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Carbachol Activates a Novel Sodium Current in Isolated Guinea Pig Ventricular Myocytes Via M_2 Muscarinic Receptors

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SUMMARY

Carbachol induces a novel tetrodotoxin-resistant Na^+ current in guinea pig ventricular myocytes bathed in Tyrode's solution with 20 mM Cs^+. This action of carbachol, which initiates a series of reactions that culminates in a catecholamine-independent positive inotropic effect, occurs through muscarinic rather than nicotinic cholinoceptive sites. The concentrations of muscarinic antagonists required to suppress the carbachol-induced current by 50% were 2.1 nM, 270 nM, and 1700 nM for atropine, AF-DX 116, and pirenzepine, respectively. These results indicate that an M_2-selective antagonist, AF-DX 116, is more potent than an M_1-selective antagonist, pirenzepine, as an inhibitor. The M_1-selective agonist McN-A-343 did not induce an inward current and blocked that caused by carbachol, in a rapid and reversible manner. This finding is also consistent with the conclusion that the muscarinic receptor involved in the regulation of myocardial Na^+ channels by carbachol cannot be distinguished from the M_2 subtype of such receptors. Treatment with pertussis toxin did not affect the ability of carbachol to induce an inward current in ventricular myocytes and reversed the current activated by carbachol in atrial cells from outward to inward. The electrophysiological and pharmacological nature of the carbachol-induced current in ventricular myocytes is very similar to that of the acetylcholine-induced current in *Xenopus* oocytes transfected with porcine M_2, but not M_1, muscarinic receptors. In both preparations, Na^+ is the dominant charge carrier, intracellular Ca^{2+} is not involved in opening the Na^+ channel, and an M_2 receptor is involved.

In atrial and ventricular muscle, the muscarinic agonists ACh, Carb, and oxotremorine exert inhibitory effects by closing voltage-dependent Ca^{2+} channels and by opening ligand-specific K^+ channels (reviewed in Refs. 1–4). The agonist-induced signals are transduced to the adenylate cyclase system connected to Ca^{2+} channels and to ligand-specific K channels by G_i/G_o, inhibitory G proteins (2–5). When these G proteins are inactivated by pertussis toxin-catalyzed ADP-ribosylation, the muscarinic inhibition of the heartbeat is suppressed.

After treatment with pertussis toxin, Carb and ACh, but not oxotremorine, depolarize atrial muscle and sinoatrial node (6–8) and evoke positive inotropic and chronotropic effects, respectively. The CCh-induced depolarization in atria from pertussis toxin-treated chicks depends on extracellular Na^+ and is not suppressed by TTX (7). Similarly, mammalian ventricular muscle exposed to either Ba (0.1 mM)- or Cs (20 mM)-containing Tyrode's solution also depolarizes (9, 10) and displays a tetrodotoxin-insensitive "background" Na^+ current that is activated by these agonists (11). This novel effect has been postulated to be the beginning of a reaction sequence that is responsible for the eventual stimulation of force development by muscarinic agonists that is catecholamine independent (9, 11, 12).

The mAChR that signals inhibition of adenylyl cyclase is of the M_2 subtype (13). This conclusion is in accordance with the view that the cardiac mAChRs that signal inhibition of the heartbeat are of the M_2 subtype (reviewed in Ref. 14). Because of the novel reaction pathway for muscarinic-induced Na^+ current and positive inotropy, which also includes PI hydrolytic products (7), we wondered whether this signaling system involves another subtype of muscarinic receptor, namely, M_1. Pharmacological experiments with selective agonists and antagonists at M_1 and M_2 receptors were done in voltage-clamped ventricular myocytes to test this hypothesis.

Materials and Methods

Cell isolation. The enzymatic method used to obtain single ventricular myocytes from the hearts of guinea pigs (250–300 g) has been described previously (11). Briefly, at 30 min after the animals were given heparin (500 units, intraperitoneally), an anesthetic dose (30 mg/

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ABBREVIATIONS: ACh, acetylcholine; Carb, carbachol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N, N, N′, N′*-tetraacetic acid; TTX, tetrodotoxin; $InsP_1$, inositol-1-phosphate; PI, phosphoinositide; G protein, guanine nucleotide-binding protein; mAChR, muscarinic acetylcholine receptor; IC_{50}, 50% inhibitory concentration.

kg) of sodium secobarbital was administered. The heart was excised, attached via the aorta to a Langendorff perfusion apparatus, and perfused with oxygenated Tyrode's solution (37°) at 6.5 ml/min for 10 min. The composition of the Tyrode's solution was (in mM): NaCl, 143; KCl, 5.4; $MgCl_2$, 1.0; $CaCl_2$, 1.8; glucose, 5.5; and HEPES, 5.0. Then, perfusion began with 50 ml of nominally Ca-free Tyrode's solution. This was followed by perfusion for 15 min at 4.5 ml/min with Ca-free Tyrode's solution to which collagenase (0.4 mg/ml; United States Biochemical Corp.) and defatted bovine serum albumin (1 mg/ml) had been added. The enzyme solution was washed out with a storage medium (see Ref. 15) and the ventricles were removed and cut into small chunks. Cells were isolated from the chunks by gentle mechanical agitation with forceps, and the cell suspension was maintained in storage solution for at least 1 hr. An aliquot of the cell suspension was placed in an experimental chamber (500-μl volume) on the stage of an inverted microscope, allowed to settle for about 10 min, and then superfused (2–4 ml/min) with Tyrode's solution at 35°. The yield of Ca-tolerant myocytes obtained by this procedure ranged from 25 to 40%.

Electrophysiological techniques. The whole-cell voltage clamp method was used for all experiments. Electrodes with a tip diameter of 1–2 μm were prepared from glass capillary tubes (i.d., 1.1 mm; o.d., 1.3 mm), as described previously (11). The electrode was filled by syringe with a pipette solution that had been passed through a Millipore filter. The composition of the pipette solution was (in mM): K^+-aspartate, 100; KCl, 50; K_2ATP, 5; EGTA, 2.5; $MgCl_2$, 1.0; and HEPES, 5.0 (pH 7.4). Electrode resistance was 2–4 MΩ; the pipette was connected, via a holder with a Ag-AgCl electrode, to the head stage of a LIST EPC-7 amplifier. The electrode tip was placed against the cell membrane, and a GΩ seal was formed by application of negative pressure to the pipette interior. The cell membrane within the pipette orifice was then ruptured by additional negative pressure. Electrode capacitance and series resistance were minimized electronically.

Drugs. The drugs used in the experiments include Carb, hexamethonium, McN-A-343 [4-(*m*-chlorophenylcarbamoyloxy)-2-butyntrimethyl-ammonium chloride], atropine sulfate, pirenzepine-HCl, and AF-DX 116 [11-[((2-diethylamino)methyl-1-piperidinyl)acetyl]-5,11-dihydro-6*H*-pyrido(2,3-*b*)(1,4)-benzodiazepine-6-one]. The sample of AF-DX 116 was a generous gift from Boehringer Ingelheim Pharmaceuticals, Inc. (Ridgefield, CT).

Results

Experiments with antagonists. Carb induces a sustained inward current that is generated by Na^+ movement through TTX-resistant background channels. We were able to test the effects of graded concentrations of antagonists, because the inward current induced by Carb persisted for up to 10 min and did not display desensitization. We initially tested the possibility that the Carb-induced inward current was initiated at nicotinic receptors. The Carb-induced current averaged −425 ± 14 nA/cm^2 (mean ± standard error; current corrected for apparent cell surface area) in the presence of 3×10^{-6} M hexamethonium ($n = 5$). This current was not significantly different ($p > 0.30$) from that induced by Carb (−480 ± 21 nA/cm^2) in the absence of this nicotinic receptor antagonist.

All of the muscarinic antagonists tested suppressed the inward current induced by Carb, in a concentration-dependent manner. With atropine, detectable inhibition occurred at 3×10^{-11} M and complete blockade was evident at 3×10^{-7} M (data not shown). When this type of experiment was done in a cell using the M_2-selective antagonist AF-DX 116, results shown in Fig. 1A were obtained. In this cell, the membrane voltage was clamped at −77 mV, which is the value of the resting potential. The inward current induced by Carb (−21 pA) was noticeably

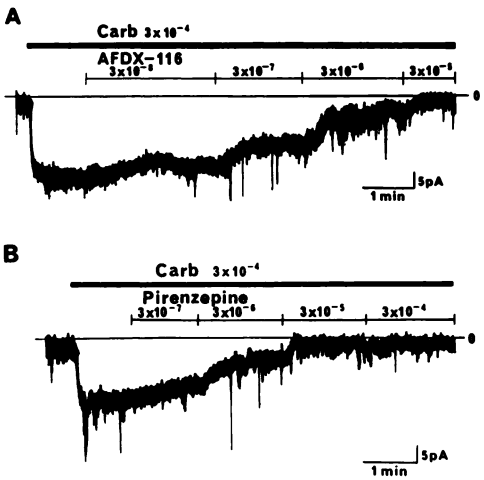

Fig. 1. Concentration-dependent block by AF-DX 116 (A) and by pirenzepine (B) of inward current induced by Carb (3×10^{-4} M). The membrane voltage was clamped at −77 mV in A and B, and the zero current level is shown by the *thin horizontal line*. Inward current is a downward deflection of the trace. Carb (3×10^{-4} M) was superfused for the period indicated by the *thick horizontal line*. Simultaneous superfusion with either AF-DX 116 (A) or pirenzepine (B) is shown, and the period at each concentration is marked by *thin vertical lines*. Vertical and horizontal calibrations, 5 pA and 1 min, respectively.

suppressed by 3×10^{-8} M AF-DX 116 to −18 pA, a 16% reduction in current magnitude. At 3×10^{-7} M AF-DX 116, the Carb-induced current amounted to −11 pA, that is, a reduction to 52% of the initial value. Increasing the antagonist concentration eventually reduced the Carb-induced current to zero, at 3×10^{-5} M AF-DX 116 (Fig. 1A).

An example of an experiment with the M_1-selective antagonist pirenzepine is shown in Fig. 1B. Addition of Carb (3×10^{-4} M) induced an inward current of about −15 pA when the membrane was clamped at −77 mV, the resting potential. At 3×10^{-7} M pirenzepine, the magnitude of the Carb-induced inward current was about −15 pA, or 71% of the value in the absence of pirenzepine. Raising the pirenzepine concentration to 3×10^{-6} M reduced the current to −4 pA, which is equivalent to 29% of that seen in the absence of pirenzepine. Blockade of the Carb-induced inward current by pirenzepine was essentially complete at 3×10^{-5} M.

The results of all experiments with atropine ($n = 6$), AF-DX 116 ($n = 6$), and pirenzepine ($n = 7$) are shown in Fig. 2. Among the antagonists, atropine was the most potent, with an IC_{50} of about 2.1×10^{-9} M. The IC_{50} for pirenzepine was the greatest (approximately 1.7×10^{-6} M), whereas that for AF-DX 116 was intermediate (approximately 2.7×10^{-7} M).

Experiments with agonists. The effect of the nonselective agonist Carb was compared with that of the M_1-selective agonist McN-A-343. In the first experiment, either Carb (3×10^{-4} M) or McN-A-343 (3×10^{-4} M) was added to the superfusion fluid bathing single myocytes. Whereas Carb induced an inward current in each of 13 cells, McN-A-343 did not. This pattern was observed whether McN-A-343 was added first ($n = 3$) and then washed out, before Carb was tested on the same cell, or vice versa. Therefore, there is no reason to suspect that exposing the cell to Carb first resulted in some interference with an action of McN-A-343.

As an alternative test of drug action, another type of experiment was done, and this is illustrated in Fig. 3. At a holding potential of −79 mV, Carb (3×10^{-4} M) promptly induced a

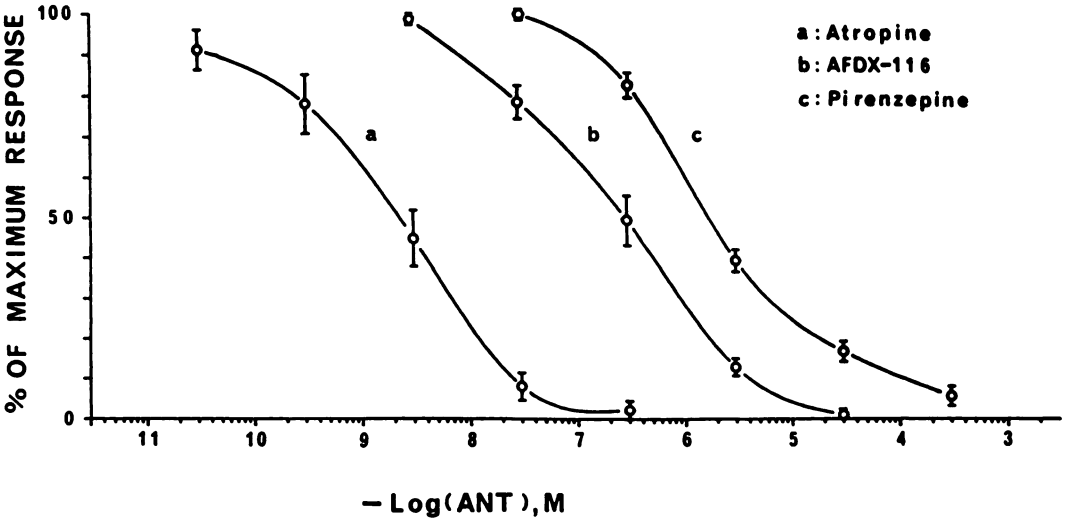

Fig. 2. Summary of experiments with muscarinic antagonists. *Ordinate*, inward current induced by Carb (3×10^{-4} M), as a percentage of maximum; *abscissa*, antagonist concentration (M), on a logarithmic scale. Blockade of the response to Carb by atropine (*curve a*), AF-DX 116 (*curve b*), and pirenzepine (*curve c*) is indicated. In the case of atropine and AF-DX 116, there are six cells; with pirenzepine, a total of seven cells were tested.

sustained inward current of about −20 pA. The addition of 3×10^{-4} M McN-A-343 completely suppressed the inward current induced by Carb within 15 sec (Fig. 3). Removal of McN-A-343 from the solution resulted in the reappearance of the Carb-induced inward current. Essentially similar results were obtained in two additional experiments of this type. When McN-A-343 (3×10^{-4} M) was added to the bathing solution 2 to 3 min before Carb (3×10^{-4} M), the inward current caused by the latter was also completely suppressed (two experiments, data not shown).

Experiments with pertussis toxin. Treatment with pertussis toxin blocks muscarinic agonist activation of K^+ channels and allows one to detect depolarization in atrial muscle (Ref. 10; reviewed in Refs. 3 and 4). Although Carb-induced depolarization and its corresponding inward current are detected in ventricular muscle without pertussis toxin, it is not known whether these actions of Carb are mediated by pertussis toxin-sensitive G proteins in ventricular myocytes. Dissociated atrial and ventricular myocytes were incubated with pertussis toxin (3.5 μg/ml) for 4 hr at room temperature in recovery solution. This treatment, as expected, blocked activation of muscarinic agonist-specific K^+ current in atrial cells (10); an inward current was detected (data not shown). In ventricular myocytes treated with pertussis toxin, Carb (3×10^{-4} M) still evoked an inward current (Fig. 4). In this cell, the inward current was about −30 pA, and the effect of Carb was reversed by washout of the drug. The Carb-induced current density averaged −540 ± 68nA/cm² in the ventricular myocytes treated with pertussis

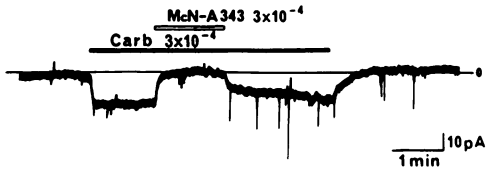

Fig. 3. Block by McN-A-343 of inward current induced by Carb. The holding potential was −75 mV, with zero current being indicated by the *thin horizontal line*. Superfusion with Carb (3×10^{-4} M) produced a prompt inward current that was sustained until McN-A-343 (3×10^{-4} M) was also present. A rapid blockade of the Carb-induced inward current was evident; the block dissipated upon washout of McN-A-343 in the continued presence of Carb. Removal of Carb was accompanied by return of current to zero level. Vertical and horizontal calibrations, 10 pA and 1 min, respectively.

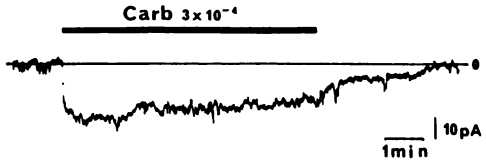

Fig. 4. Inward current evoked by Carb in pertussis toxin-treated ventricular myocyte. The holding potential was −76 mV; zero current is indicated by the *thin horizontal line*. Carb (3×10^{-4} M) induced an inward current (−30 pA) during the superfusion period shown by the *thick horizontal line*. Vertical and horizontal calibrations, 10 pA and 1 min, respectively.

toxin ($n = 4$). This current density is somewhat greater, albeit not significantly ($p > 0.3$), than the average value of −480 ± 21nA/cm² obtained when Carb (3×10^4 M) was added to the bathing solution of myocytes not treated with pertussis toxin.

Discussion

There are two conclusions that can be drawn from the present experiments. First, atropine, but not hexamethonium, prevented the Carb-induced inward current, a pattern also reported for the catecholamine-independent positive inotropic effect caused by Carb and associated with increased intracellular Na^+ in guinea pig ventricle (9). Therefore, the novel action of Carb is not initiated by a cholinoceptive site that would be unusual for heart muscle cells.

Second, experiments with selective agonists and antagonists indicated that the mAChR by which Carb activates the background Na channel cannot be distinguished from the M_2 subtype. Assuming that the ratio of dissociation constants is proportional to the IC_{50} values (16), the potency of muscarinic antagonists is atropine > AF-DX 116 > pirenzepine. This order of IC_{50} values is qualitatively and quantitatively similar to that of the K_d values reported in ligand binding experiments with guinea pig (17), rat (18), and human (19) heart membranes, where the K_d for pirenzepine was 6- to 10-fold greater than that for AF-DX 116.

Additional evidence for participation of M_2 receptors in the response to Carb is the inability of McN-A-343, an M_1-selective agonist (reviewed in Ref. 2), to induce an inward current. We tested this compound at a concentration of 3×10^{-4} M, which is about 10-fold greater than the K_d (2.88×10^{-5} M) reported in guinea pig heart (20). Moreover, the reversible blockade by

McN-A-343 of the Carb-induced current indicates that the former is not without effect and displays antagonist properties at M_2 receptors. Whereas McN-A-343 acted as a partial agonist to induce atropine-sensitive contractions in guinea pig taenia cecum (21, 22), it antagonized the Carb-induced $InsP_1$ accumulation, while failing to increase $InsP_1$ production by itself (21). In embryonic chick heart, McN-A-343 also did not stimulate $InsP_1$ accumulation, and it was concluded that the PI effect is not necessarily an expression of an M_1-selective agonist (23). If one accepts the criteria that mAChRs that are excited by McN-A-343 and sensitive to blockade by pirenzepine are M_1 (reviewed in Ref. 2), then the mAChR involved in the Carb-induced inward current is not of the M_1 subtype and is indistinguishable from M_2.

From molecular biological experiments, the human genome can synthesize at least five muscarinic receptor subtypes (24). Initial attempts to assign specific biochemical effectors to a particular receptor subtype have yielded to the view that the effects elicited by agonist action at the same mAChR are preferential rather than specific or exclusive (25). When the pig heart M_2 mAChR was expressed in Chinese hamster ovary cells, Carb inhibited cyclic AMP production at an EC_{50} approximately 100 times lower than that required to increase formation of inositol-1,4,5-trisphosphate. This conclusion was strengthened by the observation that agonists for human M_1 and M_4 mAChRs were able to promote PI hydrolysis but not inhibit cyclic AMP production (26). The M_2 and M_3 mAChRs were much more efficient at inhibiting cyclic AMP formation than at promoting PI hydrolysis (26). These results with porcine and human M_2 mAChRs are very similar to those reported in our electrophysiological and contractile experiments. Previous reports from our laboratory have shown that ACh and Carb, but not oxotremorine, depolarized atrial (7) and sinoatrial node (8) membranes from pertussis toxin-treated chicks, elicited positive inotropic and chronotropic effects, respectively, and retained the ability to increase $InsP_1$ production (7). In guinea pig ventricle (11), the depolarization and inward Na^+ current induced by Carb and ACh, but not oxotremorine, occur at the same concentrations at which there are intracellular Na^+ accumulation, positive inotropic effects (9, 12), and production of $InsP_1$ (27). All of these agonists have negative inotropic effects in guinea pig ventricle treated with isobutylmethylxanthine to increase cyclic AMP content (12).

The mAChR encoded by pig cerebral (M_1) and cardiac (M_2) cDNA yielded different results for antagonist binding and ACh-induced electrophysiological responses when expressed in *Xenopus* oocytes (28). Addition of ACh opened ion channels that admitted Na^+ and K^+ (M_2) or Cl^- (M_1). Of particular interest are the findings that the apparent reversal potential for the action of ACh via M_2 receptors was +10 mV and that the smooth inward current induced by ACh persisted when EGTA (100 mM) was present in the pipette (28). These results are quite similar to our findings concerning the ionic nature (primarily Na^+) and the apparent reversal potential (+25 mV) of the current induced by Carb in isolated guinea pig ventricular myocytes (11). The results with antagonist binding are qualitatively similar, insofar as the affinity of the M_2 receptor for AF-DX 116 was slightly greater than for pirenzepine, as indicated by IC_{50} values for displacement of (−)-[^{3}H] quinuclidinyl benzilate. As expected, atropine had the greatest affinity for the M_2 receptor (28).

In summary, our results indicate that the novel Na^+ current induced by Carb in guinea pig ventricular myocytes is initiated at a mAChR indistinguishable from the M_2 subtype found in the heart and also detected in *Xenopus* oocytes transfected with the M_2 subtype of mAChR. The ability of Carb to induce a TTX-resistant Na^+ current was not noticeably affected by pretreatment of ventricular myocytes with pertussis toxin. The concentration of pertussis toxin used was greater than that reported to prevent ACh-induced inhibition of calcium current in guinea pig ventricular myocytes (29) and was sufficient to allow Carb to induce an inward current, rather than an outward K^+ current, in guinea pig atrial cells. Therefore, the nature of the G protein, rather than the subtype of mAChR, may be the key element in the reaction pathway that permits certain muscarinic agonists to open TTX-resistant Na channels in the heart. This view is consistent with the observation that treatment with pertussis toxin does not inhibit either the activation of background Na^+ channels or the accumulation of $InsP_1$ caused by muscarinic agonists in heart muscle (7).

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