Ontogenesis of Physiological Responsiveness and Guanine Nucleotide Sensitivity of Cardiac Muscarinic Receptors during Chick Embryonic Development†

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ABSTRACT: Atria isolated from 4-day chick embryos were much less responsive to the negative chronotropic effect of muscarinic agonists than were atria from 5- or 8-day embryos, even though the density of muscarinic acetylcholine receptors (mAChR) was similar at all these ages. The mAChR in hearts from 4-day embryos were also significantly less susceptible to regulation of receptor number by in vivo agonist treatment and required a 2–5-fold greater dose of the muscarinic agonist carbachol to achieve a decrease in receptor number equivalent to that observed in 5- or 8-day embryonic hearts. When 4-day atrial membranes were assayed in physiological buffers, agonist binding to the mAChR was not regulated by GTP unless a sulfhydryl reducing agent was present. Receptors from 5- and 8-day embryos did not require addition of a sulfhydryl reducing agent in order to see guanine nucleotide effects on agonist binding. Even in the presence of a sulfhydryl reducing agent, carbachol binding to the mAChR in 4-day membranes was much less sensitive to guanyl-5′-yl imidodiphosphate (GppNHp) than binding to mAChR in 5- or 8-day membranes. In addition, forskolin-activated adenylate cyclase activity was much less sensitive to inhibition by GppNHp in membranes from 4-day atria than from 5- and 8-day atria. The GTP-binding component (N_I) which couples the mAChR to inhibition of adenylate cyclase activity was examined by covalent modification with pertussis toxin. Two polypeptides with molecular weights of 39 000 and 42 000 were observed by one-dimensional gel electrophoresis, and each molecular weight component was resolved further into two species by isoelectric focusing. There was an increase between 4 and 8 days in the relative amount of the 39 000-dalton component and also a dramatic increase in the acidic form of the 39 000-dalton component. Thus, both functional and physical changes in N_I are observed during development as the mAChR in the embryonic chick heart acquires physiological responsiveness.

Acetylcholine is released from parasympathetic nerve endings in the heart and upon binding to the muscarinic acetylcholine receptor(s) (mAChR)[1] on the cardiac muscle membrane results in a decrease in the rate and force of contraction. mAChR-mediated slowing of the pacemaker firing rate in the sinus node of the atrium is due to an increased outward K^+ current (Hutter, 1957) and may also be influenced by a decreased slow inward Ca^{2+}/Na^+ current independent of effects on K^+ conductance (Giles & Noble, 1976). However, there is no direct evidence that the mAChR itself functions as an ionophore, and the molecular mechanisms responsible for the coupling of mAChR activation to these physiological effects remain unclear.

mAChR activation also causes changes in biochemical parameters which may have a role in receptor function. In cardiac membranes, muscarinic agonists inhibit hormone-stimulated adenylate cyclase activity (Jakobs et al., 1979). Receptor-mediated inhibition of adenylate cyclase activity requires the interaction of GTP with a guanine nucleotide inhibitory regulatory component (termed N_I; Rodbell, 1980). Guanine nucleotides also decrease the apparent affinity of the mAChR for agonists in membranes from rat heart (Berrie et al., 1979; Rosenberger et al., 1980) and embryonic chick heart (Galper & Smith, 1980; Halvorsen & Nathanson, 1981a). Receptor-mediated inhibition of adenylate cyclase can be blocked by treating cells with a toxin isolated from *Bordetella pertussis* (Hazeki & Ui, 1981). This toxin catalyzes the ADP-ribosylation of a membrane component of the adenylate cyclase complex that is distinct from N_S, the guanine nucleotide regulatory protein necessary for expression of β-adrenergic receptor-mediated effects (Katada & Ui, 1982; Murayama & Ui, 1983; Hildebrandt et al., 1983). Bokoch et al. (1983) have recently purified the pertussis toxin substrate from rat liver and found that, similar to N_S (Northup et al., 1980), it functions as a heterodimer. Further work by Northup et al. (1983) has identified the pertussis toxin substrate as a subunit of N_I. Both N_I and N_S consist of a (different) guanine nucleotide binding subunit and at least one other subunit with a molecular weight of approximately 35 000 which regulates the function of both N_S and N_I (Northup et al., 1983). Kurose et al. (1983) have reported that modification of N_I by pertussis toxin prevents GTP-mediated inhibition of both adenylate cyclase activity and muscarinic agonist binding. These results are consistent with N_I mediating both guanine nucleotide regulation of agonist binding and inhibition of adenylate cyclase activity. Since there is no direct evidence to support a role for inhibition of adenylate cyclase activity in the mAChR-mediated negative chronotropic response, the role of N_I in mAChR function is not known.

The mAChR in the embryonic chick heart is a particularly attractive system to study the molecular mechanisms for the development, regulation, and function of the mAChR. The physiological responsiveness of the chick atrium to the neurotransmitter acetylcholine changes during the course of em-

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[1] Abbreviations: mAChR, muscarinic acetylcholine receptor(s); BRM, beating rate medium; GppNHp, guanyl-5′-yl imidodiphosphate; QNB, quinuclidinyl benzilate; NAD, nicotinamide adenine dinucleotide; DTT, dithiothreitol; N_S, regulatory component of adenylate cyclase required for GTP-dependent stimulation of activity; N_I, regulatory component of adenylate cyclase required for GTP-dependent inhibition of activity; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/84/0423-5813$01.50/0

bryonic development. Pappano & Skowronek (1974) first demonstrated that 3-day atria exhibited a reduced negative chronotropic response to mAChR stimulation which increased in atria from 6- to 9-day embryos. In preliminary communications (Halvorsen & Nathanson, 1981b; Halvorsen et al., 1983), we reported that the interaction of the mAChR with N_1 appeared to change during this time period of embryonic development. We report here the use of specific stages of development of the embryonic chick heart which possess mAChR at various stages of physiological and biochemical coupling in order to study the molecular mechanisms of mAChR activation and regulation.

Experimental Procedures

Materials

White leghorn chick 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 & Hamilton (1951); 4-day embryos represent stages 17–22 and 5-day embryos stages 24–26.

ATP (synthesized from adenosine), carbachol, phosphocreatine, creatine phosphokinase (rabbit muscle type I), and 1-isoproterenol were obtained from Sigma, St. Louis, MO. [1-^{3}H]QNB (specific activity 27–30 Ci/mmol) was obtained from Amersham and New England Nuclear. GTP and GppNHp were from P-L Biochemicals, Milwaukee, WI, and [^{3}H]cAMP was from ICN, Irvine, CA. [α-^{32}P]ATP (specific activity 32 Ci/mmol) was obtained from New England Nuclear. [^{32}P]NAD was synthesized from [α-^{32}P]ATP as described by Cassel & Pfeuffer (1978). Pertussis toxin was a gift from Dr. D. Storm and was a concentrate of the 48-h culture medium of *Bordetella pertussis* (Tahoma phase I). All other chemicals were of reagent grade.

Methods

Administration of Drugs in Ovo. Drugs were dissolved in phosphate-buffered saline and administered to embryos in ovo as described (Halvorsen & Nathanson, 1981a).

Assay for mAChR. Hearts were removed from embryos, atria and ventricles were separated where indicated, and washed membranes were prepared in 50 mM sodium phosphate (except as noted) as previously detailed (Halvorsen & Nathanson, 1981a). The membrane pellet was resuspended in BRM (149 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES, 5.5 mM glucose, and 0.4 mM sodium phosphate, pH 7.4) after the final sodium phosphate wash for carbachol–[^{3}H]QNB competition binding studies. The mAChR assay procedure was a modification of the filter binding method of Yamamura & Snyder (1974), as previously described (Halvorsen & Nathanson, 1981a), which utilized the highly specific muscarinic receptor antagonist [^{3}H]QNB. The concentration of [^{3}H]QNB in the binding assays was 0.65–0.75 nM, except for carbachol–[^{3}H]QNB competition studies where it was 0.30–0.35 nM. The saturation binding curves of [^{3}H]QNB to cardiac membranes were the same whether assayed in 50 mM sodium phosphate or BRM. Binding data are presented as the average of duplicate assay determinations which varied by less than 10% from the mean.

Control binding experiments were also conducted by using conditions similar to the adenylate cyclase assay (see below). Membranes were prepared as for the adenylate cyclase studies, and [^{3}H]QNB binding was assayed in 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, 0.01 mM isoproterenol, 0.05 mM sodium ascorbate, and 10 mM HEPES (pH 7.4).

Atrial Beating Rate Studies. The negative chronotropic effect of carbachol on spontaneously beating isolated atria was determined as previously described (Halvorsen & Nathanson, 1981a). Atria were dissected away from the heart and pinned onto a Sylgaard (Dow)-coated petri dish which contained oxygenated BRM maintained at 37 °C. After equilibration for 45 min, beating rates were determined visually through a dissecting microscope before and during drug exposure.

Adenylate Cyclase Assay. Atrial membranes were prepared as above except that the homogenization buffer was 1 mM DTT and 200 mM sucrose in 10 mM HEPES (pH 7.4). The assay contained 0.25 mM [α-^{32}P]ATP (16–20 Ci/mmol), an ATP and GTP regenerating system consisting of 10 mM creatine phosphate and 50 units/mL creatine phosphokinase, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM theophylline, 0.1% bovine serum albumin, 5 mM $MgCl_2$, 2 mM [^{3}H]cAMP (18 mCi/mol, included to monitor recovery), 100 mM NaCl, 0.05 mM sodium ascorbate, 80 mM sucrose, 0.4 mM DTT, 0.01–0.06 mg of membrane protein, and (as required) 0.1 mM forskolin, GTP, GppNHp, isoproterenol, or carbachol in 10 mM HEPES (pH 7.4). Control experiments showed that these concentrations of isoproterenol, $MgCl_2$, and ATP gave maximal stimulation of adenylate cyclase activity. NaCl (100 mM) is required for optimal mAChR-mediated inhibition of isoproterenol/GTP-stimulated enzyme activity (Jakobs et al., 1979; Lichtschtein et al., 1979). With these assay conditions, enzyme activity was linear with protein (5 to at least 70 μg) and time (5–15 min). Assays were carried out in triplicate for 10 min at 30 °C and were stopped by addition of 0.75 mL of 6.7% trichloroacetic acid and heating at 100 °C for 2 min. Assay tubes were centrifuged at 1300g for 20 min, and the supernatants were retained for determination of [^{32}P]cAMP and [^{3}H]cAMP content. Separation of cyclic AMP was by a modification of the method of Salomon et al. (1974). The supernatant was loaded onto columns containing 1 mL of Dowex 50AG W/X4 resin, and the eluates from this and a 3-mL water wash were discarded. Four milliliters of water was added to each column and the eluate collected onto columns containing 0.6 g of neutral alumina. The alumina columns were washed with 4 mL of 0.1 M imidazole (pH 7.5), and the eluate was collected in vials containing 15 mL of the scintillation counting fluid as used for the [^{3}H]QNB binding assay (Halvorsen & Nathanson, 1981a). Protein was determined either by the method of McKnight (1977) or by the method of Lowry et al. (1951) as modified previously (Halvorsen & Nathanson, 1981) using bovine serum albumin as a standard.

Pertussis Toxin Labeling. Atrial membranes were prepared as described above except that the homogenization buffer was 100 mM Tris-HCl (pH 8.0). In some experiments, protease inhibitors (1 mM phenylmethanesulfonyl fluoride, 0.01 mM pepstatin A, 0.5 mM diazonorleucine methyl ester, and 1 mM EDTA) were included at all steps of tissue preparation. Concentrated pertussis toxin was activated with 20 mM DTT for 20 min at 30 °C. Atrial membranes (0.3–0.4 mg of protein) were incubated with activated pertussis toxin, 0.01 mM [α-^{32}P]NAD (5–10 Ci/mmol), 10 mM thymidine, 1 mM EDTA, 5 mM $MgCl_2$, 4 mM DTT, and 1 mM ATP for 30–45 min at 30 °C. Labeling reactions were stopped with a 2-fold dilution of cold membrane buffer and centrifuged at 8800g for 8 min, the pellet was washed by suspension and centrifugation, and the final pellet was solubilized in the appropriate sample buffer as described below for one- and two-dimensional gel electrophoresis.

One-Dimensional SDS Gel Electrophoresis. Pertussis toxin labeled membranes were solubilized in SDS sample buffer (3.5% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.0005%

bromophenol blue, and 125 mM Tris-HCl, pH 6.8). An aliquot (0.03–0.05 mg of protein) of each sample was loaded onto a lane of a polyacrylamide slab gel and electrophoresed by a modification (Nathanson & Hall, 1979) of the discontinuous system described by Laemmli (1970). The following proteins were used as molecular weight (in parentheses) standards: bovine serum albumin (68 000), pyruvate kinase (57 000), fumarase (49 000), aldolase (40 000), and glyceraldehyde-3-phosphate dehydrogenase (36 000). Dried gels were autoradiographed by exposure to Kodak X-Omat film at −80 °C using a Cronex intensifying screen. Densitometric tracings of autoradiographs were obtained by using a Helena Labs Quick Scan, and the area under the curve was quantitated by cutting out and weighing the individual peaks.

Two-Dimensional Gel Electrophoresis. Two-dimensional gel electrophoresis was carried out, with some modifications, as described by O'Farrell (1975) using isoelectric focusing in the first dimension and SDS–PAGE in the second dimension. Labeled samples were solubilized in half-strength SDS sample buffer, heated at 100 °C for 2 min, and cooled to room temperature at which time ultrapure urea was added to 9.5 M, and then 2 volumes of sample dilution buffer (9.5 M urea, 5% 2-mercaptoethanol, 8% Nonidet P-40, and 3% ampholines; 0.6% pH 3–10, 1.2% pH 4–6, and 1.2% pH 5–7) was added as described by Ames & Nikaido (1976). Aliquots were loaded onto prerun (O'Farrell, 1975) isoelectric focusing tube gels (11 cm × 0.3 cm: 9.2 M urea, 4% acrylamide, 2% Nonidet P-40, and 2% ampholines; 0.4% pH 3–10, 0.8% pH 4–6, and 0.8% pH 5–7), and the gels were run for 5800–8800 V-h. Gels were then removed from the tubes and equilibrated either by soaking in "high SDS" sample buffer (as above with 10% SDS) for 30–45 min or by mounting the gel onto the second-dimension slab gel (see below) and running with high SDS electrode buffer (containing 2% SDS) in the top reservoir for 20 min at 20 mA. The high SDS electrode buffer was then replaced with regular (0.1% SDS) electrode buffer and the electrophoresis continued. The tube gels were loaded onto the second-dimension polyacrylamide slab gel as described by O'Farrell (1975) using 1% agarose in SDS sample buffer to seal the tube horizontally across the top of the slab gel and electrophoresed as described above for one-dimensional SDS–PAGE.

Results

Negative Chronotropic Response of Isolated Atria. The onset of the negative chronotropic response to muscarinic agonists was investigated by determining the inhibition of spontaneous beating of atria isolated from 4-, 5-, and 8-day embryos by applied carbachol. Consistent with previous observations, our results show that atria isolated from 4-day embryos were only minimally responsive to carbachol: as high as 10 mM carbachol inhibited the spontaneous beating rate only 30% in the isolated 4-day atria (Figure 1). Beating was never completely arrested by carbachol in atria at this stage of development ($n = 19$). In contrast, 5-day atria exhibited a greatly increased physiological response over 4-day atria. As seen in the concentrations of carbachol required to fully arrest beating, the 5-day atria were slightly less responsive than the 8-day atria (Figure 1).

[^{3}H]QNB saturation binding experiments with Scatchard analysis revealed a similar density of muscarinic antagonist binding sites in 4-day (820 fmol/mg of protein), 5-day (830 fmol/mg), and 8-day (840 fmol/mg) cardiac membrane preparations with dissociation constants of 0.08, 0.10, and 0.07 nM, respectively (data not shown). These results are consistent with those of Galper et al. (1977) and Renaud et al. (1980), which showed that unresponsive and responsive hearts have similar densities of mAChR and, thus, that 4-day embryonic atria have mAChR whose function is apparently impaired.

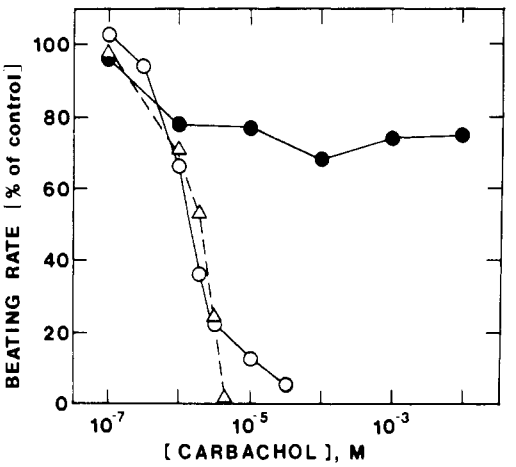

FIGURE 1: Negative chronotropic dose–response to carbachol in isolated atria. The negative chronotropic response of isolated atria to carbachol was determined in an oxygenated organ bath as described under Experimental Procedures. Curves show the response from 4-day atria (●), 5-day atria (○), and 8-day atria (△). Each point is the mean of responses from 4 to 13 preparations.

Table I: Developmental Onset of Agonist-Induced Decrease of mAChR Number[a]

	% decrease in mAChR no. for an in vivo carbachol dose of		
	1 μmol	2 μmol	10 μmol
4-day embryos	17 (±3)[b] ($n = 8$)	46 (±5)[c] ($n = 5$)	62 (±1)[b] ($n = 4$)
5-day embryos	51 (±3)[d] ($n = 6$)	70 (±2)[d] ($n = 5$)	86 (±2)[d] ($n = 5$)
8-day embryos	54 (±2) ($n = 19$)	73 (±2) ($n = 5$)	85 (±1) ($n = 9$)

[a] Embryos were pretreated in ovo with phosphate-buffered saline or the indicated amount of carbachol as described under Experimental Procedures. Hearts were removed and pooled as required (2–3 hearts/assay for 8-day embryos, 10–12 hearts/assay for 5-day embryos, and 30–40 hearts/assay for 4-day embryos) and assayed for mAChR number as described under Experimental Procedures. Table values indicate the mean percentage decrease (±SEM) in [^{3}H]QNB binding from control. The number of independent determinations (each with the number of hearts indicated above) is n. [b] Significantly different from 5- and 8-day embryos at $p < 0.0005$ (Student's t test). [c] Different from 5-day embryos at $p < 0.0025$ and 8-day embryos at $p < 0.0005$. [d] Not different from 8-day embryos at $p = 0.05$.

Agonist-Induced Decrease in mAChR Number. We have previously shown that administration of cholinergic agonists in ovo leads to a decreased number of cardiac mAChR (Halvorsen & Nathanson, 1981a). In ovo administration of 1 μmol of the stable muscarinic agonist carbachol to 8-day embryos led to a 54% decrease in mAChR number in the heart (Table I). The percent decrease in mAChR number in 5-day hearts following either 1, 2, or 10 μmol of carbachol was the same as that in 8-day embryonic hearts (Table I). However, 4-day embryos required a 2–5-fold greater dose of carbachol to achieve a similar degree of mAChR loss as 5- or 8-day embryos (Table I).

This decreased sensitivity to agonist-induced decreases in receptor number is not due to a decreased time course for receptor loss in the 4-day embryos: a similar decrease in receptor number was observed after exposure to 1 μmol of carbachol for 6.5 h (17% decrease) or for 17 h (19% decrease). Because carbachol is stable and not hydrolyzed by acetylcholinesterase, and because the decrease in receptor number

Table II: Inhibition of Adenylate Cyclase Activity in Developing Atria[a]

	activity [pmol of cAMP (mg of protein)$^{-1}$ (10 min)$^{-1}$]		
	4-day atria	5-day atria	8-day atria
no additions	370 (±30)	430 (±60)	440 (±20)
CARB	350 (±40)	390 (±30)	460 (±20)
GTP	450 (±30)	390 (±60)	370 (±40)
GTP + CARB	300 (±9)	300 (±80)	270 (±50)
GTP + ISO	590 (±20)	650 (±50)	600 (±30)
GTP + ISO + CARB	430 (±20)	440 (±10)	450 (±9)
CARB IC_{50}[b]	14 (±2)	7 (±1)	8 (±3)

[a] Adenylate cyclase activity was measured in atrial membranes prepared from embryos of the indicated age as described under Experimental Procedures. [GTP] = 1 μM; CARB = carbachol (300 μM); ISO = isoproterenol (10 μM). Table values are the mean (±SEM) of three separate experiments for each age. [b] The IC_{50} values (in micromolar) for carbachol inhibition of adenylate cyclase activity are the means (±SEM) of determinations from three to four separate experiments using increasing concentrations of carbachol in the presence of isoproterenol and GTP as above.

due to administration of carbachol in 8-day embryos is stable for at least 34 h (Halvorsen & Nathanson, 1981a), this also indicates that the diminished susceptibility of the 4-day embryos to agonist-induced receptor loss is not due to a reduced initial rate of uptake of carbachol by the 4-day embryo. As with 8-day embryos (Halvorsen & Nathanson, 1981a), there was an equivalent carbachol-induced decrease of receptor number in 4-day atria and ventricles when assayed separately (data not shown). Four-day embryonic hearts are thus less susceptible both to the negative chronotropic response of mAChR activation and to the agonist-induced decrease in receptor number. We have previously demonstrated that the muscarinic antagonists atropine and scopolamine completely prevented agonist-induced loss of receptor number, indicating that simple occupancy of the receptor by a ligand is not sufficient to induce receptor loss (Halvorsen & Nathanson, 1981a). These current results suggest that for receptor number to be decreased, additional steps beyond binding of an agonist to the mAChR are required.

Adenylate Cyclase Activity. As a biochemical measure of mAChR function, carbachol-induced inhibition of adenylate cyclase activity was investigated in embryonic atrial membranes. Isoproterenol/GTP-stimulated enzyme activity was not different at 4, 5, or 8 days, suggesting that the β-adrenergic receptor–N_S complex was coupled to adenylate cyclase equivalently within this developmental period (Table II). Carbachol inhibited isoproterenol/GTP-stimulated enzyme activity to a similar maximal degree in 4-, 5-, and 8-day atrial membranes [27% (±3%), 30% (±2%), and 26% (±2%), respectively, $n = 7$ independent experiments]. The mAChR-mediated inhibition of adenylate cyclase activity seen in our experiments was similar to the percent of inhibition reported by Jakobs et al. (1979) in rabbit myocardium and by Nathanson et al. (1978) in neuronal hybrid cells. The IC_{50} for inhibition of adenylate cyclase by carbachol was similar at 5 and 8 days (7 and 8 μM, respectively) and only slightly higher in 4-day atrial membranes (14 μM, Table II). Although the difference in the IC_{50} between 4 and 5 days was statistically significant ($p < 0.05$), it was too small a change to account for the large change in the negative chronotropic response between 4 and 5 days. Thus, at an age of development when mAChR activation elicited a much diminished negative chronotropic response and was less susceptible to an agonist-induced decrease in receptor number, mAChR stimulation nevertheless inhibited adenylate cyclase activity much the same as fully active mAChR did in the older embryos tested.

Agonist Binding to mAChR. To compare the agonist binding properties of the mAChR from the less responsive 4-day atria to the older, responsive 5- and 8-day atria, competitive binding experiments were carried out using the antagonist [^{3}H]QNB and the agonist carbachol. In physiological buffers, guanine nucleotides have been shown to decrease muscarinic agonist binding with little effect on antagonist binding in cardiac tissue from rat (Rosenberger et al., 1980) and chick (Halvorsen & Nathanson, 1981a). In our studies, the addition of GTP to the assay of mAChR in 8-day atrial membranes caused approximately a 60-fold increase in the IC_{50} for carbachol binding (Figure 2c). In striking contrast to 8-day atria, agonist binding to 4-day atrial membranes, when assayed in BRM, was not affected by the presence of GTP (10 μM, Figure 2a, circles) or the nonhydrolyzable GTP analogue GppNHp (10 μM; Halvorsen et al., 1983). The effect of GTP on agonist binding to 5-day atria was intermediate in magnitude between 4- and 8-day embryos, with GTP causing a 3-fold increase in the IC_{50} (Figure 2b).

The above results indicated that in physiological buffers the mAChR from 4-day atria was apparently not coupled to guanine nucleotide regulation, even though the results of the adenylate cyclase experiments described above indicated that the mAChR was functionally coupled to GTP-dependent inhibition of enzyme activity under the conditions of the adenylate cyclase assay. Therefore, the carbachol–[^{3}H]QNB competition experiments were repeated in a modified adenylate cyclase buffer system to further explore this apparent discrepancy. When 4-day atrial membranes were assayed under modified adenylate cyclase conditions (see Experimental Procedures), GTP induced a significant decrease in apparent agonist binding affinity, although less of a GTP effect was seen with 4-day (IC_{50} for carbachol of 0.4 and 10 μM, without and with GTP, respectively) than with 8-day membranes (0.2 and 20 μM, respectively) even under these conditions. Agonist binding to 8-day atrial membranes in modified adenylate cyclase conditions was not different from binding in BRM (data not shown).

Two major differences between the assay conditions for [^{3}H]QNB binding in BRM and for adenylate cyclase activity are the presence of isoproterenol (10 μM) and DTT (100 μM) in the adenylate cyclase assay. When DTT (100 μM) was added to the agonist binding assay in BRM, a GTP-induced decrease in carbachol binding of 2.5-fold at the IC_{50} was observed in 4-day membranes (Figure 2a, squares). Increasing the DTT concentration 3-fold did not further enhance the GTP response (data not shown). GTP effects on agonist binding to 5-day membranes were also enhanced by the presence of DTT in the assay, increasing the IC_{50} by about 10-fold (Figure 2b). DTT had no effect on agonist binding to 8-day atrial membranes (Figure 2c). Even in the presence of DTT, agonist binding to mAChR showed a greater maximum response to GTP at 8 days than at 5 or 4 days. The addition of isoproterenol (10 μM) to the binding assay in BRM did not affect the GTP response of agonist binding in either 4- or 8-day membranes in either the presence or the absence of DTT (data not shown).

As described above, guanine nucleotide regulation of agonist binding was observed in 4-day membranes only when DTT was added. As a more quantitative measure of the sensitivity of the mAChR at different stages of development to the regulation of agonist binding by guanine nucleotides, the extent of the guanine nucleotide mediated decrease in agonist binding was determined at increasing GppNHp concentrations in the presence of DTT (Figure 3). Carbachol binding to the

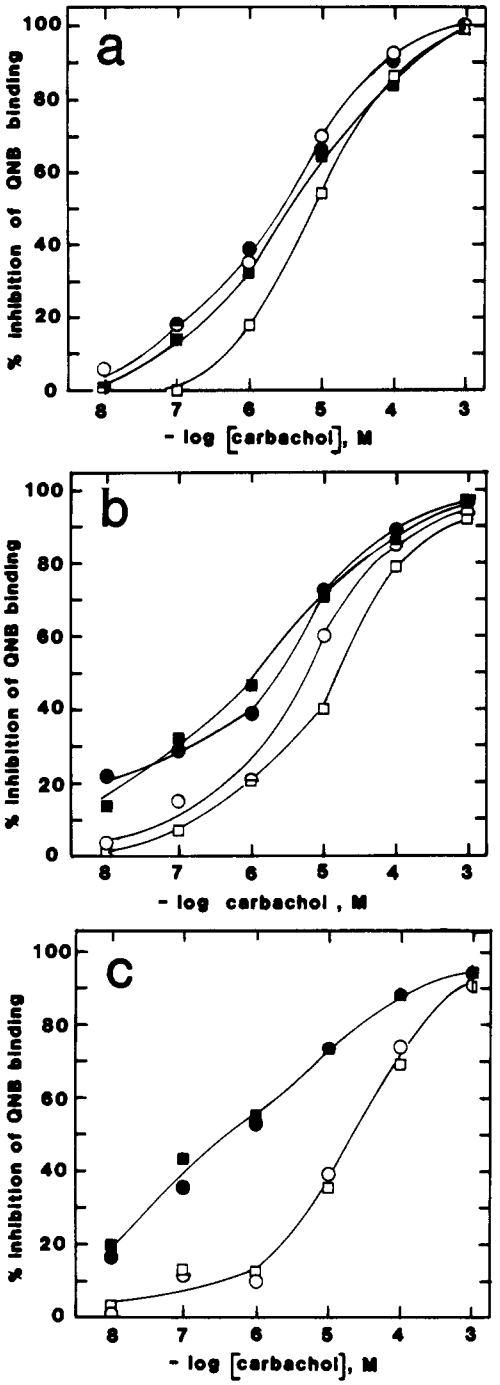

FIGURE 2: Carbachol binding to atrial membranes. Membranes from (a) 4-day, (b) 5-day, and (c) 8-day atria were prepared, and carbachol–[^{3}H]QNB competition binding assays in BRM were performed as described under Experimental Procedures. Assays were carried out with no additions (●), 10 μM GTP (○), 100 μM DTT (■), or GTP and DTT (□). Results presented are the percent inhibition of [^{3}H]QNB binding from a single experiment and were similar to at least two such experiments at each age.

mAChR in 4-day membranes was much less sensitive to GppNHp (EC_{50} = 120 ± 20 nM) than binding to membranes isolated from 5-day (EC_{50} = 31 ± 5 nM) or 8-day (EC_{50} = 20 ± 3 nM) embryos (mean ± SEM, three independent experiments; 4-day results significantly different from both 5-day and 8-day results, $p < 0.025$). The regulation of agonist binding to the mAChR by guanine nucleotides at 4 days appears to be impaired, as seen in the decreased maximal effect of and increased EC_{50} for guanine nucleotides and in the requirement for DTT treatment of the membranes in order to see guanine nucleotide effects.

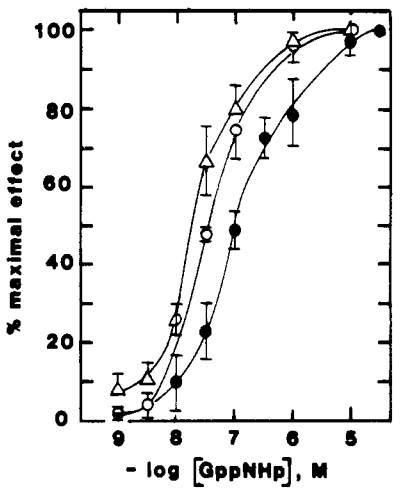

FIGURE 3: Guanine nucleotide sensitivity of agonist binding to mAChR in developing atria. The amount of [^{3}H]QNB binding to atrial membranes in the absence and presence of 1 μM carbachol was assayed in BRM and DTT (0.1 mM) with increasing concentrations of GppNHp. Results are expressed as the percent of the maximum decrease in inhibition by carbachol of [^{3}H]QNB binding due to the addition of GppNHp (as shown, for example, in Figure 2) and are the mean (±SEM) of three separate experiments for 4- (●) and 5-day (○) atria and four experiments for 8-day (△) atria.

Table III: GppNHp Inhibition of Forskolin-Activated Adenylate Cyclase[a]

	activity [pmol of cAMP (mg of protein)$^{-1}$ (10 min)$^{-1}$]		
	4-day atria	5-day atria	8-day atria
no additions	270 (±30)	400 (±20)	320 (±40)
forskolin	6640 (±140)	6790 (±210)	5640 (±190)
forskolin + GppNHp	4930 (±65)	4680 (±120)	4230 (±50)
% inhibition	29 (±3)[b]	31 (±2)	26 (±1)

[a] Adenylate cyclase activity was determined in atrial membranes as described in Table II. Table values are the mean (±SEM) from a single experiment in triplicate and are representative of three such experiments. GppNHp concentration was 3 or 10 μM, whichever resulted in greater inhibition of activity, and forskolin was present at 100 μM. Percent inhibition is the mean (±SEM) percent maximum inhibition of GppNHp determined in three independent experiments. [b] Not significantly different from 5-day or 8-day atria, $p > 0.1$.

Inhibition of Adenylate Cyclase Activity by Guanine Nucleotides. The decreased sensitivity of the mAChR–guanine nucleotide coupling at 4 days could be a result of an altered mAChR and/or guanine nucleotide regulatory component. Seamon & Daly (1982) reported that GppNHp inhibits forskolin-activated adenylate cyclase through N_I in a hormone receptor independent manner. GppNHp is thought to bind more rapidly to N_I than to N_S, so that with short assay incubation times inhibition rather than stimulation of adenylate cyclase activity is seen (Seamon & Daly, 1982). We therefore used GppNHp–mediated inhibition of forskolin-activated adenylate cyclase activity to test whether the apparent functional interaction of N_I with guanine nucleotides and adenylate cyclase was normal or impaired in 4-day embryonic atria. Both the forskolin-activated enzyme activity and the maximum inhibition by GppNHp (3–10 μM) were similar among 4-, 5-, and 8-day atria (Table III). However, adenylate cyclase activity in membranes from 5- and 8-day atria was much more sensitive to GppNHp than 4-day atria (Figure 4). The IC_{50} for GppNHp inhibition decreased from 210 ± 70 nM at 4 days to 60 ± 15 nM at 5 days and to 18 ±3 nM at 8 days (mean ± SEM, three independent experiments; 4-day results were significantly different from 5-day values at $p < 0.05$ and from 8-day values at $p < 0.01$; 5-day results were significantly

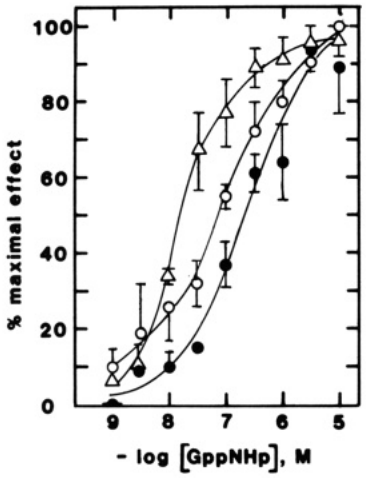

FIGURE 4: Guanine nucleotide sensitivity of forskolin-activated adenylate cyclase activity in 4-, 5-, and 8-day atria. Forskolin (100 μM)-activated adenylate cyclase activity was determined as described under Experimental Procedures in the presence of increasing concentrations of GppNHp. The results are expressed as the percent of maximum inhibition of activity (see Table III) and are the mean (±SEM) of three separate experiments for 4- (●) and 5-day (○) atria and four experiments with 8-day (△) atrial membranes.

different from 8-day results at $p < 0.025$). This increasing sensitivity to GppNHp with increasing embryonic age correlates well with the onset of mAChR activity and regulation described above.

Pertussis Toxin Labeling. In order to compare the physical characteristics of the apparently less active N_I from 4-day atria with the more active N_I from 5- and 8-day atria, pertussis toxin was used to catalyze the [^{32}P]ADP-ribosylation of N_I in 4-, 5-, and 8-day atrial membranes. When the toxin-treated membranes were solubilized and subjected to SDS–PAGE, two specifically labeled products were observed with molecular weights of 39 200 (39K; ±300 SEM, $n = 5$ independent experiments) and 42 100 (42K; ±300 SEM, $n = 5$; see Figure 5). These two products labeled by pertussis toxin were distinct from the cholera toxin specific ADP-ribosylated products in embryonic heart as determined by both SDS–PAGE and two-dimensional gel electrophoresis (cholera toxin data not shown). Four-day atria had less 39K product relative to 42K product than did 5- or 8-day atria. In the experiment shown (Figure 5), the ratio of 39K to 42K product was less at 4 days (0.45) than at 5 days (0.55) or 8 days (1.25) as determined by densitometric scanning of autoradiographs. The mobility and relative intensities of these two products were not altered when protease inhibitors were included during membrane preparation (see Experimental Procedures).

A potentially more sensitive method to separate proteins is by two-dimensional gel electrophoresis. This method separates proteins in the first dimension by isoelectric focusing, which has the capability to distinguish single charge differences in peptides that may not appear as a change in molecular weight, and separates in the second dimension by SDS–PAGE to identify proteins by molecular weight (O'Farrell, 1975). Pertussis toxin labeled products of 4-, 5-, and 8-day atrial membranes were separated by two-dimensional gel electrophoresis, and a distinct pattern of four specifically labeled 39K and 42K products was consistently seen (Figure 6). In 4-day atria, pertussis toxin labeled much less of the acidic 39K product than at 5 or 8 days (Figure 6). The amount of acidic 39K product was only 16% (±6%) of the sum of the 39K and 42K products in 4-day atria and increased to 39(±9)% at 5 days and was further increased to 55(±5)% at 8 days as determined by densitometric scanning (4-day values were significantly different from 5-day values at $p < 0.05$, $n = 3$, and 8-day values at $p < 0.005$, $n = 4$). The relative intensities of the labeled products were not affected when protease inhibitors were included during the membrane preparation steps (see Experimental Procedures). In addition, when 4-day atrial membranes were labeled with pertussis toxin and [^{32}P]NAD, washed free of unreacted NAD, and then incubated with unwashed homogenates of 8-day atria, the labeling pattern was not different from the control 4-day atrial two-dimensional pattern (data not shown). The results of the converse experiment, where 8-day atrial membranes were toxin labeled and subsequently incubated with 4-day atrial homogenates, also did not reveal any change from the control in the observed labeling pattern (data not shown). Therefore, it is unlikely that there were proteases or other factors, independent of N_I, in 4- or 8-day atrial homogenates which caused the migration of the pertussis toxin substrates to be different.

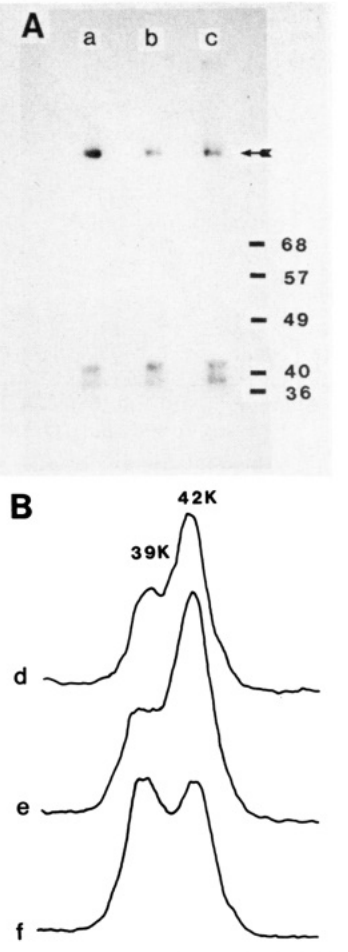

FIGURE 5: Autoradiographs of one-dimensional SDS–polyacrylamide gels of pertussis toxin labeled atrial membranes. Membranes were incubated with [^{32}P]NAD and pertussis toxin and subjected to electrophoresis as described under Experimental Procedures. (Panel A) Autoradiographs of (lane a) 4-day membranes, (lane b) 5-day membranes, and (lane c) 8-day membranes. The arrow points to a nonspecifically labeled band which is also labeled by [^{32}P]NAD in the absence of pertussis toxin. (Panel B) Densitometric tracings (see Experimental Procedures) of the 39K and 42K bands from the autoradiograph shown in panel A: (d) 4-day, (e) 5-day, and (f) 8-day membranes.

The results of these experiments revealed that two changes were detectable in the physical properties of N_I between 4 and 8 days. One was an increase in the relative amount of the 39K form of N_I as compared to 42K in 8-day compared to 4-day atria. The second was a dramatic increase in an ADP-ribosylated 39K acidic component of N_I which occurs between

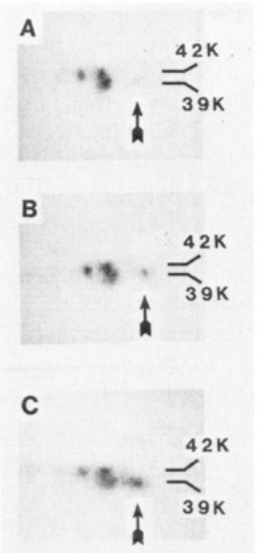

FIGURE 6: Autoradiographs of two-dimensional gels of pertussis toxin labeled atrial membranes. Membranes were incubated with [^{32}P]NAD and pertussis toxin, and two-dimensional electrophoresis was performed as described under Experimental Procedures. Only the portion of the autoradiograph that contains the 39K and 42K spots is shown. The acidic end of the gel is to the right, and the arrow indicates the 39K acidic labeled product. The most acidic component focused at pH 5.5, and the most basic component focused at pH 6.0. (A) 4-Day membranes; (B) 5-day membranes; (C) 8-day membranes. The acidic component at 4, 5, and 8 days was 5%, 24%, and 40%, respectively, of the sum of the 39K and 42K products as determined by densitometric scanning of the regions of the gels shown.

4 and 8 days. These alterations of N_I appear over the same 4–8-day developmental period as both the increased sensitivity to guanine nucleotides and the increased physiological responsiveness of mAChR activation.

Discussion

Development of the Chronotropic Effect of Muscarinic Agonists Parallels Coupling of mAChR with N_I. Muscarinic agonists have been shown to exert a minimal negative chronotropic response in isolated atria and intact hearts from 3-day embryos (Pappano & Skowronek, 1974; Galper et al., 1977; Renaud et al., 1980) and from 4-day embryos (Galper et al., 1977; Figure 1). On the fifth day, most atria were capable of a full negative chronotropic response to muscarinic agonists (Figure 1). We report here that agonist binding to the mAChR in membranes from 4-day atria was not regulated by GTP (or GppNHp) when assayed in the same physiological buffer (BRM) as used in the isolated atria studies (Figure 2). Exposure of 4-day membranes to the sulfhydryl reducing agent DTT was sufficient to permit a small but reproducible GTP effect on agonist binding (Figure 2a). GTP effects on agonist binding in 8-day membranes were greater than at 4 or 5 days and were not affected by DTT (Figure 2). Harden et al. (1982) have reported that exposure of membranes to the sulfhydryl alkylating agent *N*-ethylmaleimide blocks guanine nucleotide regulation of muscarinic agonist binding in rat heart. Together, the results of Harden et al. (1982) and those reported here (Figure 2a) suggest a role for an essential sulfhydryl group in the regulation of agonist binding by guanine nucleotides. Four-day atria were also less sensitive than 8-day atria to guanine nucleotide regulation of agonist binding when assayed with DTT (Figure 3). These results suggest that there is an impairment in the coupling of the mAChR to a guanine nucleotide regulatory component at 4 days, which is less detectable at 5 days and not apparent on the eighth embryonic day. Therefore, the time course of developmental onset of the mAChR-mediated negative chronotropic response in atria parallels the maturation of mAChR coupling to guanine nucleotide regulation. Another example of a decreased mAChR-mediated physiological response accompanied by a decreased sensitivity of agonist binding to guanine nucleotides has been reported for the cardiomyopathic Syrian hamster. Wells et al. (1981) reported that as this animal becomes spontaneously congestive the mAChR-mediated negative chronotropic response decreases and agonist binding to the mAChR is no longer regulated by guanine nucleotides.

Increased sensitivity of mAChR to agonist regulation of receptor number was also temporally correlated with development of mAChR-mediated negative chronotropy. The mAChR in 4-day hearts was less responsive than the receptor in 5- or 8-day hearts to agonist-induced decreases of receptor number (Table I). This response was increased at day 5 to a level similar to that seen in 8-day embryos (Table I). Previous work has shown that the binding of muscarinic agonists to intact heart cells more closely resembles binding of agonists to membranes in the presence rather than the absence of guanine nucleotides (Nathanson, 1983); in the presence of GTP, the 4-day mAChR had a higher apparent affinity for carbachol than receptors from 5- or 8-day embryos (Figure 2). Therefore, the decreased sensitivity at 4 days did not seem to result from a decreased affinity of the mAChR for carbachol. Agonist regulation of β-adrenergic receptor number is apparently dependent on functional receptor interaction with a guanine nucleotide regulatory subunit since mutants of S-49 lymphoma cells lacking N_S do not respond to prolonged β-adrenergic agonist exposure by decreasing receptor number as do wild-type S-49 cells (Su et al., 1980). The diminished response to muscarinic agonist-induced decreases in receptor number and the diminished negative chronotropic response at 4 days may be a reflection of the altered coupling of the mAChR due to an immature N_I.

Molecular Form of N_I Changes as Receptor Coupling Occurs. In atrial membranes, pertussis toxin catalyzed the ADP-ribosylation of polypeptides with molecular weights of 42 000 and 39 000 (Figure 5). Other investigators report a single polypeptide with a molecular weight ranging from 39 000 in human erythrocytes (Codina et al., 1983) to 41 000 in rabbit liver (Bokoch et al., 1983) which is labeled by pertussis toxin. In our results, the observed increase in the amount of the 39K substrate relative to the 42K substrate and the increase in the acidic component of the 39K product between 4 and 8 days correlate well with the increased sensitivity of guanine nucleotide regulation of both agonist binding and inhibition of adenylate cyclase. In addition, the molecular form of N_I changes over the same time course as development of the mAChR-mediated negative chronotropic response and regulation of receptor number.

These results indicate that N_I is both physically and functionally different at 4 days as compared to 5 or 8 days and that the acidic component may be the most physiologically active form. A potentially analogous situation has been described for the coupling of β-adrenergic receptors to N_S. In a preliminary report, Kassis et al. (1983) have indicated that culturing of HeLa cells in medium containing sodium butyrate induces an increase in the function of N_S as determined by its ability to reconstitute GTP activation of adenylate cyclase activity in S-49 cyc$^-$ membranes and that the isoelectric point of the 8-azido-GTP-labeled subunit of N_S is different from that for the labeled subunit of cells grown without sodium butyrate. In another system, exposure of turkey erythrocytes

to β-adrenergic agonists induces a desensitization of the β-adrenergic receptor from stimulation of adenylate cyclase activity that is distinct from agonist-induced decreases in receptor number and involves an inactivation of N_S (Briggs et al., 1983). These two situations indicate that tissues are capable of long-term modifications of the structure and function of guanine nucleotide regulatory proteins in vivo.

Our laboratory has recently found that newly synthesized receptors which reappear following an agonist-induced decrease in mAChR number in 8-day embryonic atria also exhibit a diminished mAChR-mediated negative chronotropic response. However, when some of the methods described here are used, the decreased mAChR response does not appear to be associated with a decrease in the function of N_I, suggesting that the newly synthesized mAChR is different from the mature receptor (Hunter & Nathanson, 1984). While the results reported here indicate that in 4-day atria a likely locus for the defect in the coupling of the mAChR to physiologically and biochemically measured responses residues in N_I, they do not exclude additional defects in the mAChR or other unidentified components. Several electrophysiological parameters have been reported to change in chick ventricles between the third and fifth embryonic day, such as a decrease in Na^+ permeability relative to K^+ permeability, an increase in action potential duration, and an increase in resting membrane potential (McDonald & DeHaan, 1973). The membrane-associated ($Na^+ + K^+$)-dependent ATPase activity also increases over this developmental period (Sperelakis et al., 1975). It is not known if any of these contribute to the onset of the negative chronotropic response of the atria to mAChR activation between 4 and 5 days.

Role of Cyclic Nucleotides and N_I in the mAChR-Mediated Negative Chronotropic Response. Electrophysiological studies in cardiac cells reveal a time lag (approximately 100 ms) after exposure to muscarinic agonist before onset of the response (Hill-Smith & Purves, 1978; Hartzell, 1980). This lag has been widely interpreted to suggest that production of a second messenger may be necessary for mAChR activity [see Hartzell (1981) for a review]. While mAChR activation leads to inhibition of cAMP formation in the heart, the results of studies to date have not supported a role for mAChR-induced decrease in intracellular cAMP levels mediating inhibition of nodal beating rate in cardiac tissue (Trautwein et al., 1982) or the increased potassium permeability which mediates this response (Nargeot et al., 1983). The apparent lack of effect of cyclic nucleotides suggests that coupling of mAChR to adenylate cyclase does not play a role in the negative chronotropic response.

In *Torpedo* synaptosome preparations, muscarinic agonist binding is regulated by GTP, but the mAChR is not coupled to inhibition of adenylate cyclase activity (Pinchesi & Michaelson, 1982), suggesting a role for guanine nucleotide regulation other than for coupling to adenylate cyclase. A variety of reports examining the physiological effects of pertussis toxin also suggest that N_I may have functions not directly related to alterations of cAMP levels (Nakamura & Ui, 1983; Cronin et al., 1983; Dorflinger & Schonbrunn, 1983; De Wildt et al., 1983). The data in these reports are consistent with those presented in this study and suggest that covalent modification of N_I has effects on the coupling of receptors to cellular responses independent of changes in cAMP metabolism.

In conclusion, using distinct developmental stages of the chick heart, we have shown that the onset of the mAChR-mediated negative chronotropic response and the agonist-induced decrease in receptor number are correlated with increased sensitivity of the mAChR and N_I to guanine nucleotides and with changes in the physical properties of the pertussis toxin labeled subunit of N_I. Further investigation of this system should help to elucidate the molecular components and biochemical mechanisms that are necessary for the function and regulation of the muscarinic receptor.

Acknowledgments

We thank Dr. Daniel Storm and his colleagues for helpful advice and technical assistance and Dr. William Catterall for help in the preparation of the manuscript.

Registry No. GTP, 86-01-1; GppNHp, 34273-04-6; carbachol, 51-83-2; adenylate cyclase, 9012-42-4.

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Purification of Myosin Light Chain Kinase from *Limulus* Muscle†

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ABSTRACT: It has previously been shown that the regulatory light chains of myosin from *Limulus*, the horseshoe crab, can be phosphorylated either by purified turkey gizzard smooth muscle myosin light chain (MLC) kinase or by a crude kinase fraction prepared from *Limulus* muscle [Sellers, J. R. (1981) *J. Biol. Chem. 256*, 9274–9278]. This phosphorylation was shown to be associated with a 20-fold increase in the actin-activated MgATPase activity of the myosin. We have now purified the Ca^{2+}–calmodulin-dependent MLC kinase from *Limulus* muscle to near homogeneity by using a combination of low ionic strength extraction, ammonium sulfate fractionation, and chromatography on Sephacryl S-300 and DEAE-Sephacel. The final purification was achieved by affinity chromatography on a calmodulin–Sepharose 4B column. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis showed 95% of the protein to be comprised of a doublet with M_r = 39 000 and 37 000. Electrophoresis of the kinase fraction under nondenaturing conditions resulted in a partial separation of the two major bands and demonstrated that each had catalytic activity. An SDS–polyacrylamide gel overlayed with ^{125}I-calmodulin demonstrated that both the M_r 39K and the M_r 37K proteins bind calmodulin. Neither of the bands could be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. With *Limulus* myosin light chains as a substrate, the V_{max} was 15.4 μmol min^{-1} mg^{-1}, and the K_m was 15.6 μM. The K_D for calmodulin was determined to be 6 nM. The enzyme did not phosphorylate histones, casein, actin, or tropomyosin.

Although the contraction of all types of muscle seems to be regulated by the free calcium level in the sarcoplasm, the site of direct calcium action is variable (Adelstein & Eisenberg, 1980). There are three distinct calcium regulatory systems which have been described: (1) Thin filament linked regulation works via the troponin–tropomyosin system in which a subunit of troponin reversibly binds calcium ions (Adelstein & Eisenberg, 1980). This appears to be the dominant or sole regulatory system in vertebrate skeletal muscle. (2) In certain invertebrate muscles, notably those of the mollusks, there is a myosin-linked regulation in which the myosin itself is directly regulated by reversible calcium binding (Szent-Györgyi et al., 1973; Chantler et al., 1981). In the absence of calcium, this type of myosin has a low actin-activated MgATPase activity, but when calcium is bound, the MgATPase activity of the myosin is greatly enhanced by actin (Szent-Györgyi et al., 1973). (3) Studies of scallop myosin have established that this type of regulation is mediated by a particular class of myosin

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