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Differential Cholinoceptor Subtype-Dependent Activation of Signal Transduction Pathways in Neonatal Versus Adult Rat Atria

Enri S. Borda, Claudia Perez Leiros, Juan Jose Camusso, Sandra Bacman and Leonor Sterin-Borda*

Centro de Estudios Farmacologicos y Botanicos (CEFYBO), Consejo Nacional de Investigaciones Cientificas y Tecnicas de la Republica Argentina (CONICET), y Catedra de Farmacologia de la Facultad de Odontologia de la Universidad de Buenos Aires, Buenos Aires, Argentina

ABSTRACT. In this study, we investigated the expression and distribution of muscarinic cholinergic receptors (mAChRs) and the different signaling pathways associated with mAChR activation in atria isolated from adult and neonatal rats. Carbachol stimulation of mAChRs in both neonatal and adult rat atria led to a negative inotropic response with activation of phosphoinositide hydrolysis, an increase in cyclic GMP levels, and a decrease in cyclic AMP production. However, compared with adult atria, neonatal atria showed hypersensitivity in the contractile effect induced by carbachol. Pharmacological analysis with mAChR antagonists indicated that M_1 and M_2 mAChR subtypes are important mediators of the response to carbachol in neonatal atria. In contrast, in adult atria the effect of the agonist was coupled only to the M_2 mAChR subtype. Moreover, an increased number of total mAChRs was labeled in neonatal atrial membranes compared with those of adults. Although a predominant M_2 mAChR population is expressed in atria at both stages of development studied, competition binding parameters calculated for carbachol indicated the presence of high-affinity binding sites, with higher affinity in neonates than in adults. These results suggest that the differences observed between neonatal and adult atria in their response to a cholinergic agonist may be related to differential expression of mAChR subtypes and/or changes in functional coupling of mAChR subtypes during development. BIOCHEM PHARMACOL 53;7:959–967, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. mAChR; M_1 and M_2 subtypes; cAMP; heart contractility; neonatal and adult atria; cGMP; PI hydrolysis; NOS activity

Pharmacological studies have suggested that more than one type of mAChR† might exist. On the basis of molecular cloning systems, five mAChR subtypes have been identified, but the conventional approach could only differentiate between three main mAChR subtypes [1]. There is a consensus in that M_1 receptors with high affinity for pirenzepine are located predominantly in the central nervous system and autonomic ganglia [2]; they differ from those expressed in the heart (referred to as M_2 receptors) and from those M_2 receptors characterized in exocrine glands [1].

Studies on cloned mAChRs have demonstrated that the heart M_2 and brain M_1 muscarinic receptors are related but different, presenting some degree of sequence homology, with amino acid sequences 38% identical [3, 4]. They are highly homologous in the membrane-spanning regions but exhibit considerable diversity in the third cytoplasmic loop [5, 6], where the NH_2-terminal region has been found to impart selectivity in receptor-G-protein interaction [7].

It has been postulated that the divergence in the cytoplasmic domains of mAChR subtypes may account for the diversity in the signal transduction events that are mediated by the mAChR subtypes and their selective regulation [8].

Among the intracellular events induced by mAChR activation coupled to multiple G proteins, activation of PI turnover, stimulation of NOS, increase of cGMP levels, and decrease of cAMP production have been described [9–12]. Studies of signaling events on cloned mAChR subtypes revealed differences in their ability to modulate many different signal transduction pathways. It has been proposed that M_1 mAChR subtypes are coupled to stimulation of

* Corresponding author: Enri S. Borda, M.D., CEFYBO-CONICET, Serrano 669, 1414 Buenos Aires, Argentina. FAX 54-1-856-2751.

† *Abbreviations:* mAChR, muscarinic acetylcholine receptor; PI, phosphoinositide; KRB, Krebs–Ringer bicarbonate; L-NMMA, L-arginine-N^G-monomethyl-arginine; NOS, nitric oxide synthase; GppNHp, guanosine 5′-(β,τ-imidotriphosphate; cGMP and cAMP, cyclic GMP and AMP, respectively; IP, inositol phosphate; PGE, prostaglandin E_1; [^{3}H]QNB, 1-[benzilic-4,4′-^{3}H(N)]quinuclidinyl benzilate; and NCDC, 2-nitro-4-carboxyphenyl-*N*,*N*-diphenyl-carbamate.

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polyphosphoinositide (PI) hydrolysis, whereas activation of M_2 receptors leads to attenuation of adenylate cyclase and only weak stimulation of PI hydrolysis [10, 13].

The adult rat atrium expresses only active M_2 mAChRs, and it appears that this single mAChR subtype is capable of activating more than one type of G protein affecting more than one effector [12]. However, it is still unclear if differences in the ontogeny of atrial muscarinic responsiveness is related to differential expression of, or functional linkage to, mAChR subtypes during development. It is interesting that M_1 receptors have been reported to be more abundant in neonatal than in adult murine cardiac tissue [14]. Nevertheless, little is known about the functional expression of non-M_2 cardiac mAChRs during development.

In the present study, we demonstrated that neonatal rat atrium expresses mAChR subtypes functionally different from those of adult atrium, on the basis of their ability to regulate several signal transduction pathways and to modify atrial contractility.

MATERIALS AND METHODS

Contractile Studies

Adult (70 days) and neonatal (9 days) Wistar rats were used throughout. After the rats were decapitated and bled, their chests were opened, and the hearts were excised quickly. The atria were dissected immediately and mounted on a polygraph, as described previously [15]. One end of each atrium was attached to a stationary glass holder, immersed in a tissue chamber filled with a modified KRB solution with 2.4 mM Ca^{2+} and gassed with a mixture of 95% O_2/5% CO_2, composed as reported previously [15]. The other end of each atrium was connected to a force transducer (Statham UC-3 Gold Cell).

Throughout the experiments, the preparations were subjected to a constant resting tension of 500 mg by means of a micrometric device attached to the transducer, the output of which was amplified and recorded with a direct ink-writing oscillograph. The tissue bath solution was gassed with a mixture of 5% CO_2/95% O_2 and kept at a constant temperature of 30° and pH 7.4.

The preparations were paced with a bipolar electrode and an SK4 Grass Stimulator. The stimuli had a duration of 2 msec and the voltage was 10% above threshold. Inotropic effects were determined by recording the maximum rate of isometric force (dF/dt) developed above the externally applied resting tension. Control values (= 100%) refer to the dF/dt before the addition of different drugs. The absolute values of dF/dt at the end of equilibrium (60 min) ranged between 7.4 ± 0.6 and 8.1 ± 0.7 g/sec. Cumulative concentration–response curves for carbachol on both atria were obtained according to the method of van Rossum [16]. The time interval between each concentration was that required to produce the maximal effect (5 min).

Measurement of Total Labeled IPs

Adult and neonatal atria were incubated for 120 min in 0.5 mL of KRB gassed with 5% CO_2 in oxygen with 1 μCi [*myo*-^{3}H]inositol (sp. act. 15 Ci/mmol) from Dupont/New England Nuclear (Boston, MA); LiCl (10 mM) was added for inositol monophosphate accumulation according to the technique of Berridge [17]. Carbachol was added 30 min before the end of the incubation period and the blockers 30 min before the addition of carbachol. Water-soluble IPs were extracted after 120 min of incubation following the method of Berridge [17]. Atria were washed quickly with KRB and homogenized in 0.3 mL of KRB with 10 mM LiCl and 2 mL chloroform:methanol (1:2, v/v) to stop the reaction. Then chloroform (0.62 mL) and water (1 mL) were added. Samples were centrifuged at 2000 *g* for 15 min, and the aqueous phase of the supernatants (1–2 mL) was applied to a 0.7-mL column of Bio-Rad AG 1 × 8 anion-exchange resin (100–200 mesh) suspended in 0.1 M formic acid that had been washed previously with 20 vol. of 5 mM *myo*-inositol followed by 6 vol. of water, and IPs were eluted with 1 M ammonium formate in 0.1 M formic acid. Fractions (1 mL) were recovered, and radioactivity was determined by scintillation counting. Results were expressed as the absolute values of the area under the curve following the criteria of Simpson's equation, and in order to determine the absence of [*myo*-^{3}H]inositol in the eluted peak of the IPs, chromatography on silica gel 60 F 254 sheets (Merck) was performed as described previously [15, 18]. Spots were located by spraying with freshly prepared 0.1% ferric chloride in ethanol followed, after air drying, with 1% sulfosalicylic acid in ethanol. To assay the radioactivity, a histogram was constructed by cutting up the sheet gel, placing each sample in Triton–toluene scintillation fluid, and then counting.

Determination of NOS Activity

NOS activity was measured in both atria (neonatal and adult) by production of [U-^{14}C]citrulline from [U-^{14}C]arginine according to the procedure described by Bredt and Snyder [19] and Sterin–Borda *et al.* [12]. Briefly, after a 20-min preincubation in KRB solution, atria were transferred to 500 μL of prewarmed KRB equilibrated with 5% CO_2 in oxygen in the presence of [U-^{14}C]arginine (0.5 μCi). Appropriate concentrations of drugs were added, and the atria were incubated for 20 min under 5% CO_2 in oxygen at 37°. Atria were then homogenized with an Ultraturrax in 1 mL of medium containing 20 mM HEPES, pH 7.4, 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, 1 μM leupeptin and 0.2 mM phenylmethylsulfonyl fluoride at 4°. After centrifugation at 20,000 *g* for 10 min at 4°, supernatants were applied to 2-mL columns of Dowex AG 50 WX-8 (sodium form); [^{14}C]citrulline was eluted with 3 mL of water and quantified by liquid scintillation counting. When partial purification of NOS was required, the atria were homogenized with an Ultraturrax as described above. After centrifugation at 20,000 *g* for 10 min, the supernatant was partially purified by a 2′,5′-ADP-Sepharose column equilibrated with the homogenization buffer supplemented with 1 mM $MgCl_2$ and 100 mM NaCl.

The column was washed with this medium until no more protein emerged, and then NOS activity was eluted with homogenization buffer containing NADPH and 10% glycerol. Samples containing NOS activity were incubated in a buffer with 20 mM HEPES, 10 μM [U-^{14}C]arginine (0.3 μCi), 0.5 mM NADPH, and 10 μg/mL of calmodulin (200 μL of incubation volume). After a 10-min incubation, the reaction was stopped by the addition of 1 mL of ice-cold 10 mM EGTA, 1 mM citrulline, and 100 mM PIPES, pH 5.5. Fractions containing NOS activity were concentrated by ultrafiltration through cellulose citrate membrane with a cut-off of 30 kDa. All procedures were carried out at 4°, and enzyme activity was measured. Measurement of basal NOS activity in whole atria by the above-mentioned procedure was inhibited 95% in the presence of 0.5 mM L-NMMA. The results (pmol/g tissue wet) obtained for whole atria were expressed as the difference between values in the absence (287 ± 12, N = 8) and in the presence (13 ± 2, N = 9) of L-NMMA.

cGMP Assay

Tissues were incubated in 1 mL KRB for 30 min, and carbachol was added in the last 5 min. When blockers were used, they were added 25 min before the addition of carbachol. After incubation, atria were homogenized in 2 mL of absolute ethanol and centrifuged at 6000 *g* for 15 min at 4°. Pellets were then rehomogenized in ethanol:water (2:1) and supernatants collected and evaporated to dryness as indicated above. cGMP in the residue was dissolved in 400 μL of 0.05 M sodium acetate buffer, pH 6.2. Aliquots of 100 μL were taken for nucleotide determination using the RIA procedure with a cyclic GMP ^{125}I-RIA from Dupont/New England Nuclear.

cAMP Assay

Tissues were preincubated for 20 min in the presence of 0.1 mM isobutylmethylxanthine in a final volume of 1 mL KRB solution and then for an additional 5 min with or without carbachol. PGE_1 (1×10^{-6} M) was added as an adenylate cyclase activator in the last 5 min of the incubation period [20]. After incubation, tissues were homogenized in 2 mL of absolute ethanol and centrifuged at 6000 *g* for 10 min. Supernatants were collected, and the pellets were rehomogenized with ethanol:water (2:1) and centrifuged again as before. Supernatants were then evaporated to dryness. Residues were resuspended in 5 mM Tris–HCl, pH 7.4, containing 8 mM theophylline, 0.45 mM EDTA, and 6 mM 2-mercaptoethanol. cAMP was determined by the competitive protein-binding assay described by Brown *et al.* [21], using [^{3}H]cAMP as a tracer.

Radioligand Binding Assay

Membranes from neonatal and adult atria were prepared as described previously [22]. In brief, atria were homogenized in an Ultraturrax at 4° in 6 vol. of 50 mM phosphate buffer (pH 7.5), 1 mM $MgCl_2$, 0.25 M sucrose (buffer A) supplemented with 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 5 μg/mL leupeptin, and 1 μM pepstatin A. The homogenate was centrifuged twice for 10 min at 3000 *g*, and then at 10,000 and 40,000 *g* at 4° for 15 and 90 min, respectively. The resulting pellet was resuspended in 50 mM phosphate buffer, 1 mM $MgCl_2$ with the same protease inhibitors, pH 7.5 (buffer B). Receptor ligand binding was performed as described previously [23]. Aliquots of the membrane suspension (30–50 μg protein) were incubated with different concentrations of [^{3}H]QNB (Dupont/New England Nuclear; sp. act. 44.8 Ci/mmol) for 60 min at 37° in a total volume of 500 μL of buffer B. Binding was stopped by adding 2 mL of ice-cold buffer followed by rapid filtration (Whatman GF/c). Filters were rinsed with 12 mL of ice-cold buffer, transferred into vials containing 10 mL of scintillation fluid, and counted in a liquid scintillation spectrometer. Nonspecific binding was determined in the presence of 5×10^{-6} M atropine and never exceeded 10% of total binding. Radioactivity bound was lower than 10% of total counts. For competition binding experiments, atrial preparations were incubated with increasing concentrations of muscarinic antagonists (atropine, AFD-X 116, pirenzepine) or carbachol in the presence of 0.56 nM [^{3}H]QNB. In the case of carbachol competition binding, a 0.1 mM concentration of a GTP analogue, GppNHp, was included in some experiments.

Drugs

Carbachol, atropine, NCDC, PGE_1, pirenzepine, L-arginine, L-NMMA and GppNHp were obtained from the Sigma Chemical Co. (St. Louis, MO) and AFD-X 116 was provided by Boehringer Ingelheim Pharmaceuticals Inc. (Ridgefield, CO). Stock solutions were freshly prepared in the corresponding buffers. The drugs were diluted in the bath to achieve the final concentrations stated in the text.

Statistical Analysis

Student's *t*-test for unpaired values was used to determine the levels of significance. When multiple comparisons were necessary, after analysis of variance, the Student–Newman–Keuls test was applied. Differences between means were considered significant if $P < 0.05$. Binding data were analyzed with the computer-assisted curve-fitting program LIGAND [24].

RESULTS

To determine the mAChR subtypes involved in the biological effects of carbachol on neonatal and adult rat atria, assays of binding, contractility, PI turnover, cGMP and cAMP production, and NOS activity were performed in the presence or absence of selective antagonists.

Contractile Studies

Figure 1 shows the effect of increasing concentrations of carbachol on dF/dt of atria from neonatal and adult rats. It can be seen that carbachol induced a concentration-dependent decrease in dF/dt of both groups of atria. However, while the efficacy (E_{max}) was similar in both groups, the potency as derived from EC_{50} values was greater in atria from neonatal than from adult rats (see legend of Fig. 1).

We then carried out contractile studies with selective antagonists and calculated pA_2 values: Table 1 shows that the action of carbachol on dF/dt of adult atria was preferentially associated with M_2 mAChR activation, whereas in neonatal atria this effect was mediated by both M_1 and M_2 subtypes (Table 1).

Signal Transduction Studies

As can be seen in Fig. 2, carbachol induced a concentration-dependent increase in PI hydrolysis in atria from neonatal and adult rats. The degree of stimulation did not vary between groups though significant differences were observed in basal values, basal accumulation of IP in neonates being higher than in adults (Fig. 2). Experiments conducted

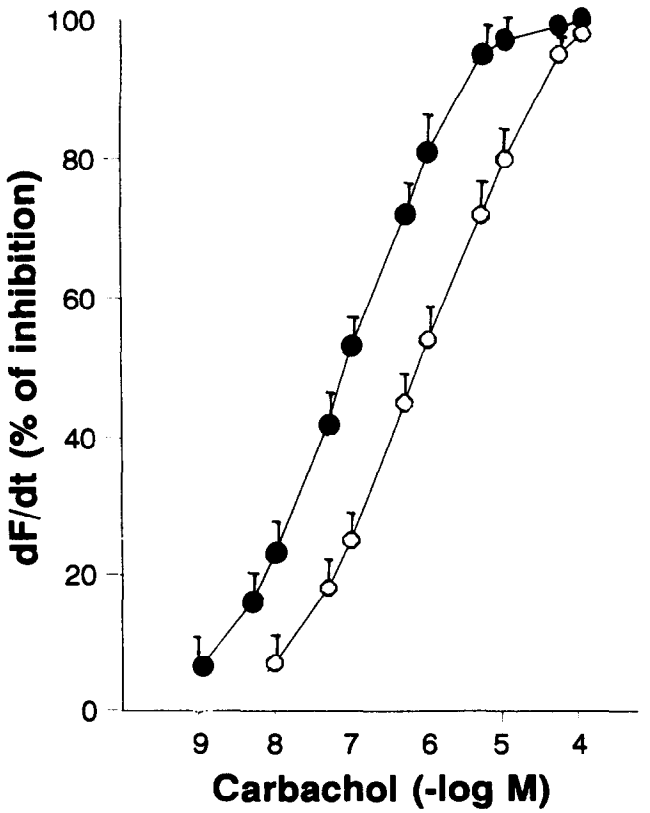

FIG. 1. Contractile effect of carbachol in neonatal (●-●) and adult (○-○) rat atria. Values of dF/dt are expressed in percentage of change versus initial controls (neonatal 7.4 ± 0.6, adult 8.1 ± 0.7 g/sec). Each point is the mean ± SEM of twelve experiments in each group. For neonatal atria, K_d ($\times 10^{-8}$ M) = 6.9 ± 0.3; and for adult atria, K_d ($\times 10^{-8}$ M) = 70.1 ± 5.1.

TABLE 1. Action of mAChR subtypes antagonists on the carbachol mechanical effect in neonatal and adult atria

	pA_2		
	Rat atria		
	Neonatal	Adult	N
Pirenzepine	8.2 (5.2 nM)	6.3 (553 nM)	8
AFD-X 116	8.9 (1.2 nM)	7.9 (11 nM)	8
Atropine	9.2 (0.6 nM)	8.1 (8.4 nM)	7

Atria were preincubated for 30 min with different antagonists before concentration-response curves for carbachol werre determined. The EC_{50} values were obtained from concentration-response curves in the presence or absence of 1×10^{-7} M pirenzepine, AFD-X 116 and atropine, and then K_i and pA_2 were calculated. Standard deviations of pA_2 values were always below 0.2. Values in parentheses are K_i.

in the presence of selective M_1 (pirenzepine) and M_2 (AFD-X 116) receptor blockers showed that both receptor subtypes were coupled to PI turnover in neonatal atria, whereas in adults only M_2 receptors were coupled to PI turnover (Table 2). Table 2 also shows that the inhibition of phospholipase C with NCDC reduced the effect of carbachol on PI hydrolysis in both neonatal and adult atria.

In addition to the activation of PI turnover, carbachol also increased in a concentration-dependent manner NOS activity (Fig. 3) and cGMP production (Fig. 4) in both groups of atria. Again, significant increases in basal values of NOS activity and cGMP levels were seen in neonates compared with adults with similar levels of stimulation in both groups. Pirenzepine inhibited the stimulatory action of carbachol on cGMP production and NOS activity in neo-

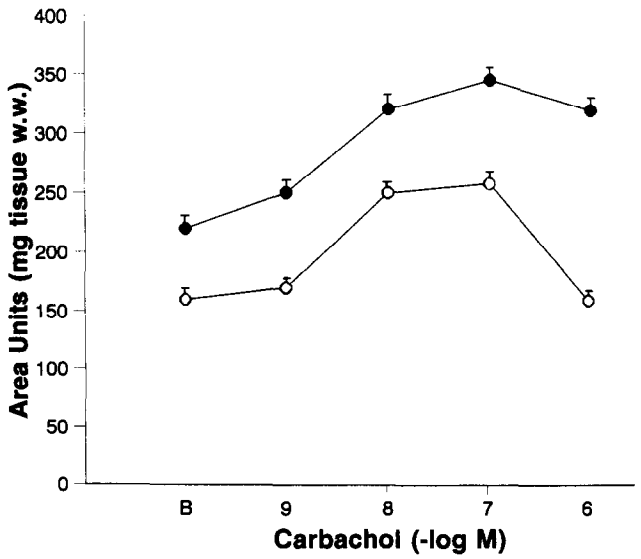

FIG. 2. Cumulative concentration–response curves for the effect of carbachol on PI accumulation in atria isolated from neonatal (●-●) and adult (○-○) rats. Accumulation of IPs was expressed as the absolute values of the area under the curve following the criteria of Simpson's equation as described in Materials and Methods. Area units were normalized by mg of tissue wet weight (mg tissue w.w.). Each point is the mean ± SEM of seven experiments performed in duplicate in each group. B = baseline.

TABLE 2. Effect of mAChR subtype antagonistic drugs on the stimulatory action of carbachol on PI hydrolysis in neonatal and adult atria

	PI area units/mg tissue wet wt.	
	Rat atria	
	Neonatal	Adult
Basal	210 ± 7.2	173 ± 4.2
Carbachol	350 ± 9.3*	241 ± 6.2*
Atropine + carbachol	200 ± 9.2	165 ± 6.2
AFD-X 116 + carbachol	218 ± 9.2	160 ± 6.4
Pirenzepine + carbachol	220 ± 9.4	238 ± 8.5*
NCDC + carbachol	220 ± 6.5	170 ± 5.3

Atria were incubated for 30 min with [*myo*-^{3}H]inositol and for an additional 30 min with or without 5×10^{-7} M mAChR blockers or 5×10^{-6} M NCDC. Tissues were then left for a further 30 min in the absence or presence of 1×10^{-7} M carbachol. Results correspond to the second peak. Values are means ± SEM of seven experiments in each group.

* Statistically different from basal values, $P < 0.001$.

natal atria, but failed to do so in adult atria. On the other hand, in both neonatal and adult atria, AFD-X 116 blocked the agonist-stimulatory action on cGMP production and NOS activity (Table 3 and Fig. 4). As a control, the inhibition of the carbachol effect by L-NMMA (1×10^{-5} M) and the reversal by L-arginine are shown in Fig. 3. The fact that in the case of PI turnover, NOS activity, and cGMP levels the basal values were significantly higher in neonates than in adults but the percent of stimulation with carbachol was similar in both groups suggests that, though differently coupled in basal conditions, the extent of cardiac responses might be limited by the amounts and activity of enzymes during neonatal life.

To determine if different mAChR subtypes were involved in carbachol-induced inhibition of cAMP production, pirenzepine and AFD-X 116 were assayed in the presence of increasing concentrations of carbachol in neonatal and adult atria. As shown in Fig. 5, carbachol decreased cAMP production stimulated by PGE_1 in both groups of atria, with higher efficacy in neonatal atria than in adults. Both AFD-X 116 and pirenzepine were effective in inhibiting the effect of carbachol on neonatal atria. In contrast, only AFD-X 116 was able to antagonize the effect on adult atria (Table 4).

mAChR Expression in Neonatal and Adult Rat Cardiac Membranes

In view of the ability of both M_1 and M_2 antagonists to inhibit carbachol-mediated biological effects in neonatal atria, we investigated the expression and distribution of mAChRs in both groups of atria.

An increased number of mAChR sites was labeled in neonatal membranes compared with those of adults with no significantly different affinities as calculated from Scatchard plots (*Kd*: neonatal 80 ± 10 pM, adult 70 ± 11 pM; B_{max}: neonatal 944 ± 36 fmol/mg protein, adult 590 ± 27 fmol/mg protein). The relative potencies of selective antagonists to compete for [^{3}H]QNB binding to both cardiac membranes shown in Table 5, indicate that a predominant M_2 mAChR population is expressed, regardless of the neonatal or adult stage of development. However, with pirenzepine two different receptor populations were evidenced. Parameters derived from the LIGAND curve-fitting program showed 11 ± 6% of pirenzepine-sensitive high-affinity binding sites and 89 ± 5% of low-affinity sites (Table 5).

To further characterize mAChR populations, we analyzed the presence of high- and low-affinity sites for the agonist carbachol in the absence and presence of GppNHp, a hydrolysis-resistant analogue of GTP. As seen in Fig. 6, both membranes expressed receptors with high- and low-affinity sites for carbachol, with the affinity of high-affinity sites significantly higher in neonates than in adults. Moreover, the GTP analogue was more effective in modifying the competition binding curve of carbachol on neonatal than adult cardiac membranes.

DISCUSSION

The present results demonstrate that neonatal rat atria exhibit an increased sensitivity to the negative inotropic effect of carbachol. This phenomenon appears to be secondary to receptor-occupancy by the agonist and was also observed in a variety of signaling events regulated by mAChRs in cardiac cells such as attenuation of adenylate cyclase, stimulation of guanylate cyclase, and NOS activity. Differences were also found in the stimulation of PI turnover at high concentrations of carbachol in neonatal atria compared with adult atria.

The pharmacological analysis with mAChR antagonists supports the hypothesis that M_1 and M_2 subtypes are important mediators of carbachol biological effects on neonatal atria. In contrast, the effects of carbachol on adult rat atria seem to be mediated only by the M_2 mAChR subtype. Therefore, our results satisfied the pharmacological criteria for the coexistence of M_1 and M_2 mAChRs in neonatal rat atria and M_2 mAChRs in adult atria mediating the decrease in contractility and cAMP production and the increased cGMP production, NOS activity, and PI turnover induced by carbachol.

The diverse signaling events mediated by M_2 mAChRs in adult rat atria shown here confirm our previous findings [12] that the negative inotropic effect of carbachol through M_2 mAChRs is associated with an increased production of nitric oxide and cGMP. The mechanism occurred secondarily to stimulation of PI turnover, involving calcium/calmodulin and protein kinase C activation.

The issue addressed in this study is the subtype selectivity of cardiac muscarinic response during development. Little is known about functionality of mAChR in immature mammalian heart. Studies in neonatal canine Purkinje fibers [25] and in neonatal rat ventricular myocytes [26] suggest that post-synaptic M_1 receptors are functionally expressed.

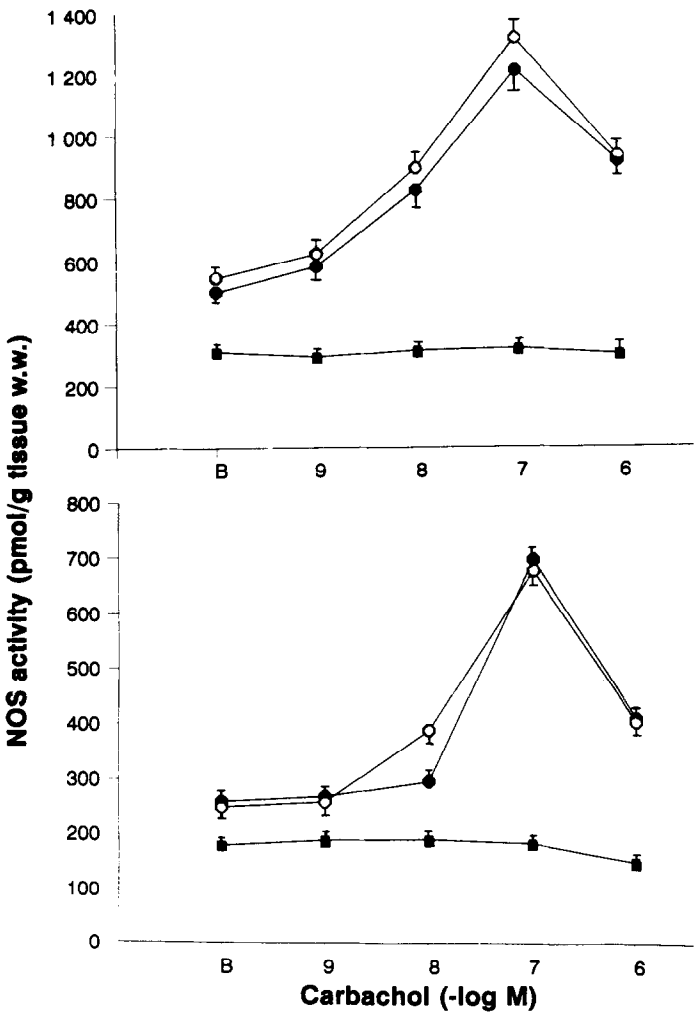

FIG. 3. Cumulative concentration-response curves for the effect of carbachol on NOS activity in atria isolated from neonatal (upper panel) and adult (lower panel) rats. The stimulatory action of carbachol alone (●-●), its inhibition by treatment of atria with 1×10^{-5} M L-NMMA (■-■), and the reversal of inhibition by 5×10^{-5} M L-arginine (○-○) is shown. Values are means ± SEM of six different determinations performed in duplicate in each group. B = baseline.

These receptors mediate responses that involve a pertussis toxin-sensitive G protein, but are not associated with changes in cAMP levels or PI hydrolysis. These observations and ours suggest that the ontogeny of myocardial muscarinic responsiveness may be related to differential expression or functional linkage of muscarinic receptor subtypes during development.

The possibility that a differential expression and/or distribution of mAChR in neonate and adult cardiac membranes was investigated by means of [^{3}H]QNB binding with selective antagonists and with the agonist in the presence or absence of a guanine-nucleotide analogue, GppNHp. Our results indicate that neonatal membranes display a higher density of mAChRs though with similar affinity compared with adult membranes. Furthermore, competition studies with selective antagonists showed that there is a detectable fraction of pirenzepine-sensitive receptors in the neonate as the competition curve for pirenzepine fitted best to a two-site model with approximately 10% of high-affinity receptors. The existence of M_1 mAChRs or pirenzepine high-affinity sites explains the most relevant finding reported here, which is the ability of pirenzepine to antagonize the intracellular signals associated with neonatal mAChR stimulation. These results are in accord with the concept that during development total mAChR density in the rat heart decreases [27] and adult rat cardiac mAChRs are predominantly of the M_2 subtype with only a very small percentage of M_1 receptors [28]. [^{3}H]Pirenzepine-labeled M_1 receptors were reported to be more abundant in neonatal than in adult murine cardiac tissues [14] and in neonatal rat cardiomyocytes the presence of a non-classical M_2 receptor population has been characterized [29]. Thus, it is conceivable that the decrease in mAChR density in the developing heart is subtype specific.

However, the high inhibitory activity of pirenzepine to blunt the effects of the agonist is hard to explain only through the discrete percentage of pirenzepine-sensitive mAChRs found in neonatal atria. To determine if differences in receptor coupling are also implicated in M_1 carbachol effects, we carried out [^{3}H]QNB competition binding experiments with carbachol on each membrane in the presence or absence of GppNHp. We actually found differences in high- and low-affinity receptor populations in neonates compared with adults. Indeed, high-affinity sites expressed in neonates displayed a significantly higher affinity than in adults with the low-affinity population being simi-

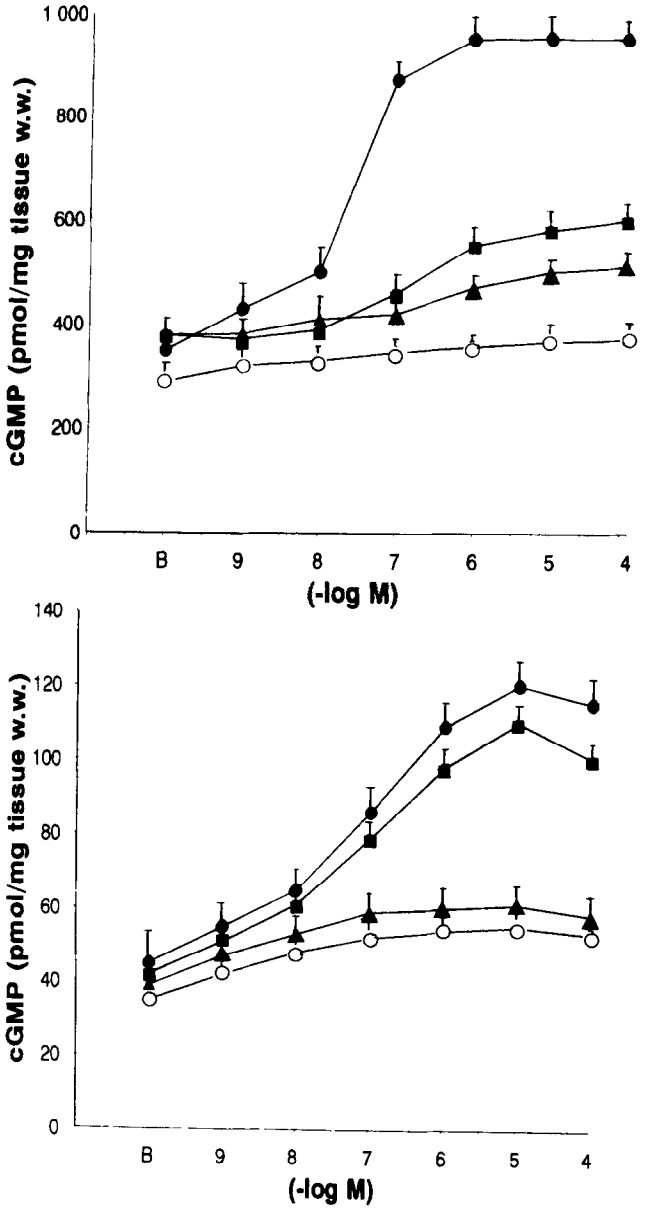

FIG. 4. Cumulative concentration–response curves for the effect of carbachol on cGMP production by atria isolated from neonatal (upper panel) and adult (lower panel) rats. The stimulatory action of carbachol (●-●) and the actions of 1 × 10^{-7} M atropine (○-○), AFD-X 116 (▲-▲), and pirenzepine (■-■) are shown. Each point is the mean ± SEM of eight experiments performed in duplicate in each group. B = baseline.

lar at both stages of development. Moreover, the addition of the GTP analogue induced a complete rightward shift of the curves, suggesting that in neonates high-affinity receptors are basally coupled to G proteins. A similar observation was made when cotransfecting G proteins and α-adrenergic receptors into NIH-3T3 cells [30]. In these cells, the relative proportion of receptors to G proteins determines the degree of coupling, with the resulting shape of the competition curves for the agonist and GTP sensitivity; a G-protein/receptor ratio of about 3 is necessary for a high-affinity state of the receptor to be detectable [30].

TABLE 3. Effect of mAChR subtype antagonistic drugs on the carbachol-stimulated NOS activity in neonatal and adult rat atria

	NOS activity (pmol/g tissue wet wt)	
	Rat atria	
Additions	Neonatal	Adult
Basal (none)	510 ± 23	275 ± 10
Carbachol	1260 ± 48*	700 ± 20*
Atropine + carbachol	500 ± 20	250 ± 11
AFD-X 116 + carbachol	580 ± 22	269 ± 14
Pirenzepine + carbachol	603 ± 31	718 ± 18*

NOS activity was measured after incubating atria with or without 5 × 10^{-7} M mAChR blockers for 30 min and then for an additional 30 min with 1 × 10^{-7} M carbachol. Results are means ± SEM of six experiments performed in duplicate in each group.

* Statistically different from basal value, $P < 0.001$.

There is growing evidence that myocardial regulation by receptors parallels the acquisition of G proteins in cardiac membranes during embryonic and neonatal life [31]. In this regard, it has been reported by others that cardiac G-protein levels vary during development with an increase at days 13–15 of embryonic life and a selective increase of pertussis-sensitive G_i as the rat cardiomyocytes develop from neonate to adult [32, 33]. Taken together, the observations of the G-protein/receptor ratio necessary to evidence receptor coupling and the increased G_i levels detected during neonatal life point to the role of G-protein–receptor stoichiometry in the selective response of the neonatal atria to carbachol reported here. The fact that in our experimental design the M_1 antagonist pirenzepine was equally as potent in inhibiting mAChR-mediated biological effects as the M-antagonist AFD-X 116, even with a similar predominant distribution of M_2 receptor subtype in

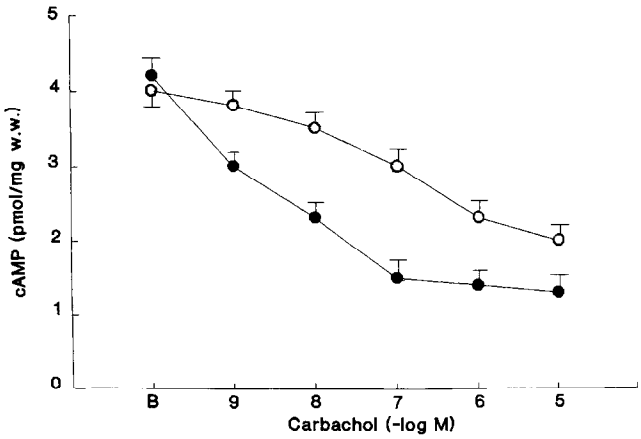

FIG. 5. Cumulative concentration–response curves for the effect of carbachol on cAMP production by atria isolated from neonatal (●-●) and adult (○-○) rats. Values were normalized by mg of tissue wet weight (mg tissue w.w.). Each point is the mean ± SEM of seven experiments, performed in duplicate.

TABLE 4. Effect of mAChR subtype blockers on the inhibition of PGE_1-stimulated cAMP production by carbachol on neonatal and adult rat atria

Additions	cAMP (pmol/mg tissue wet wt)	
	Rat atria	
	Neonatal	Adult
None (basal)	2.1 ± 0.1	1.9 ± 0.1
PGE_1	4.3 ± 0.2	4.0 ± 0.2
PGE_1 + carbachol	1.5 ± 0.06*	3.0 ± 0.1*
PGE_1 + carachol + atropine	4.2 ± 0.3	4.1 ± 0.3
PGE_1 + carbachol + AFD-X 116	4.1 ± 0.2	3.7 ± 0.2
PGE_1 + carbachol + pirenzepine	3.9 ± 0.2	3.0 ± 0.3

cAMP was measured after incubating atria with or without 5×10^{-7} M mAChR blockers for 30 min and then for an additional 30 min with 1×10^{-7} M carbachol. The inhibition of carbachol on cAMP was obtained when stimulating atria with 1×10^{-6} M PGE_1. Values are means ± SEM for six experiments performed in duplicate in each group.

* Significantly different from the age-appropriate PGE_1-stimulated values, $P < 0.001$.

each membrane, strongly suggests that though expressed in a small amount, M_1 receptors may be more coupled to G proteins than M_2 receptors in neonatal membranes. Hence, in our experiments, the differential coupling of mAChRs to G proteins deduced from carbachol displacement binding assays could account for the observed prevalence of M_1-mediated effects.

It is interesting to note that development is not the only situation where changes in G-protein expression result in differences in physiologic responses. Among experimental models of cardiovascular dysfunction, varying amounts of selectively G_i or G_s proteins have been detected [34–36]. Moreover, altered responses are related not only to the amounts of G proteins in the tissue but also to their functional integrity due to post-translational modifications [37].

The differences described here may play an important role in the functional parasympathetic regulation of the neonatal heart and could have clinical significance in heart diseases associated with neonatal life. In fact, antibodies directed against cardiac neonatal mAChRs have been described in congenital heart block [23]. The proposed changes in functional G proteins during cardiac maturation (reviewed in Ref. 31), as well as the differences in density and functional coupling of heart mAChRs in neonatal tissue reported here, could explain why those autoantibodies directed against mAChRs observed in complete congenital heart block selectively induce muscarinic cholinergic effects in the neonatal but not in the adult myocardium.

TABLE 5. Pharmacological profile of neonatal and adult mAChR as assessed by [^{3}H]QNB competition binding

Cholinoceptor agent	K_i (nM)	
	Neonatal	Adult
Atropine	2.6 ± 0.1	1.40 ± 0.08
AFD-X 116	64 ± 8	86 ± 9
Pirenzepine	K_1 1.1 ± 0.6	
	K_2 385 ± 81	690 ± 52

The equilibrium dissociation constant (K_i) values for the interaction of competing ligands were calculated with a computer-assisted curve-fitting program (LIGAND) from four sets of competition binding data for each drug. For pirenzepine competition binding, the curves for neonates but not for adults fitted better to a two-site model with P less than 0.05, and the percent of receptors in high- and low-affinity states were 11 ± 6 and 89 ± 5%, respectively. Values are means ± SD for each antagonist in each group.

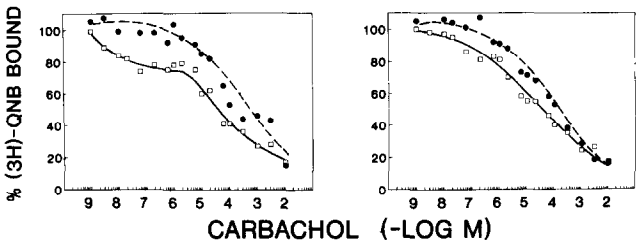

FIG. 6. Inhibition of [^{3}H]QNB binding to atria cardiac membranes (neonatal: left panel; adult: right panel) by carbachol alone (□-□) or in the presence of GppNHp (●-●). These plots are representative of five other plots from experiments performed in duplicate. Data were analyzed with the LIGAND program, and the parameters calculated correspond to simultaneous fitting of six sets of binding data for carbachol to both membranes. Values, expressed as means ± SD, are the following: neonates (nM): K_H = 5.5 ± 2.1, K_L = 14,400 ± 3,700; adults (nM): K_H = 1,100 ± 310, K_L = 20,000 ± 6,000.

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