The Role of Muscarinic K⁺ Channels in the Negative Chronotropic Effect of a Muscarinic Agonist

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ABSTRACT
Acetylcholine causes bradycardia through M2 muscarinic receptors in sinoatrial node cells. I examined with electrophysiology how the muscarinic K⁺ (K_{ACH}) channel participates in the sinus bradycardia induced by a muscarinic agonist in the Langendorff preparation of rabbit hearts. In the presence of 100 nM propranolol, 1 nM to 10 μM carbachol (CCh) induced sinus bradycardia in a concentration-dependent manner. Tertiapin (100 or 300 nM), which selectively blocks K_{ACH} channels in cardiac myocytes, significantly inhibited the effect of ~300 nM but not ≤100 nM CCh. The effect of CCh was divided into tertiapin-sensitive and -insensitive components. The former component was induced by >100 nM CCh in a concentration-dependent manner and accounted for ~75% of the maximum effect of CCh. The K_{ACH} channel in atrial myocytes was also activated by this range of concentrations of CCh as measured with the patch-clamp method. The tertiapin-insensitive component was induced by 1 to 300 nM CCh in a concentration-dependent manner and accounted for ~25% of the maximum effect of CCh. The sinus rate in the presence of 1 μM CCh and 300 nM tertiapin was similar to that in the presence of 2 mM CsCl, a blocker of the hyperpolarization-activated I_f current. Furthermore, no tertiapin-insensitive component existed in the presence of 2 mM CsCl. Therefore, the negative chronotropic effect of ≥300 nM CCh is mainly mediated by K_{ACH} channels, whereas that of ≤100 nM CCh may result from suppression of the I_f current.

Parasympathetic regulation of the heart rate is mediated by acetylcholine (ACh) (Loewi and Navratil, 1926; Löeffelholz and Pappano, 1985). ACh activates M2 muscarinic receptors and the heterotrimeric G_{12, G_{13}} and/or G_s proteins in cardiac myocytes (Luetje et al., 1988). The α subunits of the G_s proteins inhibit the adenyl cyclase (Sunahara et al., 1996), whereas the βγ subunits of the G_s proteins directly activate the inwardly rectifying muscarinic K⁺ (K_{ACH}) channel in sinoatrial (SA), atrial, and atrioventricular (AV) nodes, and Purkinje myocytes (Logothetis et al., 1987; Sowell et al., 1997; Yamada et al., 1998).

K_{ACH} channels cause the negative chronotropic effect by hyperpolarizing SA node cells (del Castillo and Katz, 1955; Hutter and Trautwein, 1955; Noma and Trautwein, 1978; Sakmann et al., 1983). On the other hand, the suppression of adenyl cyclase decreases the heart rate by inhibiting such inward currents in SA node cells as the hyperpolarization-activated (I_f) current, the L-type calcium current, and the sustained inward current, all of which are activated by cAMP or cAMP-dependent protein kinase, especially in the presence of β-adrenergic stimulation (Irisawa et al., 1993). It is unknown to what extent each of the signal transduction pathways is responsible for the negative chronotropic effect of ACh. This is partly because there have been no selective inhibitors of every signal transduction pathway evoked by muscarinic stimulation.

A peptidyl honeybee toxin tertiapin was recently found to selectively inhibit K_{ACH} channels in cardiac myocytes (Jin and Lu, 1998; Drici et al., 2000; Kitamura et al., 2000). Tertiapin fully inhibits ACh-induced whole-cell K_{ACH} channel currents in rabbit atrial myocytes in a concentration-dependent manner in a range of concentrations between 10 pM and 10 μM through ~1:1 stoichiometry (Kitamura et al., 2000). The half-maximum IC_{50} of tertiapin is ~8 nM independent of the membrane potential. Tertiapin also inhibits the K_{ACH} channel current activated by a GTP analog applied to the cytosol, indicating that the effect of tertiapin is not mediated by a muscarinic receptor. At the single-channel level, tertiapin inhibits the K_{ACH} channel from the outside of the membrane by reducing the P_o (N is the number of functional channels, and P_o is the open probability of each channel) without affecting the single-channel conductance or fast open-close kinetics. Thus, tertiapin is a slow blocker of the K_{ACH} channel.

ABBREVIATIONS: ACh, acetylcholine; CCh, carbachol; I_f currents, hyperpolarization-activated currents; SA, sinoatrial; AV, atrioventricular; K_{ACH}, muscarinic K⁺.
In this study, I examined the effect of tertiapin on the sinus bradycardia caused by carbachol (CCh) in the presence of a β-adrenergic blocker in the Langendorff preparation of rabbit hearts with ECG. I divided the effect of CCh into the tertiapin-sensitive (i.e., K_{ACCh} current-dependent) and insensitive (i.e., K_{ACCh} current-independent) components. The tertiapin-sensitive component was ~10 times less sensitive to CCh but ~3 times more effective than the tertiapin-insensitive component in decreasing the sinus rate. The tertiapin-insensitive component seemed to be mediated by inhibition of I_f currents probably through suppression of the basal CAMP level.

Materials and Methods

Langendorff Preparation of Rabbit Hearts. Male Japanese-White rabbits weighing 1.5 to 1.7 kg were injected with heparin sodium (200 U/kg b.wt.) through an ear vein. Approximately 20 min later, the rabbits were anesthetized with pentobarbital sodium or thiopental sodium (30 mg/kg b.wt.) injected through a vein in another ear. As soon as the rabbits lost a nociceptive response, the heart was quickly removed and immediately perfused with oxygenated Tyrode’s solution (for composition, see below) at 37°C in the Langendorff apparatus in a retrograde manner.

Recording Electrocardiogram from Isolated Rabbit Hearts. The isolated heart was hung over a glass funnel ~5 cm in diameter in such a way that its apex lightly touched the inner surface of the lowest and narrowest part of the funnel. A few sheets of thin paper (Kimwipes; Kimberly-Clark Co., Tokyo, Japan) filled the space between the ventricular wall and the inner surface of the funnel. After the paper was wetted with the cardiac effluent (i.e., the modified Tyrode’s solution), the standard bipolar lead ECG was recorded from the electrodes connected to the paper with a conventional ECG recorder (Cardiofax; Nihon Kohden Co. Ltd., Tokyo, Japan). After the heart exhibited a stable sinus rhythm, various drugs were applied to the heart through coronary arteries by continuously monitoring ECG. ECG was recorded on paper once every 1 to 5 min, at which the PP, PQ, QRS, and QT intervals were measured. The sinus rate was calculated as follows: sinus rate (min⁻¹) = 60/ x, where x is the duration of n PP intervals in seconds, and n is an integer between 3 and 10. The QT intervals were corrected with Bazett’s formula (QTc = QT/RR^{1/2}).

Isolation of Atrial Myocytes and Patch-Clamp Study. The methods of isolation of atrial myocytes and patch-clamp experiments were precisely described in a previous paper (Kitamura et al., 2000). Briefly, the heart in the Langendorff apparatus was perfused with 150 ml of the nominally calcium-free Tyrode’s solution (for composition, see below) at 37°C. The pipette solution was prepared in the KB solution (for composition, see below) at 4°C. The pipette solution contained 136.5 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.53 mM MgCl2, 5.5 mM glucose, and 5.5 mM HEPES (pH adjusted to 7.4 with NaOH). For the nominally calcium-free Tyrode’s solution, CaCl2 was simply omitted from the modified Tyrode’s solution. The KB solution contained 10 mM taurine, 10 mM oxalic acid, 70 mM glutamic acid, 25 mM KCl, 10 mM KH₂PO₄, 11 mM glucose, 0.5 mM EGTA, and 10 mM HEPES (pH adjusted to 7.3 with KOH). The pipette solution contained 140 mM KCl, 5 mM MgCl₂, 5 mM EGTA, 3 mM ATP, 0.1 mM GTP, and 5 mM HEPES (pH adjusted to 7.4 with KOH). The calculated free Mg²⁺ concentration in this solution was 1.6 mM. In the ECG experiments, 5 mM ascorbic acid was added to the modified Tyrode’s solution (pH readjusted to 7.4 with NaOH) to prevent oxygenation of tertiapin. Ascorbic acid (5 mM) alone did not affect the ECG. Tertiapin was purchased from Peptide Institute Inc. (Osaka, Japan). Tertiapin was dissolved in distilled water at 100 μM immediately before use, further diluted to desired concentrations with the oxygenated modified Tyrode’s solution with ascorbic acid, and immediately applied to the hearts. CCh and propranolol were purchased from Sigma Chemical Co. (St. Louis, MO). Atropine was from Wako Pure Chemical (Osaka, Japan).

Statistical Analysis. All statistical values are indicated as mean ± S.E. The statistical difference was evaluated by Student’s t test. For multiple comparison between pairs, difference was assessed with analysis of variance and the Bonferroni method. A value of p < 0.05 was considered statistically significant.

Results

Concentration-Dependent Effect of Carbachol on the Sinus Rate of a Rabbit Heart. I first examined the concentration-dependent effect of CCh on the sinus rate in the Langendorff preparation of a rabbit heart by monitoring the ECG (Fig. 1). At the beginning of each experiment, a β-adrenergic blocker propranolol (100 nM) was applied to eliminate the effect of residual catecholamine in the heart (Fig. 1A). Propranolol (100 nM) decreased the sinus rate to 85.7 ± 1.9% of the control on average and increased the PQ interval significantly (Table 1). After the sinus rate became stable (from recordings 1 and 2), CCh was applied in a cumulative manner. CCh decreased the sinus rate in a concentration-dependent manner in the range of concentration between 10 nM and 1 μM (recordings 3–6). CCh caused sinus arrest in eight of nine hearts at 1 μM and in all four hearts at 10 μM. The PQ interval slightly but significantly increased from 51.12 ± 2.78 ms in controls to 59.22 ± 1.98 ms in the presence of 300 nM CCh (n = 9). However, CCh did not cause the second or third degree of AV conduction block before inducing the sinus arrest in 39 of 40 hearts examined. Thus, I did not further analyze the effect of CCh and/or tertiapin on the AV conduction.

As shown in Fig. 1, tertiapin (100 nM) applied in the presence of 1 μM CCh, reversed the sinus rate to 59% of the controls (recording 7). From a previous study (Kitamura et al., 2000), 100 nM tertiapin inhibits ACh (1 μM) induced K_{ACCh} channel currents by ~89% in rabbit atrial myocytes. Thus, K_{ACCh} channels might not be entirely responsible for the bradycardic effect of 1 μM CCh. Atropine (10 μM) applied at the end of the experiment almost completely restored the sinus rate to the value before application of CCh (recording 8).

Effect of Tertiapin on the Sinus Bradycardia Induced by Lower Concentrations of Carbachol. I further examined the effect of tertiapin in the presence of lower
concentrations of CCh (Fig. 2). Tertiapin (100 nM) reversed the negative chronotropic effect of 300 nM CCh by 41% (Fig. 2A). The same concentration of tertiapin, however, antagonized the effect of 100 nM CCh only by 16% (Fig. 2B) and barely antagonized that of 30 nM CCh (Fig. 2C). In the absence of CCh, 100 or 300 nM tertiapin did not significantly alter the sinus rate, although it reduced the effect of subsequently applied 1 μM CCh by 73% (Fig. 2D and data not shown). Therefore, the effect of tertiapin was less as the concentration of CCh decreased.

Figure 3A summarizes the concentration-response relationship of the CCh-induced sinus bradycardia in the absence and presence of 100 or 300 nM tertiapin. Under control conditions, CCh decreased the sinus rate in a concentration-dependent manner in the range of concentrations between 1 nM and 10 μM CCh with IC50 of ~300 nM. Tertiapin (100 nM) significantly increased the sinus rate only in the presence of 300 nM and 1 μM CCh. Tertiapin (300 nM), which inhibits ACh (1 μM) induced KACCh, channel currents by ~95% (Kitamura et al., 2000), further increased the sinus rate in the presence of 1 μM CCh. Tertiapin (300 nM) also significantly increased the sinus rate in the presence of 10 μM CCh.

The inset of Fig. 3A depicts the negative chronotropic effect of CCh, and its tertiapin-sensitive and -insensitive components as a percentage of the maximum effect of CCh. The tertiapin-sensitive component exhibited a steep concentration-response curve in the range of concentrations between 100 nM and 1 μM and accounted for 75% of the maximum effect of CCh. In contrast, the tertiapin-insensitive component showed a less steep concentration-response curve in the range of concentrations between 1 and 300 nM and accounted for 25% of the maximum effect of CCh.

Concentration-Dependent Effect of Carbachol on the KACCh Channel. I next examined the effect of CCh on KACCh channel currents in atrial myocytes (Fig. 3B). In the whole-cell configuration of the patch-clamp method, CCh (>10 nM) induced KACCh channel currents with characteristic slow relaxation (Fig. 3B, a). The current flowed inward at ~100 mV and outward at ~40 and +10 mV. CCh induced KACCh channel currents in a concentration-dependent manner in the range of concentrations between 1 nM and 10 μM (Fig. 3B, b). The line indicates the fit of the results with the Hill equation (see figure legend), which provided an estimate of the half-maximum effective concentration of 435 nM and the
Hill coefficient of 1.52. This curve is replotted in the inset of Fig. 3A. This curve was very similar to that of the tertiapin-sensitive component in a range of CCh concentrations of \(1 \text{ nM} \) to \(1 \mu \text{M}\). However, in the presence of \(>1 \mu \text{M}\) CCh, the K\(\text{ACh}\) current increased in a concentration-dependent manner, whereas that for the tertiapin-sensitive component reached a plateau because \(>1 \mu \text{M}\) CCh caused sinus arrest.

**Effect of CsCl on the Sinus Rate and Carbachol-Induced Bradycardia.** The tertiapin-insensitive component may be mediated by suppression of the basal cAMP level. It is reported that ACh inhibits \(I_f\) currents at lower concentrations than activating K\(\text{ACh}\) channels by reducing the basal cAMP level (DiFrancesco and Tromba, 1988; DiFrancesco et al., 1989). Thus, I examined the effect of CsCl on the heart rate because CsCl is reported to selectively inhibit \(I_f\) currents at 1 to 2 mM in rabbit SA node cells (Denyer and Brown, 1990). As shown in Fig. 4A, 2 mM CsCl reduced the sinus rate to \(\sim66\%\) of the control (from recordings 1 and 2), indicating that \(I_f\) currents contribute to but are not essential for sinus automaticity (Irisawa et al., 1993). CsCl (2 mM) did not significantly change the QRS or QTc intervals (Table 1), indicating that it did not block the voltage-dependent or inwardly rectifying K\(^+\) currents in ventricular and Purkinje myocytes at least effectively in the physiological range of membrane potentials. CCh (1 \(\mu \text{M}\)) applied after washout of CsCl caused sinus arrest (recording 3), and 300 nM tertiapin applied under this condition increased the sinus rate to the level similar to that in the presence of 2 mM CsCl alone (recordings 2–4). On average, the sinus rate was \(78.5 \pm 4.2\%\) of controls in the presence of 2 mM CsCl alone (\(n = 12\)) and \(72.1 \pm 4.3\%\) in the presence of 1 \(\mu \text{M}\) CCh and 300 nM tertiapin (\(n = 5\)) (Fig. 3A). These values were not significantly different (\(p = 0.382\)). In Fig. 4B, 1 \(\mu \text{M}\) CCh was added in the presence of 2 mM CsCl. CCh decreased the sinus rate more slowly than in the absence of CsCl probably because K\(\text{ACh}\) channels were blocked by CsCl more potently as the membrane potential was hyperpolarized (Argibay et al., 1983). In the presence of 2 mM CsCl, 300 nM tertiapin completely antagonized the effect of 1 \(\mu \text{M}\) CCh (recordings 2–4). Thus, it is likely that the tertiapin-insensitive component of the CCh-induced sinus bradycardia results from inhibition of the \(I_f\) current.

**Discussion**

By using tertiapin as a selective K\(\text{ACh}\) channel blocker in the heart (Kitamura et al., 2000), I divided the negative chronotropic effect of CCh into the tertiapin-sensitive (i.e., K\(\text{ACh}\) current-dependent) and -insensitive (i.e., K\(\text{ACh}\) current-independent) components. The K\(\text{ACh}\) current-dependent component exhibited a steep concentration-response curve in the range of CCh concentrations between 100 nM and 1 \(\mu \text{M}\), and accounted for \(\sim75\%\) of the maximum effect of CCh. On the other hand, the K\(\text{ACh}\) current-independent component was induced by 1 to 300 nM CCh and accounted for \(\sim25\%\) of the maximum effect of CCh. Thus, the K\(\text{ACh}\) current-dependent...
The component was 10 times less sensitive to CCh but 3 times more effective than the K_ACh current-independent component in decreasing the sinus rate.

The concentration-response curve for the CCh-induced K_ACh current closely resembled that for the tertiapin-sensitive component in a range of CCh concentrations of 10^{-9} M to 10^{-5} M with a similar steepness (Figs. 3A, inset; the Hill coefficient $n = 1$). This positive cooperativity may arise from the interaction between the G protein subunits and K_ACh channels (Hosoya et al., 1996). In the presence of 10^{-5} M CCh, the CCh-induced K_ACh current increased in a concentration-dependent manner, whereas that for the tertiapin-sensitive component reached a plateau. This is because >1 $\mu$M CCh caused sinus arrest.

Recently, Drici et al. (2000) identified the role of K_ACh channels in the parasympathetic regulation of AV conduction by showing that tertiapin attenuated the ACh-induced advanced AV block in guinea pig hearts. They also briefly mentioned the effect of tertiapin on the negative chronotropic effect of ACh. In guinea pig hearts, they found that tertiapin almost completely reversed the effect of 0.5 $\mu$M ACh. In rabbit hearts, however, they found that 5 $\mu$M ACh reduced the sinus rate by only 20%, and that this response was insensitive to tertiapin. This is very different from my observation. I found in preliminary experiments that >1 $\mu$M CCh usually caused sinus arrest as is the case for CCh. At lower concentrations, however, ACh caused the negative chronotropic effect of ACh in their rabbit heart preparation might have been much higher than 5 $\mu$M.

It is widely accepted that the negative chronotropic effect of vagal nerve activity or parasympathomimetics results from hyperpolarization of sinus node cells due to activation of K_ACh channels (Gaskell, 1887; Burgen and Terroux, 1953; del Castillo and Katz, 1955; Hutter and Trautwein, 1955 and 1956; Harris and Hutter, 1956; Hutter, 1957; Trautwein an Dudel, 1958; Noma and Trautwein, 1978; Sakmann et al., 1983). On the other hand, weak vagal stimulation or low concentrations of ACh is known to cause the negative chro-
notropic effect by decreasing the slope of diastolic depolarization without causing hyperpolarization of the maximum diastolic potential (Hutter and Trautwein, 1955 and 1956; West, 1955; Bouman et al., 1963; Shibata et al., 1985; Campbell et al., 1989; DiFrancesco, 1993). This phenomenon may be at least in part explainable in terms of the slow relaxation of K<sub>ACh</sub> channels (Yamada et al., 1998). ACh-induced inhibition of the L-type calcium channel current and/or the sustained inward current may also be responsible for this phenomenon (Irisawa et al., 1993). On the other hand, DiFrancesco et al. ascribed the phenomenon to inhibition of the I<sub>f</sub> current resulting from the ACh-induced suppression of the basal cAMP level (DiFrancesco and Tromba, 1988; DiFrancesco et al., 1989; DiFrancesco, 1993). They showed that ACh inhibited I<sub>f</sub> currents and slowed the spontaneous firing rate of isolated rabbit SA node cells at ~20 times lower concentrations than activating K<sub>ACh</sub> channels. Although there is some controversy on their hypothesis and contribution of I<sub>f</sub> currents to the basal sinus automaticity (Irisawa et al., 1993), 2 mM CsCl indeed reduced the sinus rate by ~20% (Fig. 4) without causing a significant change in QRS or QTc intervals (Table 1). Thus, the I<sub>f</sub> current seems to contribute to the basal cardiac pacemaking probably by counteracting the hyperpolarizing influence of the atrial myocytes connected to SA node cells (DiFrancesco, 1993; Irisawa et al., 1993). Furthermore, the sinus rate was similar in the presence of 2 mM CsCl alone and in the presence of 1 μM CCh plus 300 nM tertiapin (Fig. 4A), and there was no tertiapin-insensitive component in the presence of 2 mM CsCl (Fig. 4B). Therefore, the tertiapin-insensitive component seems to result from suppression of I<sub>f</sub> currents.

It was recently demonstrated that GIRK4 knock-out mice deficient of K<sub>ACh</sub> channels exhibited a normal mean resting heart rate with impaired baroreflex (Wickman et al., 1998). In view of the present study, the mean resting heart rate may be regulated by relatively low concentrations of ACh through the suppression of I<sub>f</sub> currents (Fig. 3A). The study also indicates that the vagal activity under baroreflex provides sufficiently high concentrations of ACh to activate K<sub>ACh</sub> channels in the SA node cells (Fig. 3A). In the baroreflex, the efferent cardiac vagal activity occurs more or less fixed to the cardiac cycle (Jewett, 1964; Katona et al., 1970), and the effectiveness of vagal activity on the sinus automaticity is strongly dependent on the relationship between the pacemaker cycle and the duration, amplitude, and timing of the hyperpolarizing effect of vagal impulses (Jalife and Moe, 1979). K<sub>ACh</sub> channels possess a sufficiently fast response time to ACh to mediate such a phasic effect of vagal activity (Breitwieser and Szabo, 1988; Inomata et al., 1989), at least in part due to the direct coupling of the channels with G proteins and the regulator of G protein signaling proteins (Doupnik et al., 1997; Yamada et al., 1998). The positive cooperativity found in activation of K<sub>ACh</sub> channels (Fig. 4B) will also help the channels to promptly open when ACh concentration rises and suddenly close when it decreases even slightly. It is, therefore, plausible that K<sub>ACh</sub> channels play an essential role in the baroreflex.

To summarize, I quantitatively assessed the contribution of K<sub>ACh</sub> channels to the negative chronotropic effect of CCh in rabbit hearts by using tertiapin. I found that K<sub>ACh</sub> currents mediate the negative chronotropic effect of ~300 nM CCh and account for up to ~75% of the maximum effect of CCh.
However, suppression of I_h currents might play a major role for the effect of 1 to 100 nM CCh and account for ~25% of the maximum effect of CCh. The K_ACh current-dependent mechanism may play an important role in the heat-to-heat regulation of the sinus rate under baroreflex, whereas suppression of I_h currents may participate in regulation of the mean resting heart rate. Tertiapin is a useful pharmacological tool to identify the physiological and pathophysiological roles of K_ACh channels in parasympathetic regulation of the heart rate of various animals under ex vivo and in vivo conditions.

References


Gaskell WH (1887) On the action of muscarin upon the heart and on the electrical changes in the non-beating cardiac muscle brought about by stimulation of the inhibitory and augmentor nerves. *J Physiol (Lond)* 9:404–415.


Harris ED and Hutter OF (1956) The action of acetylcholine on the movements of potassium ions in the sinus venous of the heart. *J Physiol (Lond)* 133:587–599.


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