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Carbachol inhibition of Ca^{2+} currents in ventricular cells obtained from neonatal and adult rats

Yasuhiro Katsube [a], Hisashi Yokoshiki [b], Masanori Sunagawa [b], Takashi Seki [b], Masao Yamamoto [a], Nicholas Sperelakis [b,*]

[a] *Department of Pediatrics, Nippon Medical School Hospital, 1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113-8603, Japan*

[b] *Department of Molecular and Cellular Physiology, College of Medicine, University of Cincinnati, P.O. Box 670576 Cincinnati, OH 45267-0576, USA*

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Abstract

We investigated the postnatal developmental changes produced by the muscarinic receptor agonist, carbachol, on the L-type Ca^{2+} current ($I_{Ca(L)}$) in neonatal (aged 5 to 7 days) and adult (aged 2 to 5 months) rat ventricular cells by using the whole-cell voltage clamp technique. Carbachol inhibited the isoproterenol-stimulated $I_{Ca(L)}$. The maximal inhibition was $89.3 \pm 4.8\%$ ($n = 5$) in neonatal cells and $17.7 \pm 7.7\%$ ($n = 9$) in adult cells. Carbachol inhibited the forskolin-stimulated $I_{Ca(L)}$ to almost same extent as the isoproterenol-stimulated $I_{Ca(L)}$. In the cells pretreated with pertussis toxin, carbachol failed to inhibit the isoproterenol-stimulated $I_{Ca(L)}$, indicating that carbachol produced its effect via a pertussis toxin-sensitive G-protein pathway. The effects of carbachol in adult cells became more pronounced, increasing from 17.7% to 54.8% ($n = 11$), with the addition of the synthetic inhibitory G-protein α subunit ($G_{i\alpha}$) (1 μM) to the reaction. Conversely, the α subunit of another pertussis toxin-sensitive synthetic G-protein ($G_{o\alpha}$, 1 μM) failed to mimic the effect of $G_{i\alpha}$. These results suggest that, in rat ventricular cells, (1) the action of carbachol on $I_{Ca(L)}$ showed a marked decrease during development; (2) the decrease in the effect of carbachol in adult cells is in part due to a decrease in the activity of pertussis toxin-sensitive G protein, especially $G_{i\alpha}$.

Keywords: Muscarinic acetylcholine receptor; Carbachol; Heart, neonatal; Ca^{2+} current; G-protein, pertussis toxin-sensitive; Voltage clamp, whole-cell

1. Introduction

The sympathetic and parasympathetic nervous systems regulate myocardial contractility by releasing of their respective neurotransmitters that act on the β-adrenoreceptors and muscarinic acetylcholine receptors. The slow-type Ca^{2+} current ($I_{Ca(L)}$), which is involved in the regulation of the excitation–contraction coupling in the heart, is strongly regulated by the autonomic nervous system (for reviews, see Hartzell, 1988; Lindemann and Watanabe, 1995; Robinson, 1996).

We previously reported that developmental changes in the regulation of $I_{Ca(L)}$ occur in neonatal and adult rat ventricular myocytes when treated with the β-adrenoreceptor agonist, isoproterenol (Katsube et al., 1996). We showed that the stimulatory effect of isoproterenol on $I_{Ca(L)}$ was significantly greater in neonatal ventricular cells than in adult, suggesting a greater activity of the stimulatory G protein (G_s) in the neonatal cells. There was no developmental changes in the effect of forskolin, suggesting no developmental changes in the activity of adenylyl cyclase and thereafter.

In contrast to the stimulatory effect of β-adrenoreceptor agonist on the heart, the effect of muscarinic receptor agonist is inhibitory. The primary effect of muscarinic receptor agonists on the heart is to inhibit supraventricular (sinoatrial node, atrium, atrioventricular node), but not ventricular, electrical and mechanical activity. In ventricular cells, the effect of muscarinic receptor agonist is most evident in the presence of simultaneous β-adrenergic stimulation (Levy, 1971).

It is postulated that the binding of carbachol to the muscarinic acetylcholine receptors inhibits adenylyl cyclase activity by stimulating a G protein that is sensitive to pertussis toxin (Mubagwa et al., 1993; Osaka et al., 1993; Petit-Jacques et al., 1993). Thus, two factors may influence developmental changes, resulting in altered effects of mus-

* Corresponding author. Tel.: +1-513-558-3126; Fax: +1-513-558-2668; E-mail: spereln@ucmail.uc.edu

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carinic acetylcholine receptor agonists on $I_{Ca(L)}$. One is the density of muscarinic acetylcholine receptors on the sarcolemma, while the other is the activity of the pertussis toxin-sensitive G protein.

Binding studies with a radiolabelled muscarinic receptor antagonist, [^{3}H]quinuclidinyl benzilate, has demonstrated that the density of the muscarinic acetylcholine receptors decreases during postnatal development (Nedoma et al., 1986; Kojima et al., 1990).

The levels of pertussis toxin-sensitive G-protein measured by immunoblot analysis or [^{32}P]ADP ribosylation with pertussis toxin, have been shown to decrease during development (Kojima et al., 1988; McMahon, 1989; Kumar et al., 1994; Bartel et al., 1996). Luetje et al. (1988) reported that comparisons of neonatal rat ventricular tissue with adult tissue using immunoblot and northern blot analyses showed a developmental decrease in $G_{i\alpha}$-protein, as well as a drop in mRNA for $G_{i\alpha 2}$. There was no developmental change in $G_{i\alpha 3}$, and $G_{i\alpha 1}$ was not detected. Developmental changes were also observed in the amount of mRNA encoding $G_{o\alpha}$ in the ventricular tissue.

Osaka et al. (1993) reported using the patch-clamp technique that there were developmental changes in the modulation of $I_{Ca(L)}$ by muscarinic receptor agonists in rabbit heart. 10 μM carbachol shifted the isoproterenol dose–response to the right 2.8-fold in adult ventricular cells; the same concentration completely abolished the effect of isoproterenol in the neonatal ventricular cells. Those authors attributed the decreased effectiveness of carbachol in the mature ventricular cells to a reduced contribution of a pertussis toxin-sensitive G-protein, G_i, toward the inhibition of adenylyl cyclase.

The present study attempted to determine whether the regulation of $I_{Ca(L)}$ by muscarinic acetylcholine receptor agonists changes functionally with development, and which factors may be responsible for the changes.

2. Materials and methods

2.1. Cell preparation

Freshly-isolated single cells were prepared from atria (right and left appendages) and ventricles of neonatal (5 to 7 days-old) and adult (2 to 5 months-old) Sprague–Dawley rats. The rats were decapitated under CO_2 or pentobarbital anesthesia (100 mg/kg administered intraperitoneally). The hearts were removed and rinsed in oxygenated Tyrode's solution, then immersed in Ca^{2+}-free Tyrode's solution for 30 min at room temperature. The atria and ventricles were dissected after spontaneous beating had ceased. Small pieces of the heart tissue were then enzymatically digested, for 20 to 40 min for neonatal cells and 60 min for adult cells, in Ca^{2+}-free Tyrode's solution (37°C) that contained collagenase (0.3 to 0.5 mg/ml for neonatal cells and 0.8 mg/ml for adult cells, Worthington Chemicals, USA). Following digestion, the cells were mechanically dispersed in modified KB (Kraftbrühe) solution by using a Pasteur pipette. The cell suspension was stored at 4°C until used.

The isolated cells were rinsed three times in M199 (GIBCO, USA) culture medium, containing 10% fetal bovine serum, and plated on glass coverslips in plastic 35 mm Petri dishes. The cell cultures were stored for 48 h in a humidified air/CO_2 incubator at 37°C and pH 7.4.

2.2. Solution and drugs

Normal Tyrode's solution contained (in mM): NaCl 143, KCl 5.4, NaH_2PO_4 0.33, $MgCl_2$ 0.5, $CaCl_2$ 1.8, glucose 5.5, HEPES 5, and pH was adjusted to 7.4 with NaOH. The modified KB solution contained (in mM): L-glutamate 50, KOH 20, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 3, glucose 10, EGTA 0.5, HEPES 10 and was adjusted to pH 7.4 with KOH. The external test solution (Na^+- and K^+-free) contained (in mM): tetraethylammonium chloride 150, $CaCl_2$ 1.8, $MgCl_2$ 0.5, 4-aminopyridine 3, HEPES 5, and glucose 5.5 and was adjusted to pH 7.4 with HCl. The pipette solution contained (in mM): CsOH 110, CsCl 20, L-glutamate 90, $MgCl_2$ 3, adenosine triphosphate disodium salt 5, creatine phosphate disodium salt 5, EGTA 10, HEPES 5, and was adjusted to pH 7.2 with CsOH.

The β-adrenoreceptor agonist, (−)-isoproterenol hydrochloride (Sigma, USA), was dissolved in distilled water to a final concentration of 1 mM with equimolar ascorbic acid and was made fresh daily. Forskolin (Sigma), a direct activator of adenylyl cyclase, was dissolved in dimethyl sulfoxide to provide a 25 mM stock solution. The final concentration of dimethyl sulfoxide (0.04%) did not affect Ca^{2+} currents. Carbamylcholine chloride (carbachol) (Sigma), a stable analogue of acetylcholine, was dissolved in distilled water to provide a 50 mM stock solution, and was prepared fresh daily. The drugs were diluted with appropriate volumes of test solution to yield the final working concentrations. Proteins composed of the C-terminal region of the α subunit of G_{i1}/G_{i2} and G_{i3} (G_i, inhibitory guanine nucleotide-binding regulatory protein) were obtained from Calbiochem (USA). The fragment of the α subunit of guanine nucleotide-binding regulatory protein, $G_{o\alpha}$ was obtained from Peninsula Laboratories (USA). The α subunits of G_{i1}/G_{i2}, G_{i3} and G_o were dissolved directly in the pipette solution at a concentration of 1 μM.

2.3. Whole-cell voltage clamp recording

Voltage-clamp recordings were performed by using the whole-cell configuration of the patch-clamp method with a patch clamp amplifier (Axopatch-1D, Axon Instruments, USA) and heat-polished borosilicate glass pipettes (World Precision Instruments, USA). These pipettes had resis-

Table 1
Developmental changes in cell capacitance (C_m) (pF), peak current (pA), and current density (pA/pF) of the basal $I_{Ca(L)}$ in ventricular cells from neonatal and adult rats and the percent increase of $I_{Ca(L)}$ produced by isoproterenol (1 μM) and forskolin (10 μM)

	$I_{Ca(L)}$			Percent increase by	
	C_m (pF)	Peak current (pA)	Current density (pA/pF)	Isoproterenol (1 μM)	Forskolin (10 μM)
Neonate	27 ± 1.3 (41)	200 ± 13 (41)	7.6 ± 0.4 (41)	151 ± 11 (18)	104 ± 25 (6)
Adult	155 ± 10 (18)[a]	1239 ± 115 (18)[a]	8.1 ± 0.6 (18)	105 ± 13 (6)[a]	94 ± 13 (4)

Data are expressed as mean ± S.E.
n Value is given in parentheses.
[a] $P < 0.05$ compared to the cells from neonate.

tances of 1.5 to 5 MΩ when filled with the pipette solution. The cell suspension was placed into a small chamber (containing 1.4 ml of external test solution) that rested on the stage of an inverted microscope (Diaphot, Nikon, Japan). These suspensions were constantly perfused with the external test solution at a rate of 1.8 ml/min.

The $I_{Ca(L)}$ currents were elicited from a holding potential of −40 mV to a test potential of +10 mV or +20 mV, depending on the peak currents, for 300 ms (every 15 s). The currents were abolished completely by adding 2 mM Co^{2+} or 1 mM Cd^{2+}, consistent with currents that are carried through a Ca^{2+} channel.

The leak and residual capacitative currents were subtracted from the Ca^{2+} currents by using currents elicited by small hyperpolarizing pulses (P/4 protocol). The current and voltage signals were filtered with a cut-off frequency of 1 kHz, digitized by an A/D converter (TL-1, Axon Instruments, USA), and sampled at 2.5 kHz. They were stored in an IBM-AT personal computer by using the pCLAMP software (ver. 5.5 or 6.0, Axon Instruments).

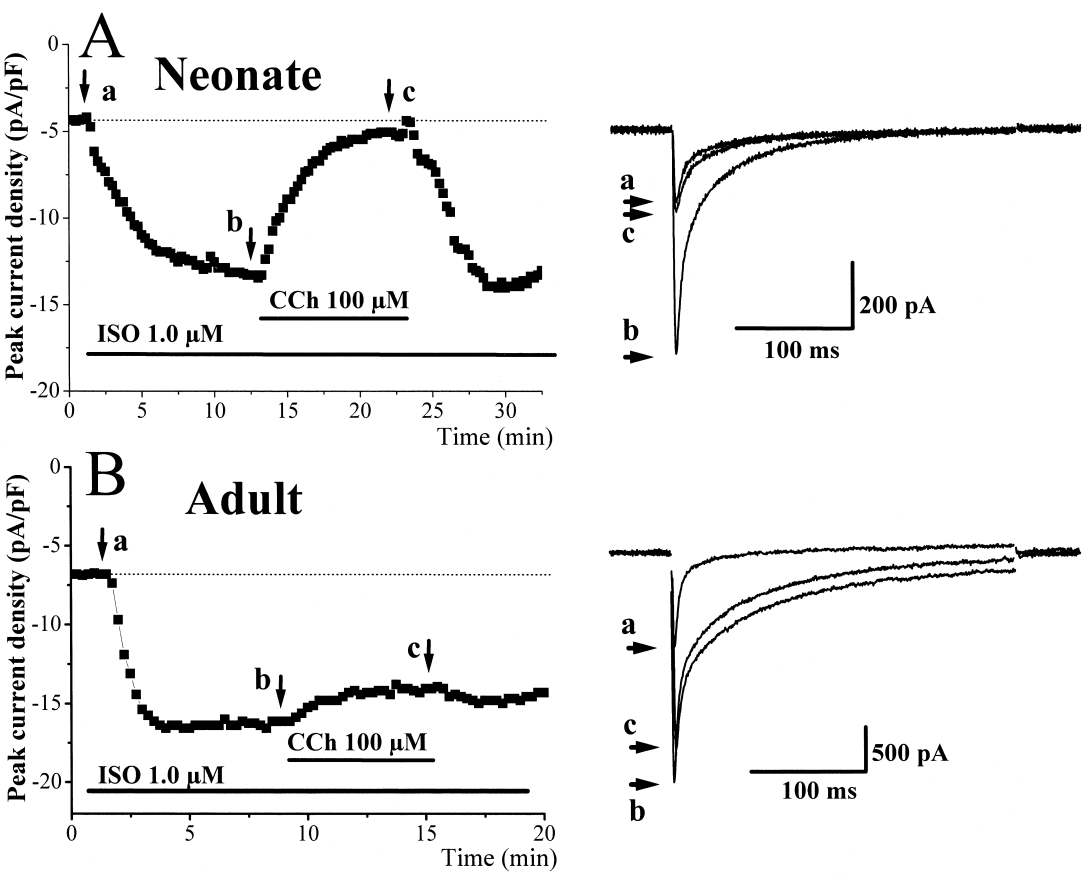

Fig. 1. Effect of carbachol (CCh, 100 μM) on isoproterenol-stimulated $I_{Ca(L)}$ in neonatal (6 day-old; C_m = 51 pF) and adult (C_m = 158 pF) rat ventricular cells. $I_{Ca(L)}$ was measured as the peak inward current from a holding potential of −40 mV to a test potential of +10 mV. 1.8 mM Ca^{2+} was used as the charge carrier. A: Carbachol inhibited the isoproterenol-stimulated $I_{Ca(L)}$ by 93.0% in this neonatal cell. Dotted lines indicate the control level of $I_{Ca(L)}$. B: In this adult cell, carbachol only inhibited the isoproterenol stimulation by 25.7%. Selected current tracings are illustrated at the right side at the points indicated (a, b, c).

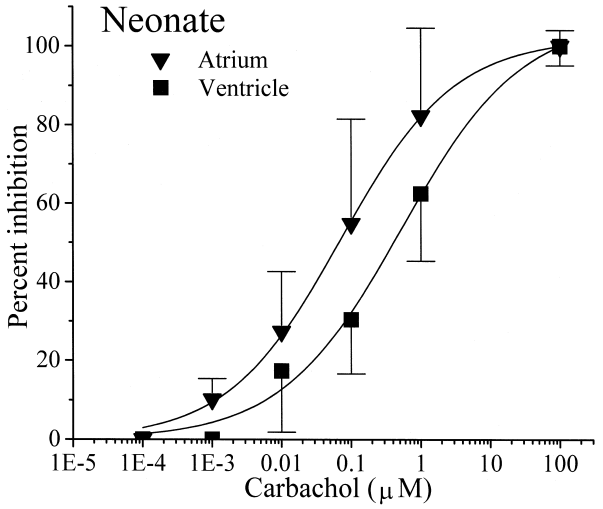

Fig. 2. Dose–response curves for carbachol inhibition of isoproterenol-stimulated $I_{Ca(L)}$ in atrial and ventricular cells of neonatal rats (1–7 days). The ordinate represents the relative inhibition of $I_{Ca(L)}$ pre-stimulated by isoproterenol (1 μM). Each data point is the mean (±S.E.) of 3–7 cells. The data points were well-fitted to the Hill equation (solid curves). The half-inhibition (IC_{50}) was 0.072 μM in atrial cells and 0.53 μM in ventricular cells.

The membrane capacitance was determined from the current amplitude elicited in response to a depolarizing and hyperpolarizing ramp pulse of 0.8 V/s (first phase, depolarize to −30 mV from a holding potential of −50 mV; second phase, return to a holding potential of −50 mV). Currents were recorded after $I_{Ca(L)}$ completely stabilized, usually 5 to 10 min after breaking the membrane. All experiments were carried out at room temperature (22–24°C).

2.4. Statistical analyses

Data are expressed as mean ± S.E. Student's unpaired *t*-test was used to evaluate differences between data sets. A level of $P < 0.05$ was accepted as statistically significant.

3. Results

The cell capacitance (C_m), peak current amplitude (pA), and peak current density (pA/pF), along with the effects of isoproterenol and forskolin in the ventricular cells from neonatal and adult rats are summarized in Table 1.

3.1. Effect of carbachol on isoproterenol-stimulated $I_{Ca(L)}$

The time course of the effects of carbachol on the isoproterenol-stimulated $I_{Ca(L)}$ levels in the neonatal and adult rat ventricular cells is shown in Fig. 1. The effect of carbachol is expressed as percent inhibition ($\% = (b - c)/(b - a) \times 100$); a, b, and c are the peak current amplitudes for control, isoproterenol, and carbachol applications, respectively. In the neonatal cells, carbachol almost completely reversed the stimulation of $I_{Ca(L)}$ produced by isoproterenol ($a = 216$ pA, $b = 686$ pA, $c = 249$ pA). In the adult cells, the application of carbachol inhibited the isoproterenol-stimulated $I_{Ca(L)}$ only by 25.7% ($a = 1085$ pA, $b = 2636$ pA, $c = 2236$ pA).

3.2. Dose dependent effect of carbachol

Fig. 2 shows the dose–response relationships for the inhibitory effect of carbachol on isoproterenol-stimulated $I_{Ca(L)}$ in atrial and ventricular cells from neonatal rats. Each data point is the mean (±S.E.) of 3–7 cells. Data points were fitted to the Hill equation; that is, inhibition $\% = \{[\text{carbachol}]^{n_H}/([\text{carbachol}]^{n_H} + IC_{50}^{n_H})\} \times E_{max}$, where E_{max} is the maximum effect, n_H is the Hill coefficient, IC_{50} is the concentration for half-maximum inhibition by carbachol. IC_{50} was 0.072 μM and 0.53 μM in atrial and ventricular cells, respectively. That is, IC_{50} was about 7 times higher in ventricular cells than in atrial cells.

3.3. Effect of carbachol on forskolin-stimulated $I_{Ca(L)}$

We also examined the effects of carbachol on forskolin-stimulated $I_{Ca(L)}$. Carbachol inhibited the forskolin-stimulated $I_{Ca(L)}$ to similar degree as for the isoproterenol-stimulated $I_{Ca(L)}$ in both the neonatal and adult cells (Table 2). These findings support the results of Wahler and Sperelakis (1986), who reported that acetylcholine depressed or abolished slow action potentials induced by isoproterenol or forskolin, each yielding a similar degree of inhibition.

Table 2
Percent of inhibition of carbachol on isoproterenol- and forskolin-stimulated $I_{Ca(L)}$ in ventricular cells from neonatal and adult rats

	Isoproterenol	Forskolin	Pertussis toxin-pretreatment	$G_{i\alpha}$-in pipette	$G_{o\alpha}$-in pipette
Neonate	89.3 ± 4.8 (5)	88.8 ± 6.1 (7)	1.5 ± 1.5 (4)	–	–
Adult	17.7 ± 7.7 (9)[a]	23.4 ± 7.7 (4)[a]	–	54.8 ± 7.2 (11)[b]	23.8 ± 5.8 (10)[c]

Data are expressed as mean ± S.E.
n Value is given in parentheses.
[a] $P < 0.05$ compared to the cells from neonate.
[b] $P < 0.05$ compared to the isoproterenol-stimulated currents in adult rats.
[c] Not significant compared to the isoproterenol-stimulated currents in adult rats.

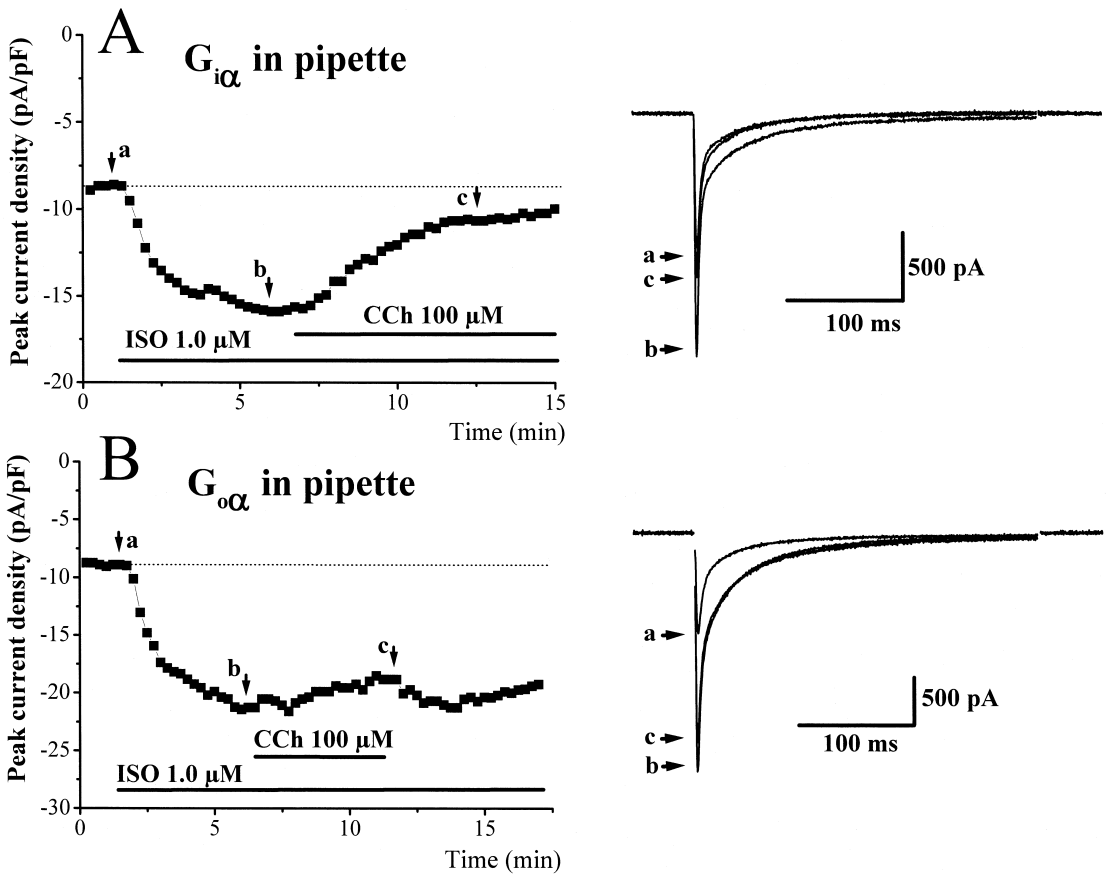

Fig. 3. The involvement of G-protein in the effect of carbachol on isoproterenol-stimulated $I_{Ca(L)}$ in adult ventricular cells. Time-course of the effects of carbachol on two adult cells (panel A, $C_m = 115$ pF; panel B, $C_m = 124$ pF). A: When 2 μM synthetic $G_{i\alpha}$-protein was present in the pipette, the application of 100 μM carbachol strongly inhibited the isoproterenol-stimulated $I_{Ca(L)}$ by 72.9%. B: However, when the pipette was filled with 1 μM $G_{o\alpha}$-protein (synthetic), the application of carbachol inhibited the isoproterenol-stimulated $I_{Ca(L)}$ by only 21.6%.

According to our new preliminary data, there was no developmental changes in the effect of carbachol inhibition on isoproterenol- and forskolin-stimulated Ca^{2+} current in atrial cells (in neonatal atrial cells, 78.5 ± 5.7 ($n = 7$) and 71.7 ± 9.0 ($n = 4$) in isoproterenol- and forskolin-stimulated $I_{Ca(L)}$, respectively; in adult atrial cells, 77.6 ± 10.0 ($n = 6$) and 86.3 ± 4.0 ($n = 5$) in isoproterenol and forskolin stimulated $I_{Ca(L)}$, respectively).

3.4. The involvement of G-protein in the effect of carbachol

To evaluate the effect of pertussis toxin on that of carbachol, we incubated the cells in KB solution containing 5 μg/ml pertussis toxin for at least 8 h as previously reported. However, due to difficulties in harvesting usable cells, particularly from neonatal tissue, we used cells cultured for these experiments. As being generally accepted, carbachol had no effect on the isoproterenol-stimulated $I_{Ca(L)}$ in pertussis toxin-pretreated neonatal cell, which suggests that carbachol exerts its effect through pertussis toxin-sensitive G-protein (Table 2).

We hypothesized that the weak effect of carbachol in adult ventricular cells may be due to the weak inhibition of the adenylyl cyclase by pertussis toxin-sensitive G-protein. To evaluate this hypothesis, synthetic $G_{i\alpha}$ or $G_{o\alpha}$-protein was added to the pipette. Fig. 3A shows the time-course of the effect of carbachol in the presence of 2 μM $G_{i\alpha}$-protein (1 μM $G_{i\alpha 1}/G_{i\alpha 2}$ and 1 μM $G_{i\alpha 3}$) in the pipette. In adult cells, the presence of $G_{i\alpha}$ allowed carbachol to inhibit the isoproterenol-stimulated $I_{Ca(L)}$, as was seen in the neonatal cells under normal conditions. The isoproterenol-stimulated $I_{Ca(L)}$ was inhibited 72.9% by carbachol ($a = 986$ pA, $b = 1802$ pA, $c = 1207$ pA). $G_{o\alpha}$ was not effective in this regard. Fig. 3B shows the lack of effect of carbachol in the presence of 1 μM $G_{o\alpha}$-protein in the pipette.

4. Discussion

The present study examined the postnatal changes that occur in the muscarinic acetylcholine receptor agonist modulation of $I_{Ca(L)}$ in rat ventricular myocytes. Our results suggest that the neonatal ventricular cells are

strongly regulated by the muscarinic acetylcholine receptor agonists. That is, the ventricular cells from adult are much less responsive to the muscarinic receptor agonists. A tissue-dependent difference of IC_{50} was also reported in acetylcholine-sensitive muscarinic K^+ current ($I_{K(ACh)}$) in isolated feline atrial and ventricular cells in cell-attached patch recordings (Koumi et al., 1995). According to their report, the IC_{50} values were 0.03 μM in atrial cells and 0.15 μM in ventricular cells; that is, $I_{K(ACh)}$ in atrial cells is 5 times more sensitive to acetylcholine than are ventricular cells.

The reduced effect of carbachol in adult cells may be due, in part, to a decrease in the amount of pertussis toxin-sensitive G-protein, especially in the $G_{i\alpha}$ present in these cells. It is generally accepted that in the cardiac cells, the antagonistic effect of muscarinic acetylcholine receptor agonists on isoproterenol- or forskolin-stimulated $I_{Ca(L)}$ is mediated by the inhibition of adenylyl cyclase via the G_i-protein. It has been reported (Hescheler et al., 1987) that opioid peptide agonists reduce the Ca^{2+} currents in neuroblastoma-glioma hybrid cells. This effect is pertussis toxin-sensitive and is restored by the intracellular application of $G_{o\alpha}$ and $G_{i\alpha}$, with $G_{o\alpha}$ being more potent under these conditions. $G_{o\alpha}$ also regulates the apical Na^+ channels in mouse mandibular gland duct cells (Komwatana et al., 1996). In addition, it was recently reported that in heart cells from transgenic mice, the effect of carbachol on the $I_{Ca(L)}$ level is mediated by $G_{o\alpha}$ (Valenzuela et al., 1997). According to that report, isoproterenol stimulated the $I_{Ca(L)}$ equally in both the wild-type and the $G_{o\alpha}$ knockout mice: wild-type = 241 ± 27% of control in absence of isoproterenol; $G_{o\alpha}$ knockout = 244 ± 29%. In addition, the inhibitory effect of 1 μM carbachol on the isoproterenol-stimulated $I_{Ca(L)}$ was markedly lessened in the $G_{o\alpha}$ knockout mice: wild-type = 106 ± 14% of control in absence of isoproterenol; $G_{o\alpha}$ knockout = 212 ± 37%.

Adenylyl cyclase is constituted by several isoforms, and there is tissue dependency. In heart, Types V and VI are major adenylyl cyclase isoforms (types II, IV, and VII form the ubiquitous subgroup, which is also detectable in the heart) (reviewed by Ishikawa and Homcy, 1997). In addition to the tissue dependent isoforms of adenylyl cyclase, there are developmental changes in the isoforms. Type V appears in the adult, whereas type VI is more highly expressed in the fetus and neonate (Espinasse et al., 1995). The inhibition produced by $G_{i\alpha}$ varies with the isoform. That is, all $G_{i\alpha}$ subtypes ($G_{i\alpha 1}$ to $G_{i\alpha 3}$) elicited a similar degree of inhibition of the type V and VI isoforms (Taussig et al., 1994). $G_{o\alpha}$ inhibits type I adenylyl cyclase while minimally affecting type VI. Taking into the account these biochemical findings and the report of Valenzuela et al. (1997), the developmental changes of the effect of G-protein on muscarinic antagonism of isoproterenol stimulation of $I_{Ca(L)}$ have to be carefully explored.

Interestingly, Kumar et al. (1996) reported that adenosine, one of the most important inhibitory modulators in the heart, had no effect on isoproterenol-stimulated $I_{Ca(L)}$ in adult cells, while it completely blocked the isoproterenol-stimulated $I_{Ca(L)}$ with a inhibitory potency similar to carbachol in neonatal cells. They previously reported (1994) that $G_{i\alpha}$ was present in neonatal cells, but not detectable in adult cells. Taking into account these results and the experiment of the use of the synthetic $G_{i\alpha}$ in the patch pipette, they concluded that the inhibitory action of adenosine on $I_{Ca(L)}$ is mediated primary through $G_{i\alpha}$ pathway, especially $G_{i\alpha 3}$. Their conclusion supports the results obtained from this study.

In summary, the present study demonstrated a marked developmentally related decrease in the effect of carbachol on rat ventricular cells. The lesser effect of carbachol on adult cells may be due to the decrease in the activity of $G_{i\alpha}$-protein.

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