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Choline Modulates Cardiac Membrane Repolarization by Activating an M_3 Muscarinic Receptor and its Coupled K^+ Channel

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Abstract. Choline is a necessary substrate of the lipid membrane and for acetylcholine synthesis. Accumulating evidence indicates that besides being a structural component, choline is also a functional modulator of the membrane. It has been shown to be a muscarinic acetylcholine receptor (mAChR) agonist and can induce a novel K^+ current in cardiac cells. However, the potential role of choline in modulating cardiac functions remained unstudied despite that mAChRs are known to be important in regulating heart functions. With microelectrode techniques, we found that choline produced concentration-dependent (0.1~10 mM) decreases in sinus rhythm and action potential duration in isolated guinea pig atria. The effects were reversed by 2 nM 4DAMP (an M_3-selective antagonist). Whole-cell patch-clamp recordings in dispersed myocytes from guinea pig and canine atria revealed that choline is able to induce a K^+ current with delayed rectifying properties. The choline-induced current was suppressed by low concentrations of 4DAMP (2~10 nM). Antagonists toward other subtypes (M_1, M_2 or M_4) all failed to alter the current. The affinity of choline (K_d) at mAChRs derived from displacement binding of [^{3}H]-NMS in the homogenates from dog atria was 0.9 mM, consistent with the concentration needed for the current induction and for the HR and APD modulation. Our data indicate that choline modulates the cellular electrical properties of the hearts, likely by activating a K^+ current via stimulation of M_3 receptors.

Key words: M_3 muscarinic acetylcholine receptor — Choline — Heart rate — Action potential duration — K^+ current — Receptor binding assay

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Choline, a member of the vitamin B complex family, is well known to be a necessary substrate of membrane lipid, and a precursor and metabolite of acetylcholine. Studies have demonstrated that choline is not limited to be a structural component of cellular membrane but it also has the ability to functionally affect the membrane physiology. Choline as a muscarinic receptor agonist has also been documented. Drake, Glavinovic & Trifaro (1992) described blockade of Ca^{2+}-activated K^+ channels by choline in bovine chromaffin cells. More recently, Fermini and Nattel presented an intriguing finding on the activation of a novel K^+ current by choline in dog atrial myocytes. The ability of choline to activate a K^+ current was found to be associated with mAChR stimulation. This finding indicates that choline can potentially modulate cardiac electrical activity by activating a K^+ current via stimulation of mAChRs. Indeed, a study performed by Podzuweit (1982) showed that choline abolished the ventricular tachycardia induced by subepicardial infusion of norepinephrine and Ca^{2+} in open-chest pigs. It appears conceivable that the role of choline is not limited to being a structural component but it is also a functional modulator of the membrane. However, it was still unclear what the physiological implications of choline-induced K^+ current are, and the potential mechanisms underlying the K^+ current activation by choline remained unstudied as well.

Choline has received more and more attention because a growing body of evidence indicates that it plays an important role in the prevention of many pathologic conditions, such as cirrhosis of the liver, arteriosclerosis, certain deficiencies of brain function and memory, and the pathogenesis of Alzheimer's disease. However, the potential role of choline in modulating cardiac functions has not been characterized, although this notion is quite

plausible in the light of its ability to induce a K^+ current and to activate mAChRs in the heart.

To date, at least four different subtypes of mAChRs have been pharmacologically and functionally defined in primary tissues, designated M_1, M_2, M_3, and M_4 (Goyal, 1989; Eglen & Whiting, 1990; Hulme, Birdsall & Buckley, 1990; Mutschler et al., 1995; van Zwieten & Doods, 1995). Each subtype has its own characteristic distribution and function. Recently, we obtained functional and molecular evidence indicating the presence of M_3 receptors in canine atrial myocytes. We also found that the cardiac M_3 receptors are functionally coupled to a novel type of delayed rectifier K^+ channel (Shi, Wang & Wang, 1998) and we named this K^+ current I_{KM3} (M_3 receptor-mediated K^+ current). While we have confirmed that the cardiac M_3 receptors and I_{KM3} can be activated by tetramethylammonium (Shi et al., 1998*a*,*b*) and pilocarpine (Wang, Shi & Wang, 1998), it was unknown what endogenous substances could stimulate the M_3 receptors. It has been reported by our laboratory (Shi et al., 1998*c*) and others (Fermini & Nattel, 1994) that choline can activate a K^+ current with properties almost identical to TMA- or pilocarpine-induced current in dog atrial cells and stimulation of mAChRs is required for the current activation. We have elucidated that the TMA- or pilocarpine-induced K^+ currents are the consequence of M_3 receptor stimulation, it remains unknown whether the same mechanism or subtype specificity of mAChRs can be applied to choline induction of the K^+ current.

The objectives of this study are twofold: (i) to investigate whether choline-induced current contributes to cardiac electrical activity and (ii) to explore the mAChR subtype mechanisms of choline induction of the K^+ currents. Standard microelectrode techniques were applied to isolated guinea pig heart preparations and whole-cell patch clamp techniques to dispersed myocytes from guinea pig and dog atria. Preliminary results have been presented in an abstract form in *Life Science.*

Materials and Methods

Preparations

Guinea pig atrial preparation procedures have been described in detail elsewhere (Wang et al., 1990; Wang, Fermini & Nattel, 1991). Briefly, adult guinea pigs of either sex weighing about 300 g were killed by a blow on the head. Their hearts were quickly removed, washed in cool, oxygenated Tyrode solution, then cannulated to a Langendorff perfusion device via the aortic artery. The hearts were placed horizontally onto a Sylgard-covered bottom of a 20-ml Lucite chamber and perfused with Tyrode solution at 5 ml/min. The superfusion solution contained (mM): 120 NaCl, 4 KCl, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 10 glucose and 2 $CaCl_2$; pH was adjusted to 7.4 with NaOH. The superfusate was aerated with 95% O_2–5% CO_2, and the bath temperature was maintained at 36°C by a heating element and proportional power supply (Hanna Instruments, Philadelphia, PA). One hour was allowed for heart equilibration before experiments were begun.

Standard Microelectrode Techniques

The microelectrode techniques used have been described in detail previously (Wang et al., 1990, 1991, 1993*a*). Glass microelectrodes filled with 3 M KCl and with tip resistances of 8–20 MΩ were coupled by a headstage to an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Command pulses were generated with Axoptape in Tape software (Axon Instruments, Foster City, CA) running on an IBM AT type computer interfaced with a D/A converter. The Bandwidth for recordings was set to 10 kHz.

Action potential characteristics, including resting potential, action potential duration to 50% and 90% repolarization (APD_{50} and APD_{90}, respectively), were determined. To monitor the heart rate, action potentials were recorded at sinus rhythm. After baseline measurements, choline chloride (Crystalline, 99.9%, cell culture tested, Sigma) was added to the superfusate, and action potential characteristics were monitored over time. Different concentrations of choline chloride (0.1, 0.5, 1 and 10 mM) were given sequentially. The measurements made under control conditions were repeated after 20 min of drug perfusion at each concentration and after 30 min of washout. In experiments aimed at exploring the role of the M_3 subtype of mAChRs, atropine (1 μM) or 4DAMP (2 nM or 5 nM, an M_3-selective antagonist) was co-applied with choline. Recordings were obtained from at least ten cells under each different situation (with or without drugs and washout) for comparison.

Cell Isolation

Single canine atrial myocytes were isolated with techniques as previously described (Yue et al., 1997; Shi et al., 1998*a*). Briefly, the right atrium from adult mongrel dogs (18–28 kg) of either sex was quickly dissected and mounted via the right coronary artery to a Langendorff perfusion system. The preparation was perfused with Ca^{2+}-containing Tyrode solution (composition same as the solution described below for whole-cell patch-clamp recording) at 37°C until the effluent was clear of blood, and then switched to Ca^{2+}-free Tyrode solution for 20 min at a constant rate of 12 ml/min, followed by perfusion with the same solution containing collagenase (110 U/ml CLS II collagenase; Worthington Biochemical, Freehold, NJ) and 0.1% bovine serum albumin (Sigma Chemicals, St. Louis, MO). The dispersed cells were stored in KB medium at 4°C for later electrophysiological experiments. The KB medium for cell storage contained (mM): 20 KCl, 10 KH_2PO_4, 25 glucose, 70 potassium glutamate, 10 β-hydroxybutyric acid, 20 taurine, 10 EGTA, 0.1% albumin and 40 mannitol; pH was adjusted to 7.4 with KOH.

For isolation of guinea pig atrial myocytes, hearts were quickly removed from adult guinea pig of about 300 g and cannulated to a Langendorff perfusion system via an aortic artery. Cell isolation procedures were same as described above for canine atrial myocytes.

Patch-Clamp Techniques

Patch-clamp recording techniques used have been described in detail elsewhere (Wang et al., 1993*b*, 1994; Shi et al., 1998*a*). Ionic currents were recorded with the whole-cell voltage-clamp methods, using an Axopatch −200B amplifier (Axon Instruments, Foster City, CA). Borosilicate glass electrodes (1 mm O.D.) had tip resistances of 1–3 MΩ when filled with pipette solution of following composition (mM): 0.1 GTP, 110 potassium aspartate, 20 KCl, 1 $MgCl_2$, 5 Mg-ATP, 10 HEPES, 10 EGTA, 5 phosphocreatine; pH adjusted to 7.3 with KOH. Junction potentials were zeroed before formation of the membrane-pipette seal in Tyrode solution containing (mM): 136 NaCl, 5.4

KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 5 HEPES, 10 glucose and 1 $CaCl_2$; pH was adjusted to 7.4 with NaOH. Mean seal resistance averaged 17 ± 1 GΩ. Five minutes after seal formation, the membrane was ruptured by gentle suction to establish the whole-cell configuration.

Command pulses were generated with pCLAMP6 software running on an IBM AT type computer interfaced with a D/A converter. Recordings were low-pass filtered at 1 kHz. The capacitance and series resistance were electrically compensated to minimize the duration of the capacitive surge on the current recording and the voltage drop across the clamped cell membrane. Cells with changing leak current (indicated by >10 pA changes in holding current at −50 mV) were rejected. Experiments were conducted at 36 ± 1°C.

Contamination by sodium current was prevented by holding the cells at −50 mV. Potential contamination by other currents was minimized by including the following compounds in the bath solution: dofetilide (1 μM, to inhibit I_{Kr}), 293B (20 μM, to block I_{Ks}), glyburide (10 μM, to prevent ATP-sensitive K^+ current), and Cd^{2+} (200 μM, to suppress calcium current). Chemicals used for microelectrode and patch-clamp recordings were purchased from SIGMA Chemical (St. Louis, MO), except for 293B which was a kind gift from Hoechst Pharmaceuticals.

Membrane Receptor Binding Assay

Methods for receptor binding used in this study were the same as described in detail elsewhere (Shi et al., 1998*a*). Fresh atrial tissues dissected from canine heart were minced and washed with ice-cold PBS buffer. The tissues were then homogenized with a polytron in 15-ml of ice-cold lysis buffer containing Tris-HCl 5 mM, EDTA 2 mM (pH7.4), plus a protease inhibitor cocktail consisting (in μg/ml): phenylmethylsufony fluoride 5, benzamidine 10, and soybean trypsin inhibitor 5. The homogenate was centrifuged at 500 × *g* for 15 min at 4°C. The pellets were then homogenized as before, spun again and the supernatants pooled. The supernatants were centrifuged at 45,000 × *g* for 15 min and the pellets washed twice in the same buffer. The membrane fractions were resuspended in a buffer containing (in mM): Tris-HCl 75 (pH7.4), $MgCl_2$ 12.5, and EDTA 5. The protein content was determined with Bio-Rad Protein Assay Kit (Bio-Rad, Mississauga, ON) using bovine serum albumin as the standard.

Saturation binding assays were performed using eight concentrations of [^{3}H]-NMS ([N-methyl-3H]-scopolamine methylchloride) ranging from 2 to 2500 pM. Nonspecific binding was measured in the presence of 1 μM atropine. Experiments were carried out in triplicate for each experiment with total of four individual preparations. Incubations (90 min at room temperature) were terminated by rapid filtration using GF/B filters (Xymotech, Montreal, PQ), and radioactivity was counted with an LS6500 Scintillation Counter (Beckman, Fullerton, CA) with average efficiency of 58%.

Competition binding assays were carried out as follows: Homogenates were incubated with 400 pM of [^{3}H]-NMS with choline chloride (10 nM–10 mM) or 4DAMP (0.1 nM–100 μM), respectively. Fixed amounts of membrane protein (100 μg) were used for each sample in the binding study. Seven individual experiments were performed with each determination performed in duplicate for each compound. Chemicals and reagents for the binding study were purchased from Research Biochemicals International (Natick, MA).

Data Analysis

Group data are expressed as mean ± SE. Statistical comparisons between two groups were performed on raw data with Student's *t* test, with a two-tailed $P < 0.05$ taken to indicate a statistically significant difference. Comparisons for multiple groups were performed with analysis of variance (ANOVA) with Scheffé's contrasts. Binding data were analyzed using curve-fitting functions of the GraphPad Prism software (GraphPad Software, CA). To assure validity and accuracy of displacement binding studies, linear regression was performed on the percentage bound *vs.* the ratio of bound over free ligand and only data with a regression coefficient of ≥0.9 was accepted for analyses. The *F* test was used to compare fits for the competition binding data, and the best fit (one-site binding *vs.* two-site binding) was determined by the probability value for the *F* test and by the change in the residual sum of squares for the two different fits. One- and two-site models were tested, and the model yielding the least residual sum of squares was taken to describe the data.

Results

Choline Modulation of Heart Rate and APD in Guinea Pig Atrial Preparations

To assure there is no time-dependent decline of the effects produced by choline, the heart rate and action potential characteristics were monitored with prolonged incubation time up to 30 minutes. The data obtained after 20 min were used for analysis, although the effects of choline at the lowest concentration used (0.1 mM) reached steady-state level after 10 minutes superfusion with the compound. Figure 1 illustrates the effects of choline (1 mM) on the heart rate (the rate of sinus rhythm) in guinea pig atrial preparations. Choline produced reversible slowing of HR. Exchange of superfusate back to control solution without choline completely restored the HR to the pre-drug level. Effects of choline on the HR were nearly abolished by 1 μM atropine (*data not shown*), a nonselective mAChR antagonist. Since we had obtained data suggesting that choline can induce a K^+ current via stimulating M_3 receptors in canine hearts (Shi et al., 1998*c*), we evaluated the effects of 4DAMP (an M_3-selective antagonist, Barlow & Shepherd, 1986; Michel, Stefanich & Whiting, 1989; Araujo, Lapchak & Quirion, 1991; van Zwieten & Doods, 1995) on choline-induced changes of HR. To prevent potential contribution of M_2 receptors, 100 nM methoctramine (Michel & Whiting 1988; van Zwieten & Doods, 1995) was present in the bathing solution throughout the experiments to inhibit the M_2 receptors. 4DAMP (2 nM) considerably reversed choline slowing of HR, indicating a role of M_3 receptors in mediating choline's action (Fig. 1*A*). Mean data are presented in Fig. 1*B*. Under control conditions, the HR was 139 ± 6 beat/min. The values were changed to 107 ± 4 beat/min 10 minutes after bath application of choline ($p < 0.05$, $n = 5$ hearts) and to 128 ± 2 Beat/min after addition of 4DAMP to choline-containing solution. Complete conversion was achieved with 5 nM 4DAMP.

The effects of choline on APD were also evaluated and the results are illustrated in Fig. 2. Remarkable changes in AP, particularly the morphology and APD

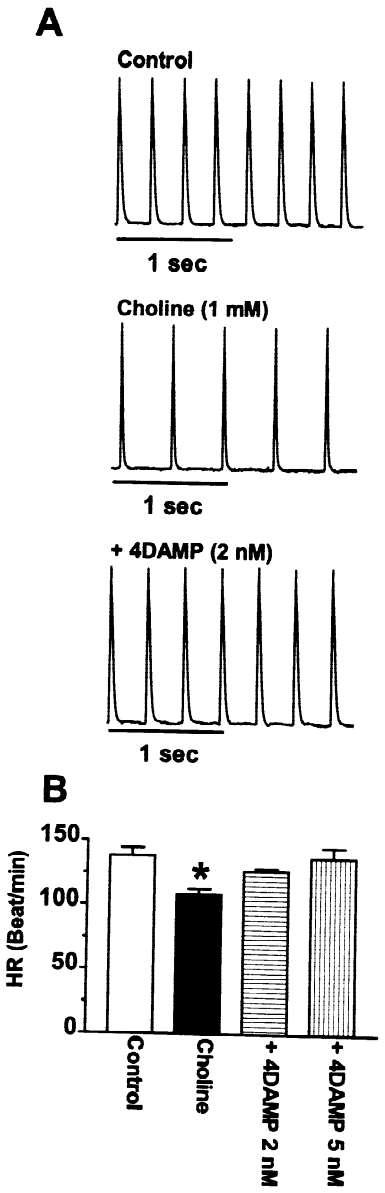

Fig. 1. Choline modulation of sinus rate via stimulation of mAChRs in guinea pig atria. Sinus rate was determined as the firing frequency of action potentials (AP) recorded in atrial preparations with intact sinus nodes. (*A*) Trains of action potentials recorded under control conditions (top panel), in the presence of 1 mM choline chloride (middle panel), and 20 min after concomitant superfusion of choline and 4DAMP (2 nM, an M_3-selective antagonist, bottom panel). Note that the interval between APs is clearly longer than that before choline perfusion and 4DAMP nearly completely restores the control heart rate. (*B*) Averaged data ($n = 5$ hearts) of sinus rate from baseline recording, in the presence of choline (1 mM), and after co-application of choline and 4DAMP (2 and 5 nM). *$P < 0.05$ *vs.* control values.

were consistently observed upon exposure of cells to choline. Before choline, AP had a typical shape with a rapid phase 1 repolarization, a clear phase 2 plateau and a slow phase 3 repolarization. In the presence of the drug, AP configuration became triangular-shaped due to a loss of plateau phase. APD was strikingly shortened relative to baseline recordings. All these alterations caused by choline were readily restored by washout of the choline or by inclusion of 4DAMP (2 nM) in the perfusion solution. Effects of choline (1 mM) on APD were quantified by comparing the changes of APD_{50} and APD_{90}. The mean data ($n = 25$ cells from 5 hearts) are summarized in Fig. 2*C*.

Effects of choline on HR and APD were concentration dependent. Appreciable effects were seen at a concentration of 0.1 mM and higher concentrations (0.5–10 mM) produced statistically significant effects. For example, choline caused 9.4 ± 2.6% slowing of HR at 0.1 mM and 19.3 ± 2.8% at 1 mM. The concentration-dependent APD shortening by choline was analyzed with the Hill equation and the calculated IC_{50} values were 400 μM and 1 mM for APD_{50} and APD_{90}, respectively. Obviously, choline affects more on the plateau phase than on the final phase of action potentials. The results are summarized in Fig. 3*B*. On the other hand, the conversion of the effects of choline by 4DAMP was nearly complete with a concentration of 5 nM.

Choline Induction of a Novel K^+ Current in Isolated Guinea Pig Atrial Myocytes

To understand the potential molecular mechanisms by which choline modulates cardiac electrical activity, we performed whole-cell patch-clamp experiments in isolated guinea pig atrial myocytes. Dofetilide (1 μM) and 293B (20 μM) were included in the superfusate to antecedently block the rapid and the slow components of the delayed rectifier K^+ current (I_{Kr} and I_{Ks}), respectively. In some cells (20% of the cells tested), complete suppression of I_{Kr} and I_{Ks} was achieved and choline at a concentration of 10 mM was applied to the bath. In these cells, a delayed rectifier type of current was activated with choline by depolarizing voltage steps (Fig. 4). This current was abolished by 1 μM atropine, a nonselective mAChR antagonist. More importantly, 4DAMP at a concentration as low as 2 nM remarkably diminished the current, as shown in Fig. 4*B*. To ensure that 4DAMP really acts on mAChRs without direct effects on I_{Kr} and I_{Ks} or other nonspecific effects, the experiments were carried out in the absence of dofetilide and 293B. In all five cells examined, 4DAMP up to 10 nM did not affect I_{Kr} or I_{Ks}, indicating that these two currents were not involved in the choline-induced currents.

The choline-induced currents in guinea pig atrial myocytes resemble the novel K^+ current mediated M_3 receptors first described in canine atrial cells (Fermini & Nattel, 1994; Shi et al., 1998*a*). We decided to perform the experiments with canine atrial cells to further characterize the choline-induced current, because I_{Kr} and I_{Ks}

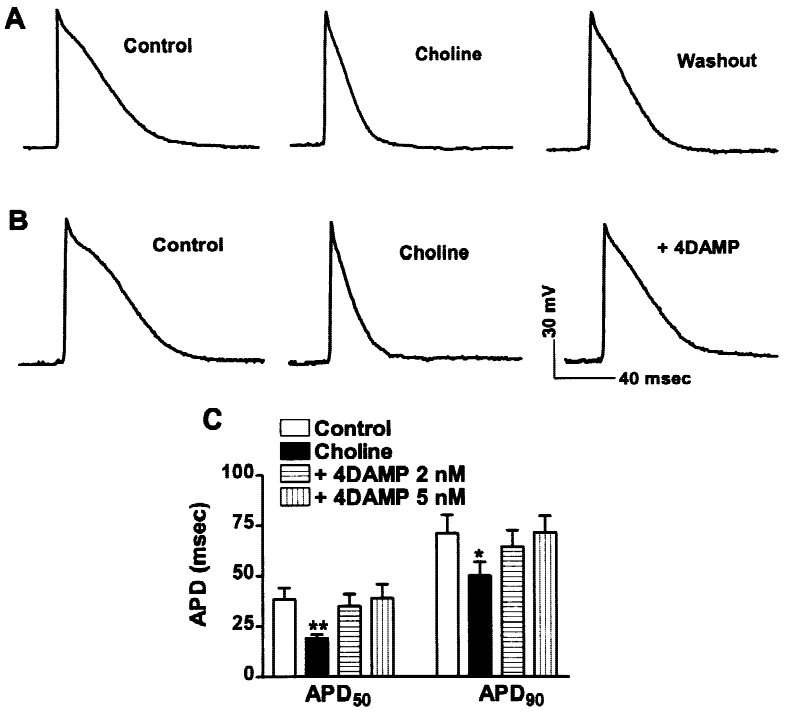

Fig. 2. Choline modulation of action potential duration (APD) by activation of mAChRs in guinea pig atria. (*A*) Choline (1 mM) reversibly shortens APD in a representative preparation. (*B*) 4DAMP (2 nM) restoration of APD shortening caused by choline, recorded in the same preparation as in A. (*C*) Quantification of choline effects on APD. Length of APD was determined at 50% (APD_{50}) and 90% (APD_{90}) repolarization. Shown are data averaged from five hearts. *P < 0.05 and **$P < 0.01$ compared to control.

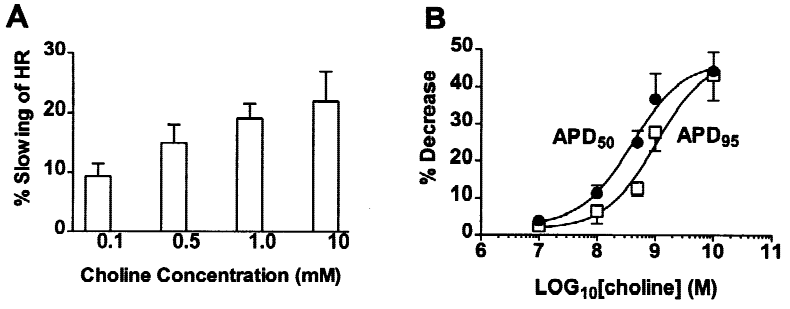

Fig. 3. Concentration-dependence of choline effects on heart rate and action potential duration in guinea pig atria. Concentrations of choline tested were 0.01, 0.1, 0.5, 1 and 10 mM. (*A*) Effects of choline on HR expressed as percent slowing of HR ($n = 5$ hearts). (*B*) Concentration response curves showing effects of choline on APD. Symbols are experimental data from a total of five hearts and lines represent best fits to Hill equation: $B(\%) = 100/[1 + (IC_{50}/D)^n]$, where B(%) is the percent changes of heart rate or APD at a drug concentration D, IC_{50} is the concentration of choline that produces 50% effects, and n is the Hill coefficient. The calculated IC_{50} values for APD_{50} and APD_{90} are 400 μM and 1 mM, respectively.

might contaminate the currents of interest and confound the analysis, and complete elimination of these two currents is hard in guinea pig myocytes, if not impossible, as already stated above. Based on our experience and previous experiments, removal of all contaminating currents is consistently achievable in dog myocytes.

Choline Induction of a K^+ Current in Isolated Canine Atrial Myocytes

As in guinea pig atrial cells, a delayed rectifier type of K^+ current was consistently activated in the presence of choline when all other potential contaminating currents (such as I_{Kr}, I_{Ks}, I_{KATP}, I_{Ca}, and I_{Na}) had been effectively minimized. The minimal concentration of choline required for the current induction was 0.1 mM and a maximal level of the current activation was observed with 10 mM choline. We therefore used 10 mM choline for the rest of our experiments.

Although choline-induced current has been demonstrated to be carried by K^+. We performed experiments to confirm this finding. We found that the reversal potential of the current, as determined by the tail currents elicited upon repolarization to various test potentials ranging from −120 to 0 mV from a prepulse at +50 mV, was dependent on the external K^+ concentrations (ranging from 5.4 to 130 mM). The reversal potential was shifted to less negative potentials with elevating $[K^+]_o$ (*data not shown*).

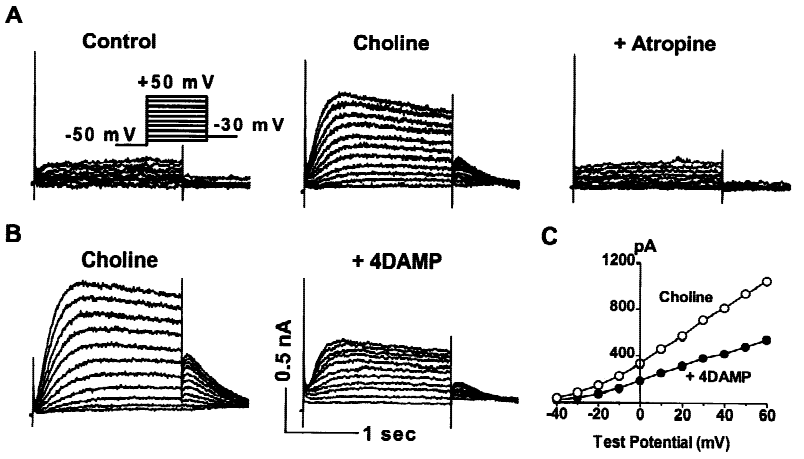

Fig. 4. Choline-induction of a novel K^+ current via stimulation of mAChRs in isolated single guinea pig atrial myocytes. Currents were elicited by 2-sec pulses to potentials ranging from −40 to +50 mV with 10-mV increment followed by a 1-sec step to −30 mV. Voltage steps were delivered from a holding potential (HP) of −50 mV at an interpulse interval of 5 sec. (*A*) An example showing the effects of atropine (1 μM) on choline-induced K^+ currents. The choline concentration was 10 mM. (*B*) Analog data showing the effects of an M_3-selective antagonist 4DAMP (2 nM) on choline-induced K^+ currents from a representative cell. Left panel: currents induced by choline (10 mM) before wash-in of 4DAMP; Right panel: currents in the presence of 4DAMP (2 nM). (*C*) Current-voltage relationship of the baseline currents and after exposure of cells to 4DAMP at a concentration of 2 nM from the same cell in (*B*). Similar data were obtained from another three cells.

Since I_{Kr} and I_{Ks} share some similarities with choline-activated K^+ current, possible contribution of these two currents to choline-induced current was examined. Recordings were made with the solution which did not contain dofetilide and 293B. Following choline activation of the current to steady state levels (usually 10 minutes after starting of the superfusion with choline), dofetilide (1 μM) or 293B (20 μM) was added to the choline-containing solution and the whole-cell recordings were repeated 20 minutes after exposure. Our results showed that neither of the drugs produced any changes of choline-induced current, excluding the involvement of I_{Kr} and I_{Ks} in the choline-induced K^+ current. For the dofetilide experiments, the current density measured at +50 mV was 10.8 ± 2.1 pA/pF in the absence of dofetilide and 10.6 ± 1.9 pA/pF in the presence of dofetilide. For 293B, the values were 9.7 ± 1.3 and 9.5 ± 2.3 pA/pF without and with 293B, respectively.

It seems that choline, like TMA (Shi et al., 1998*a*,*b*) and pilocarpine (Wang et al., 1998), activates the K^+ currents via stimulation of M_3 receptors. This was verified by the following experiments. Effects of atropine on the currents were first evaluated and one example is illustrated in Fig. 5*A*. Atropine at 1 μM eliminated the choline-induced currents, suggesting a role of mAChRs in the current induction. To explore whether M_3 receptors were responsible for mediating choline activation of the K^+ currents, we assessed the effects of 4DAMP. As depicted in Fig. 5*B*, *C* and *D*, 4DAMP, concomitantly applied with choline, substantially depressed the current at all test potentials studied (ranging from −40 to +50 mV). Like in guinea pig myocytes, 2 nM of 4DAMP produced approximately 50% inhibition (48.2 ± 6.1% at +50 mV, $n = 6$). Greater percentage of block was observed with higher concentrations of the drug: 75% by 5 nM and 93% by 10 nM.

To investigate whether the choline-induced current also responds to the antagonists selective towards other subtypes ($M_1/M_2/M_4$) of mAChRs, effects of pirenzepine (PZ, for M_1, Watson, Yamgmur & Roeske, 1983), methoctramine (MA, for M_2, Wess et al., 1988; Michel & Whiting, 1988), and tropicamide (TA, for M_4, Lazareno, Buckley & Roberts, 1990; Lazareno & Birdsall, 1993) were evaluated. The results are presented in Fig. 6. None of these compounds exhibited any blocking actions on the choline-induced K^+ current. To ensure that the failure of these compounds to inhibit the currents were not due to insufficient concentrations used, we further tested the effects of elevated concentrations of PZ (100 nM) or MA (100 nM) on the current. However, we still failed to observe any changes of the current. Higher concentrations of the drugs were not studied because they may cause cross-actions on different subtypes.

To ensure that the effects of choline were not the consequence of enhanced release of catecholamine from presynaptic ganglions, additional experiments were performed to evaluate the effects of α_1- and β-adrenergic receptor antagonist prazosin (2 μM) and propralonol (2 μM). However, no changes were observed under such conditions and choline demonstrated the same potency for K^+ current induction (*data not shown*), excluding the contribution of adrenoceptor activation. This was further precluded by our preliminary study showing that phenylephrine (an α_1-adrenoceptor agonist) and isoproterelol (a β-adrenoceptor agonist) both cause significant decrease in the current, instead of increase as expected if these receptors are involved in choline induction of the K^+ current.

Choline Binding to mAChRs in Membrane Homogenates from Canine Atria

If, as our functional study suggested, choline modulates heart functions by activating a K^+ current via stimulation

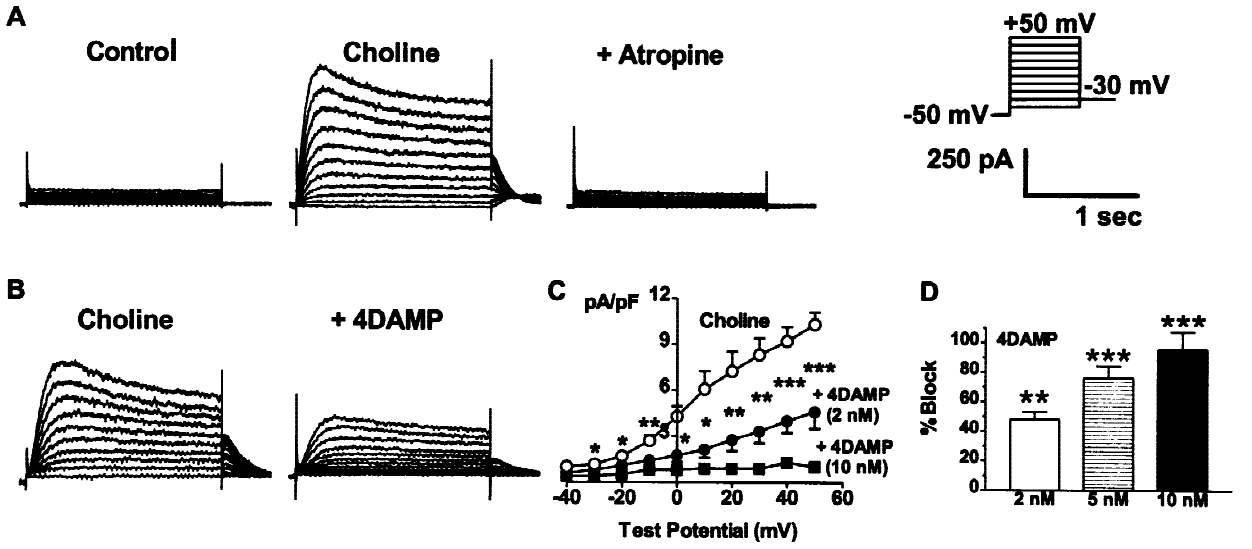

Fig. 5. Inhibition of choline-induced K^+ currents by 4DAMP (an M_3-selective antagonist) in canine atrial myocytes. (*A*) A family of traces recorded before drug application, in the presence of choline (10 mM) and after concomitant application of choline and atropine (1 μM). (*B*) Analog data showing inhibition of choline-induced K^+ currents by 4DAMP (2 nM). (*C*) Current density-voltage relationship choline-induced currents before and after application of 4DAMP (2 and 5 nM). Shown are averaged data from a total of 14 cells. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, Scheffe's contrast after *F* tests, comparison between choline and choline + 2 nM 4DAMP only. (*D*) Percentage block of choline-induced K^+ currents by varying concentrations of 4DAMP, as determined at +50 mV. $**P < 0.01$ and $***P < 0.001$, student *t*-test, comparison between choline and choline + 4DAMP ($n = 5$).

of an M_3 receptor, then it should be able to displace [^{3}H]-NMS binding in a competitive fashion. To clarify this issue and also to verify the validity of the choline concentrations used in our experiments, mAChR binding assay was carried out. Saturation binding of [^{3}H]-NMS to the membrane homogenates from canine atria yielded a maximum binding value, or M-receptor density of 282 ± 26 fmol/mg protein and a dissociation constant K_d of 223 ± 24 pM. The experiments for competition binding were performed with 400 pM [^{3}H]-NMS and thirteen concentrations of choline ranging from 10 nM to 10 mM. Choline binding yielded displacement curves best described with a two-site binding model. The data are shown in Fig. 7*B*. The K_d values were 5.5 ± 1.2 μM (34%, percentage of high affinity binding) and 0.9 ± 0.2 mM (65%, percentage of low affinity binding) for choline. The low affinity binding was quite compatible with the concentrations at which choline produced induction of the K^+ current and alteration of the HR and APD in both canine cells and guinea pig cells. For comparison, displacement binding of [^{3}H]-NMS with 4DAMP was also performed and the results are shown in Fig. 7*B*. The K_d values were 2.5 ± 0.6 nM and 79.4 ± 9.6 nM. The high affinity binding K_d was in good agreement with the concentration of 4DAMP (2 nM) required to produce ~50% conversion of the effects of choline.

Discussion

Choline is known to be a structural component of the cell membrane. We demonstrated here that choline is also a

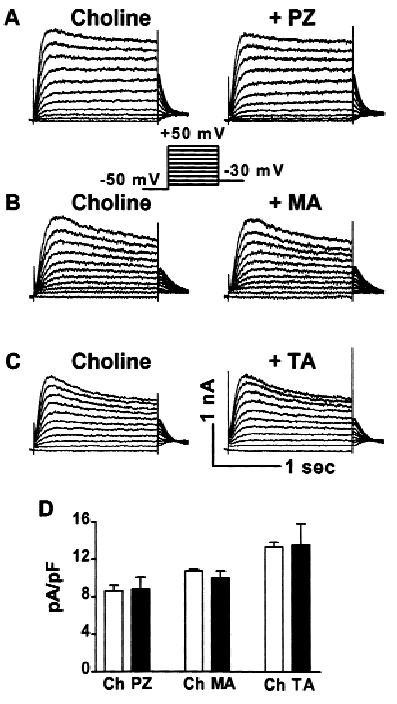

Fig. 6. Lack of effects of mAChR subtype-selective antagonists on choline-induced K^+ currents in canine atrial myocytes. Currents were elicited in the presence of 10 mM choline, with voltage protocols same as described in Fig. 4. Analog data from representative myocytes showing effects of pirenzepine (PZ, 10 nM (*A*)), methoctramine (MA, 20 nM (*B*)), and tropicamide (TA, 200 nM (*C*)). (*D*) Averaged data of choline-induced K^+ currents before and after and antagonists, measured at +50 mV ($n = 5$ for PZ, $n = 5$ for MA, and $n = 4$ for TA).

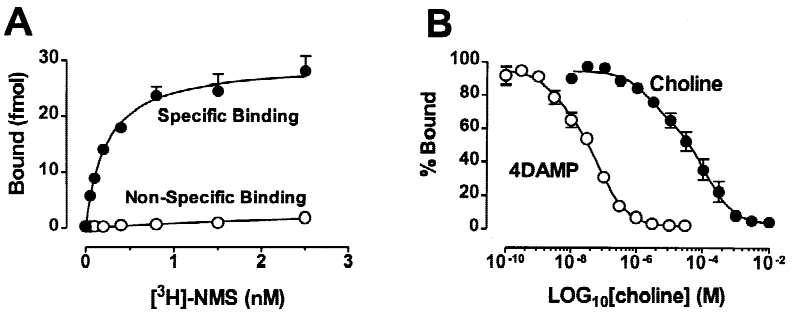

Fig. 7. M-receptor binding with [^{3}H]-NMS to homogenates from canine atrial tissues. (*A*) Saturation binding. Shown are the specific and nonspecific data averaged from four individual preparations carried out in triplicate for each experiment. Symbols are experimental data and lines represent the fit with a one-site binding model. (*B*) Displacement binding of [^{3}H]-NMS with choline (10 nM–10 mM) or 4DAMP (0.1 nM–100 μM). Each point represents the mean ± SEM from six experiments assayed in duplicate. Curves are best fits of the experimental data to a two-site binding model.

functional modulator of the cell membrane. Choline slowed the heart rate and accelerated the membrane repolarization. These effects could be explained at least partially by its ability to bind to mAChRs and to activate a novel K^+ current most likely via stimulating the M_3 receptors in mammalian heart cells.

The ability of choline to induce a K^+ current with delayed rectifier properties distinct from the classical delayed rectifier K^+ current (I_{Kr}/I_{Ks}, Wang et al., 1995) was first described by Fermini and Nattel (1994). They found that choline induction of the K^+ current relied on stimulation of mAChRs. And their data argued against the role of M_1 receptor subtype or nicotinic receptors in this function. Since their results could not be readily explained by M_2 receptors, they proposed that other subtypes may be responsible for choline induction of the K^+ current. Unfortunately, no further characterization was performed in their study.

In contrast to most peripheral tissues, the myocardium has been considered to possess a single mAChR subtype: M_2 receptors have long been believed to be the only functional mAChR subtype in the heart (Watson et al., 1983; Bonner et al., 1987; Mizushima et al., 1987; Peralta et al., 1987). However, the concept that the heart possesses a single M_2 subtype of mAChR is now being challenged. Expression of multiple isoforms of mRNAs encoding for different subtypes of mAChRs ($M_1/M_2/M_3/M_4$) in chick hearts has been confirmed by two groups (Tietje et al. 1990; Tietje & Nathanson, 1991; Gadbut & Galper, 1994; McKinnon & Nathanson, 1995). Sun et al. (1996), by studying the antagonism of carbachol-induced chronotropy and IP1 accumulation, found evidence for a heterogeneous population of muscarinic receptors including M_2 and M_3 subtypes in neonatal rat ventricular myocytes. Sharma et al. (1996) used single-cell PCR, subtype-specific antibodies and the measurement of Ca^{2+} transients to provide convincing molecular and functional evidence for the presence of M_1 receptors in rat ventricular myocytes. Ford, Eglen and Whiting (1992) analyzed mAChR mediated PI hydrolysis in guinea pig atria and ventricles. The inhibition of the response to agonist by several antagonists including HHSiD and *p*-F-HHSiD generated an affinity profile dissimilar to the pure M_2 response, suggesting "a second population of muscarinic sites." In isolated blood-perfused dog atria, Akahane (1990) compared the inhibitory potency of carbachol-induced negative chrono- and inotropic responses by 4-DAMP, AF-DX 116 and pirenzepine. They found that the potency of 4-DAMP was equal to atropine but greater than AF-DX 116 (an M_2-preferring antagonist) and much greater than pirenzepine (an M_1-selective inhibitor), suggesting a role of the M_3 subtype. Yang et al. (1992) performed detailed pharmacological characterization of mAChR subtypes in membrane homogenates from dog left ventricular tissues and their data seemed to exclude the presence of M_1 receptors but favored the existence of M_2 and M_3 subtypes. They further found in their functional study that the inhibition of carbachol-mediated PI hydrolysis by pirenzepine, AF-DX 116 and 4DAMP generated an affinity profile dissimilar to the classical cardiac M_2 response. The authors interpreted these data as favoring the existence of a second population of mAChRs distinct from M_2. We have previously identified the cardiac M_3 receptors in dog atrial myocytes with both functional and molecular evidence (Shi et al., 1998*a*,*b*). We found that stimulation of the cardiac M_3 receptors by TMA (Shi et al., 1998*a*) or pilocarpine (Wang et al., 1998) activates a delayed rectifier type of K^+ current, which is sensitive to several M_3-preferential antagonists but not to the antagonists selective toward other subtypes of mAChRs. Radioligand binding data pointed to the existence of M_3 subtype in dog atrial membrane homogenates. A cDNA fragment of the cardiac M_3 receptor gene has also been isolated from the dog heart. More recently, M_3 receptors have also been detected at the protein level with Western blotting from our preliminary experiments. We have further found using antibodies that M_3 receptors are linked to the K^+ channels via G_q proteins. Yet the physiological functions of this cardiac M_3 receptor and its coupled K^+ channel were unclear. The present study provided evidence indicating that activation of the cardiac M_3 receptor and its coupled K^+ channel may contribute significantly to the regulation of HR and membrane repolarization. Slowing of HR can be beneficial in many clinical settings such as myocardial ischemia, arrhythmias, atrial fibrillation, etc. Short-

ening of APD particularly the plateau phase could be either arrhythmogenic (for re-entrant type of arrhythmias) or antiarrhythmic (for early afterdepolarization), depending on different situations. But decrease in Ca^{2+} entry may be caused indirectly by shortened APD, which could lead to weakening of contraction and reduced oxygen consumption. The fact that choline is able to abolish the norepinephrine and Ca^{2+} induced ventricular tachycardia (Podzuweit, 1982) may be related to its ability to shorten APD. Choline is known to have a neuroprotective effect in situations of hypoxia and ischemia of the brain. Whether the same protective effects are also applied to cardiac hypoxia and ischemia is unknown. Furthermore, choline has been demonstrated to be able to prevent occurrence of apoptosis (Albright et al., 1996) and both choline and M-receptors play important role in Alzheimer's disease (Elble, Giacobini & Higgins, 1989). It is not unreasonable to speculate that the effects of choline in various tissues and systems as in the heart can also be mediated by M_3 receptors too. It has been found that M_3 receptors are present in various neuronal populations throughout the brain and also have their wide distribution in the peripheral tissues (Buckley, Bonner & Brann, 1988). Whether the M_3 receptors in these tissues also couple to K^+ channels, as in the heart, remains unclear. Future studies are warranted to test these notions and to clarify whether choline can affect the heart functions in vivo.

Given that high concentrations of choline (0.1~10 mM), relative to its physiological blood levels, are required to activate M_3 receptors and the K^+ current, choline is unlikely an endogenous agonist of M_3 receptors. Nonetheless, choline should at least be considered an effective pharmacological probe for studying the cardiac M_3 receptors and the novel K^+ current.

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