (Figure 5A, EC_{50} = 53.2 ± 4.2 and 53.4 ± 18.9 nM, respectively) and day 15E ventricular membranes (Figure 5B, EC_{50} = 52.1 ± 10.5 nM). Basal adenylate cyclase activity in day 15E atrial membranes was much less responsive to GTP (Figure 5B). This loss of GTP sensitivity temporally correlates with the atrial specific increase in the β-subunit level (see Figure 3A).

Next, mAChR regulation of basal adenylate cyclase activity was examined. The concentration-effect curves for carbachol inhibition of basal adenylate cyclase activity in day 10E, 13E, and 15E atrial and ventricular membranes are shown in Figure 6. On day 10E (Figure 6A), atrial basal adenylate cyclase activity was 7.2-fold more sensitive to carbachol than was ventricular activity (EC_{50} = 1.9 ± 0.2 and 13.7 ± 5.7 μM,

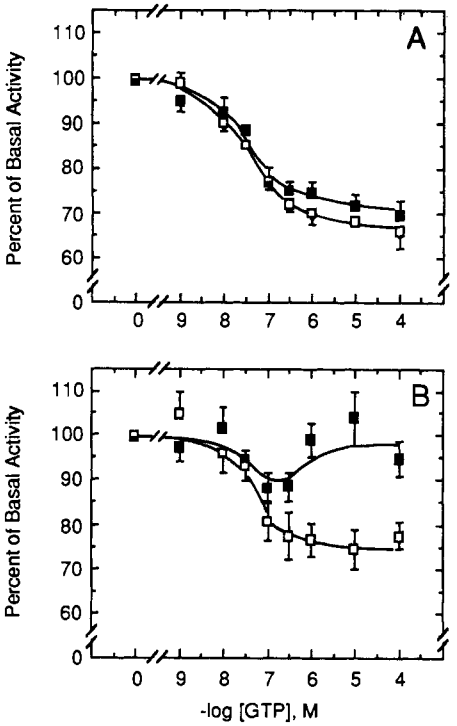

FIGURE 5: GTP inhibition of basal adenylate cyclase activity in atrial and ventricular membranes. Adenylate cyclase activity was determined in atrial (■) and ventricular (□) membranes from day 13E (A) and day 15E (B) chicken embryos in the absence or presence of the indicated concentrations of GTP. Each point represents the mean ± SEM of three to seven separate experiments, each consisting of triplicate determinations. Results are expressed as the percent of basal activity, which was 232.7 ± 17.1 and 177.7 ± 9.7 pmol of cAMP (10 min)$^{-1}$ (mg of membrane protein)$^{-1}$ in day 13E atrial and ventricular membranes, respectively, and 168.3 ± 6.0 and 172.2 ± 13.4 pmol of cAMP (10 min)$^{-1}$ (mg of membrane protein)$^{-1}$ in day 15E atrial and ventricular membranes, respectivley (mean ± SEM of four to five separate experiments, each in triplicate). On day 15E (B) atrial and ventricular activities were significantly different in the presence of 1 μM GTP ($p < 0.01$), 10 μM GTP ($p < 0.02$), and 100 μM GTP ($p < 0.05$).

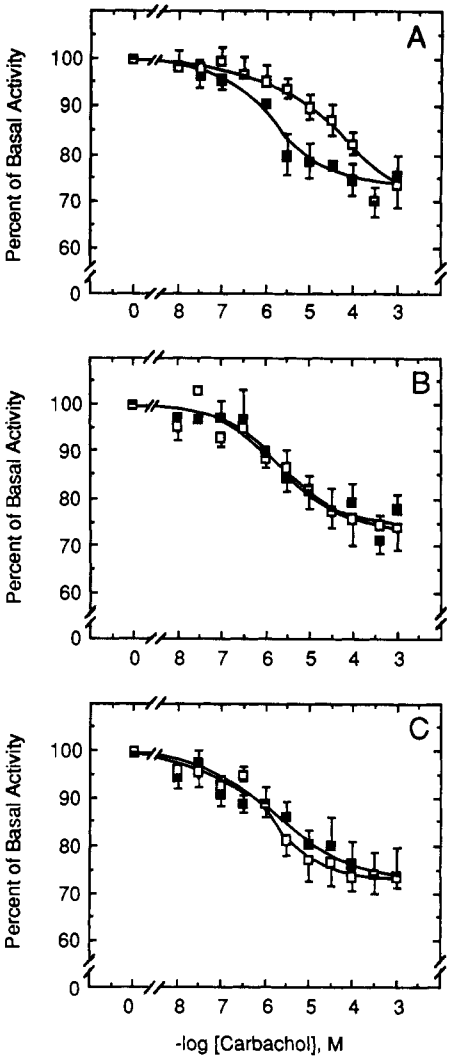

FIGURE 6: Carbachol inhibition of basal adenylate cyclase activity in atrial and ventricular membranes. Adenylate cyclase activity was determined in atrial (■) and ventricular (□) membranes from day 10E (A), day 13E (B), and day 15E (C) chicken embryos. Assays were performed in the presence of 1 μM GTP as well as the indicated concentrations of carbachol. Each point represents the mean ± SEM of three to six separate experiments, each consisting of triplicate determinations. Results are presented as the percent of basal activity in the presence of 1 μM GTP, which was 146.6 ± 24.1 and 115.9 ± 41.6 pmol of cAMP (10 min)$^{-1}$ (mg of membrane protein)$^{-1}$ in day 10E atrial and ventricular membranes, respectively, 184.1 ± 15.0 and 136.2 ± 14.1 pmol of cAMP (10 min)$^{-1}$ (mg of membrane protein)$^{-1}$ in day 13E atrial and ventricular membranes, respectively, and 166.1 ± 7.0 and 129.8 ± 11.3 pmol of cAMP (10 min)$^{-1}$ (mg of membrane protein)$^{-1}$ in day 15E atrial and ventricular membranes, respectively (mean ± SEM of three to five separate experiments, each in triplicate). On day 10E (A) the atrial and ventricular activities were significantly different ($p < 0.05$) in the presence of 3 and 10 μM carbachol.

respectively, significantly different, $p < 0.05$), while the same maximal effect was achieved. This difference in carbachol sensitivity temporally correlates with the 44% higher level of $G_i\alpha$-subunit in atrial than in ventricular membranes on day 10E (see Figure 3C). Atrial and ventricular basal adenylate cyclase activities were equally sensitive to carbachol on day 13E (Figure 6B, EC_{50} = 1.3 ± 0.6 and 2.0 ± 1.2 μM, respectively) and on day 15E (Figure 6C, EC_{50} = 1.3 ± 0.5 and 1.1 ± 0.5 μM, respectively). Thus, the carbachol sensitivity of ventricular basal adenylate cyclase activity increased 6.8-fold between day 10E and day 13E. This does not correlate with changes in $G_i\alpha$ levels since the atrial level decreases between day 10E and day 13E, while the ventricular level does not change.

Discussion

Functional parasympathetic innervation of the embryonic chicken heart can first be detected on day 12E or on day 10E in the presence of an acetylcholinesterase inhibitor (Pappano, 1977; Pappano & Loffelholz, 1974). An atrial-specific increase in mAChR level has been reported to coincide with the onset of functional parasympathetic innervation (Kirby & Aronstam, 1983). In this study we have used a quantitative immunoblot assay to determine whether subunits of the G-proteins with which the mAChR interacts are similarly regulated. We also investigated the functional effects of this regulation by examining the regulation of adenylate cyclase activity, one of the effector systems to which the mAChR are coupled by G-proteins.

Monospecific antibodies were purified from antiserum RV3 and AS7 by elution from specific bands on immunoblots (Smith & Fisher, 1984). The anti-β and anti-$G_0\alpha$ antibodies each recognized single bands corresponding to the β-subunit and the $G_0\alpha$-subunit, respectively, in a purified G-protein sample, bovine cerebral cortex membranes, and embryonic chicken cardiac membranes. The anti-Tα antibodies recognized a single band corresponding to the α-subunit of transducin in a sample of purified bovine transducin and to the $G_i\alpha$-subunit in a purified G-protein sample. This cross-reactivity is not surprising since the amino acid sequence of the

C-terminal decapeptide of bovine $G_i\alpha$ differs from that of bovine $T\alpha$ by only one amino acid (Lochrie et al., 1985; Michel et al., 1986). In samples of embryonic chicken cardiac membranes two bands were recognized, a 40 000-Da band, which corresponds to $G_i\alpha$, as well as a 39 000-Da band. The same is true in samples of embryonic chicken brain membranes.

The 39 000-Da polypeptide recognized by the anti-$T\alpha$ antibodies may be the α-subunit of G_0, which might share an immunological similarity with $G_i\alpha$ in chicken tissues but not in bovine tissues. It is also possible that this polypeptide represents the α-subunit of an additional G_i-like protein. The possibility that this polypeptide is not $G_0\alpha$ is supported by quantitative immunoblot analysis, which showed that there was no difference between the atrial and ventricular membrane levels of 39 000-Da polypeptide on day 15E. In contrast, the level of $G_0\alpha$, as determined by using affinity-purified anti-$G_0\alpha$ antibodies, was 49% greater in atrial than in ventricular membranes on day 15E. The existence of multiple forms of $G_i\alpha$-like polypeptides has been suggested by others. A third IAP substrate has been observed in bovine brain (Neer et al., 1984), and a cDNA clone encoding a $G_i\alpha$-like protein has been isolated from a bovine pituitary cDNA library (Michel et al., 1986). It has also been suggested that an IAP substrate that is neither $G_i\alpha$ nor $G_0\alpha$ is present in NG108-15 and C6 glioma cells (Milligan et al., 1986) and human neutrophils (Gierschik et al., 1986a).

The quantitative immunoblot assay described here takes advantage of the use of an alkaline phosphatase conjugated second antibody to eliminate the need for a radiolabeled second antibody. While comparison of the absolute level of one chick G-protein subunit with that of another may not be appropriate due to the use of antibodies raised against bovine antigens, this assay provides an accurate way to measure relative changes in G-protein subunit levels in the developing chick heart. A linear relationship was observed between optical density and the amount of protein loaded onto the gel. This was true when either purified G-protein subunits or crude membrane preparations were used. To assure that the proteins recognized in chick cardiac tissue were in fact immunologically cross-reactive with the bovine G-protein standards, antibodies binding to the chick cardiac protein bands were eluted from the nitrocellulose and shown to recognize the purified bovine G-protein subunits being used as quantitation standards.

We also wished to show that the use of antibodies produced against bovine cerebral cortex G-protein subunits and a bovine transducin peptide sequence would not affect the accuracy of quantitating G-protein subunits in chicken cardiac membranes. For example, the chicken cardiac G-protein subunits might be recognized by only a subset of the antibodies recognizing the bovine G-protein subunit standards, resulting in an underestimation of the chicken cardiac G-protein subunit level. The antibodies could also bind with differing affinities to the standards and the chicken cardiac G-protein subunits. It was found in each case that all immunoreactivity, toward each of the bovine G-protein subunits used as quantitation standards, could be removed by prior adsorption of the affinity-purified antibodies with the corresponding chicken cardiac G-protein subunit immobilized on nitrocellulose. This demonstrated that the chicken cardiac G-protein subunits and bovine cerebral cortex G-protein subunits were recognized by the same set of antibodies. Possible problems caused by the purified antibodies having differing affinities for the quantitation standards and the chick cardiac G-protein subunits were avoided by using concentrations of the affinity-purified antibodies that were determined in preliminary experiments to be saturating.

Atrial specific regulation of the levels of mAChR, β-subunit, $G_0\alpha$-subunit, and $G_i\alpha$-subunit was observed. The level of mAChR in atria increased by 61% between days 10E and 12E, slightly earlier than the increase reported by Kirby and Aronstam (1983), which occurred between days 12E and 15E. The level of β-subunit in atria increased by 80% between days 13E and 15E, and the level of $G_0\alpha$ in atria increased by 46% between days 10E and 15E. The ventricular levels of these proteins did not change between days 10E and 15E. In contrast, the $G_i\alpha$-subunit level was 44% greater in atria than in ventricles on day 10E and then decreased to the ventricular level by day 13E. No changes were seen in G-protein subunit or mAChR levels between days 15E and 20E, except for the ventricular $G_0\alpha$ level, which increased by 47%. The atrial-specific changes in the mAChR, $G_0\alpha$, and $G_i\alpha$ levels occurred during, and the atrial-specific change in β-subunit level occurred just after, the onset of functional parasympathetic innervation of the chick heart. This suggests that the process of innervation may play a role in the regulation of these proteins. This is supported by the observation that the atrial increase in mAChR number was prevented by in ovo administration of the muscarinic antagonist atropine (Kirby & Aronstam, 1983). In addition, coculture of neonatal rat ventricle cells with sympathetic neurons has been shown to cause an increase in the level of a 41 000-Da IAP substrate (Steinberg et al., 1985). The increase in the level of IAP substrate correlated with the acquisition of an α-adrenergic negative chronotropic response in the nerve–muscle cultures, suggesting that alterations in the levels of G-protein subunits may have functional consequences.

To investigate the possible functional effects that the changes in G-protein subunit and mAChR levels might have, we examined the regulation of basal adenylate cyclase activity by receptor-dependent and receptor-independent mechanisms. Because the mAChR is thought to be coupled to adenylate cyclase by G_i, changes in the levels of $G_i\alpha$-subunit, β-subunit, or mAChR might have an effect on adenylate cyclase activity. The function of $G_0\alpha$ in the chick heart is unknown, making it difficult to assess what effects changes in the $G_0\alpha$ level might cause.

On days 10E and 13E we found that GTP inhibited basal adenylate cyclase activity to an equal extent in both atrial and ventricular membranes. Similar inhibition by GTP has been observed in atria and ventricles on day 8E (E. Subers and N. Nathanson, unpublished observations) and in atria on day 5E (Halvorsen & Nathanson, 1984). On day 15E, GTP inhibited ventricular basal adenylate cyclase activity similarly to that at earlier ages but was unable to inhibit atrial basal adenylate cyclase activity. This loss of GTP inhibition was examined in detail in Figure 5. The EC_{50} for GTP inhibition in day 15E ventricles was similar to that at earlier ages, while atrial basal adenylate cyclase activity was unresponsive to GTP at concentrations as high as 100 μM. The loss of responsiveness to GTP correlates with the 80% increase in β-subunit concentration that occurs between days 13E and 15E (Figure 3A). A possible mechanism by which GTP causes inhibition of basal adenylate cyclase activity is that binding of GTP to the α-subunit of $G_i\alpha$ causes dissociation of the $\alpha\beta\gamma$-complex, the free $G_i\alpha$-GTP then being able to inhibit the catalytic subunit of adenylate cyclase (Roof et al., 1985; Katada et al., 1986). The α-subunit is inactivated by hydrolysis of the GTP and by reassociation with the $\beta\gamma$-subunits (Gilman, 1984). We have shown that an increase in β-subunit appears to result in a loss of responsiveness of adenylate cyclase activity to GTP. Due to the increased β-subunit level, $\alpha\beta\gamma$-complexes may be more

likely to form, inactivating the α-subunit.

We also examined the ability of mAChR to inhibit basal adenylate cyclase activity (Figure 6). We found that on day 10E atrial basal adenylate cyclase activity was 7.2-fold more sensitive to carbachol than was ventricular activity. This difference correlated with the 44% higher level of $G_i\alpha$ in atrial than in ventricular membranes on day 10E (Figure 3C). The carbachol sensitivity of ventricular basal adenylate cyclase activity increased to that of atria by day 13E. In contrast, the level of $G_i\alpha$ in atria decreased to equal that of ventricles by day 13E. Between days 10E and 13E the atrial sensitivity to carbachol did not change, even though the level of $G_i\alpha$ decreased. It may be that $G_i\alpha$ is not a limiting component in the carbachol inhibition of adenylate cyclase activity or that the coincident increase in atrial mAChR level (Figure 4) balances the loss of $G_i\alpha$. The increase in ventricular carbachol sensitivity occurs over a period of time during which no change was observed in mAChR or $G_i\alpha$-, $G_0\alpha$-, or β-subunit levels in ventricular membranes. This difference in carbachol sensitivity between atrial and ventricular membranes and the subsequent increase in ventricular sensitivity might be due to a change in the affinity of the ventricular mAChR for agonist. This seems unlikely since on day 8E and soon after hatching the affinities of the atrial and ventricular mAChR for carbachol were similar (Martin et al., 1987; Sorota et al., 1986). The increase in sensitivity could also be due to a change in the amount or properties of the adenylate cyclase.

Liang et al. (1986) have recently examined the regulation of adenylate cyclase activity and G-protein subunit and mAChR levels in whole heart homogenates during embryonic chick development. They measured the levels of $G_0\alpha$ and $G_i\alpha$ using IAP-mediated ADP-ribosylation and the levels β and $G_0\alpha$ using specific antisera. They found that the levels of $G_0\alpha$-, $G_i\alpha$-, and β-subunit increased in whole hearts between day 2.5E and day 10E and then remained constant through day 2 after hatching. An increase in the ability of carbachol to inhibit isoproterenol-stimulated adenylate cyclase activity was shown to occur in the same time period during which the levels of $G_0\alpha$-, $G_i\alpha$-, and β-subunit increased. We observed atrial specific changes in G-protein subunit levels between days 10E and 15E. It is not surprising that these changes were not observed by Liang et al. since whole heart homogenates consist primarily of ventricular proteins. The levels of ventricular G-protein subunits do not change during this time period.

Others have also observed correlations between changes in G-protein subunit levels and hormone responsiveness and adenylate cyclase activity. Ludford and Talamo (1983) found that during neonatal rat parotid gland development an increase in β-adrenergic sensitivity of secretion correlated with an increase in the cholera toxin substrate ($G_s\alpha$) level. As discussed above, nerve–cardiac muscle coculture-induced acquistion of an α-adrenergic negative chronotropic response correlated with an increase in IAP substrate level (Steinberg et al., 1985). Dexamethasone treatment of an osteosarcoma cell line resulted in an increase in β-adrenergic sensitivity of adenylate cyclase activity and an increase in β-adrenergic receptor and $G_s\alpha$ levels (Rodan & Rodan, 1986). It has also been reported that decreased levels of $G_i\alpha$-, $G_0\alpha$-, and β-subunit correlate with increased adenylate cyclase activity in differentiated 3T3-L1 fibroblasts (Gierschik et al., 1986c), while an increase in β-subunit level may be responsible for decreased adenylate cyclase activity in chicken embryo fibroblasts transformed with Rous sarcoma virus (Woolkalis et al., 1986).

In conclusion, we have developed and used a quantitative immunoblot assay to measure the levels of the α-subunits of G_i and G_0, as well as the common β-subunit, in atria and ventricles during embryonic chicken development. The regulation of these G-protein subunits was compared to that of the mAChR, with which G_i and G_0 interact. Atrial-specific regulation of the levels of the $G_i\alpha$-, $G_0\alpha$-, and β-subunits and mAChR occurred at times coinciding with or closely following the onset of functional parasympathetic innervation of the chick heart. Our results and the work of others have shown that changes in G-protein and mAChR levels are associated with changes in both G-protein- and mAChR-mediated regulation of adenylate cyclase activity. These results suggest that the process of innervation may play a role in the regulation of both the number and the function of the mAChR and the G-proteins with which it interacts.

Registry No. GTP, 86-01-1; adenylate cyclase, 9012-42-4; carbachol, 51-83-2.

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Topography of the *Dictyostelium discoideum* Plasma Membrane: Analysis of Membrane Asymmetry and Intermolecular Disulfide Bonds†

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ABSTRACT: Through the application of a unique method for isolating plasma membranes, it was possible to specifically iodinate cytoplasm-exposed plasma membrane proteins in vegetative cells of the cellular slime mold *Dictyostelium discoideum*. The original procedure [Chaney, L. K., & Jacobson, B. S. (1983) *J. Biol. Chem. 258*, 10062] which involved coating cells with colloidal silica has been modified to yield a more pure preparation. The presence of the continuous and dense silica pellicle on the outside surface of the isolated plasma membrane permitted the specific labeling of cytoplasm-exposed membrane proteins. Lactoperoxidase-catalyzed iodination was employed to label cell-surface and cytoplasm-exposed membrane proteins. The isolated and radioiodinated membranes were then compared and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The cell-surface and cytoplasmic face labeling patterns were distinct. A total of 65 proteins were found to be accessible to at least one surface of the membrane. Sixteen intermolecular disulfide bond complexes were observed in the plasma membrane of *Dictyostelium*; most of these complexes involved glycoproteins and, hence, were exposed to the cell surface.

For the elucidation of the function of membrane components at the molecular level, a comprehensive analysis of the composition and topography of the membrane in question is essential. The composition of purified membranes can be determined by one-dimensional sodium dodecyl sulfate (Na-

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[1] Abbreviations: bis(acrylamide), *N,N'*-methylenebis(acrylamide); BSA, bovine serum albumin, fraction V; CCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; Con A, concanavalin A; Con A buffer, 50 mM Tris-HCl, 0.1 M NaCl, 1 mM $MnCl_2$, and 1 mM $CaCl_2$, pH 7.0; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; NaDodSO_4, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PM, plasma membrane(s); PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane; Mes, 2-(*N*-morpholino)ethanesulfonic acid; kDa, kilodalton(s).

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