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RESEARCH ARTICLE

A new potassium ion current induced by stimulation of M_2 cholinoreceptors in fish atrial myocytes

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ABSTRACT

A novel potassium ion current induced by muscarinic stimulation (I_{KACh2}) is characterized in atrial cardiomyocytes of teleost fishes (crucian carp, *Carassius carassius*; rainbow trout, *Oncorhynchus mykiss*) by means of the whole-cell patch-clamp technique. The current is elicited in atrial, but not ventricular, cells by application of carbamylcholine (CCh) in moderate to high concentrations (10^{-7}–10^{-4} mol l^{-1}). It can be distinguished from the classic I_{KACh}, activated by the βγ-subunit of the G_i-protein, because of its low sensitivity to Ba^{2+} ions and distinct current–voltage relationship with a very small inward current component. Ni^{2+} ions (5 mmol l^{-1}) and KB-R7943 (10^{-5} mol l^{-1}), non-selective blockers of the sodium–calcium exchange current (I_{NCX}), strongly reduced and completely abolished, respectively, the I_{KACh2}. Therefore, I_{KACh2} was initially regarded as a CCh-induced outward component of the I_{NCX}. However, the current is not affected by either exclusion of intracellular Na^+ or extracellular Ca^{2+}, but is completely abolished by intracellular perfusion with K^+-free solution. Atropine (10^{-6} mol l^{-1}), a non-selective muscarinic blocker, completely eliminated the I_{KACh2}. A selective antagonist of M_2 cholinoreceptors, AF-DX 116 (2×10^{-7} mol l^{-1}) and an M_3 antagonist, 4-DAMP (10^{-9} mol l^{-1}), decreased I_{KACh2} by 84.4% and 16.6%, respectively. Pertussis toxin, which irreversibly inhibits G_i-protein coupled to M_2 receptors, reduced the current by 95%, when applied into the pipette solution. It is concluded that I_{KACh2}, induced by stimulation of M_2 cholinoceptors and subsequent G_i-protein activation, represents a new molecular target for the cardiac parasympathetic innervation.

KEY WORDS: Acetylcholine, Muscarinic receptors, Fish heart, Atrial myocytes, Ionic currents, I_{KACh}.

INTRODUCTION

Cardiac contractility is adjusted to circulatory demands on a beat-to-beat basis by the interaction of sympathetic and parasympathetic nervous systems via β-adrenergic receptors and muscarinic (M_2) cholinergic receptors, respectively (Hartzell, 1988). Effects of sympathetic activation are rapidly reversed by parasympathetic stimulation, despite the continued sympathetic tone via the 'accentuated antagonism' of the autonomic nerves (Levy, 1971). It is widely recognized that the main parasympathetic neurotransmitter, acetylcholine (ACh), induces negative chronotropic and inotropic effects mainly via the activation of the second subtype (M_2) of muscarinic cholinoreceptors, which prevails in the mammalian myocardium (Dhein et al., 2001), even though the involvement of M_3 cholinoreceptors in mediation of ACh effects has been recently confirmed (Abramochkin et al., 2012; Wang et al., 2007).

The crucial cardiotropic effects of M_2 cholinoreceptors are mediated by activation of the ACh-gated inwardly rectifying K^+ current (I_{KACh}) via G_i proteins. Binding of ACh to M_2 receptors leads to dissociation of G_i proteins and subsequent direct activation of K_{ACh} channels by their βγ-subunits (Hibino et al., 2010). I_{KACh} belongs to the family of cardiac inward rectifiers (I_{Kir}), which includes two other K^+ current systems: the background inward rectifier current (I_{K1}) and the ATP-sensitive current. Consistent with its physiological function in maintaining a stable negative resting membrane potential, the I_{K1} is significant in ventricular myocytes, less prominent in atrial cardiomyocytes and practically absent in the pacemaker cells of the sinoatrial node. Opposite to the I_{K1}, the density of I_{KACh} is much higher in supraventricular cardiac tissues than in ventricular myocardium, which appears in electric activity as high sensitivity of atrial and pacemaker action potentials (APs) to muscarinic modulation. Induction of the I_{KACh} by stimulation of M_2 receptors leads to hyperpolarisation, which is particularly distinct in the pacemaker cells, and AP shortening, which is more pronounced in the atrial myocardium than in the pacemakers (Boyett et al., 1995).

In the present study, we demonstrate the presence of another K^+ current associated with muscarinic stimulation in atrial cardiomyocytes of fish. It can be clearly distinguished from the conventional I_{KACh} by its low sensitivity to Ba^{2+}. The outward component of the novel current (I_{KACh2}) has a current–voltage dependence very similar to that of the I_{KACh}, but in contrast to the I_{KACh} the inward component of I_{KACh2} is very small. Activation of this novel carbamylcholine chloride (CCh)-sensitive current seems to be mediated by a pertussis toxin (PTX)-dependent pathway involving M_2 receptors.

RESULTS

Current induced by CCh in fish atrial myocytes and its ionic nature

When studying the Na–Ca exchange current (I_{NCX}) in fish cardiac myocytes, we noticed that the recorded current was strongly and quickly increased by CCh, a muscarinic agonist. Conditions for I_{NCX} recording require Cs^+-based pipette and external solutions (for composition, see Materials and methods) and various ion channel blockers (tetrodotoxin, nifedipine, E-4032 and $BaCl_2$ for sodium, calcium, delayed rectifier and inward rectifier currents, respectively) applied into the external saline solution. Because of the experimental setting for I_{NCX} recording, it was initially thought that activation of the current represented parasympathetic modulation of the I_{NCX}. Indeed, both atrial and ventricular myocytes of crucian carp and rainbow trout hearts demonstrate a definite background I_{NCX} before CCh addition (Fig. 1). During the experimental protocol, both inward and outward components of the I_{NCX} always increased during the first 2–3 min after gaining access to the whole-cell

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List of symbols and abbreviations

ACh	acetylcholine
AP	action potential
CCh	carbamylcholine chloride
I_{K1}	background inward rectifier K^+ current
I_{KACh}	acetylcholine-activated inward rectifier current
I_{NCX}	NCX current
M-receptor	muscarinic receptor
NCX	sodium–calcium exchanger
PTX	pertussis toxin
SK_{Ca}	small conductance Ca^{2+}-activated K^+ channel

recording, and then remained relatively stable for up to 30 min or more. This background current could be completely blocked by an organic blocker KB-R7943 (10^{-5} mol l^{-1}) or suppressed by 60–80% with 5 mmol l^{-1} Ni^{2+} (data not shown).

CCh at concentrations of 10^{-6}–10^{-4} mol l^{-1} induced a marked increase in the recorded current in atrial myocytes of both crucian carp and rainbow trout (Fig. 1). We define the CCh-induced current as the difference between the current in the presence of CCh and the current recorded just before CCh application (see current–voltage curves in Fig. 1C,D). In contrast to the basal KB-R7943-sensitive current, which had both inward and outward current components, the CCh-induced current was exclusively in the outward direction. CCh-induced current had the same shape in atrial myocytes of both fish species (Fig. 1C,D). However, the density of the current produced by 10^{-4} mol l^{-1} CCh was ~42% larger in crucian carp (10.48±1.89 pA pF^{-1}) than in rainbow trout (7.39±1.62 pA pF^{-1}) myocytes ($P<0.05$, Mann–Whitney test). In ventricular myocytes of both species, CCh up to the concentration of 10^{-4} mol l^{-1} failed to produce any distinguishable effects. This is not surprising in light of the well-known insensitivity of fish ventricular myocardium to muscarinic agonists (Vornanen and Tuomennoro, 1999).

KB-R7943 (10^{-5} mol l^{-1}), a non-selective blocker of NCX, abolished both the CCh-sensitive and the background current (I_{NCX}), leaving only a tiny leakage current (Fig. 1A). A concentration of 5 mmol l^{-1} Ni^{2+}, a non-specific inorganic blocker of the NCX, strongly reduced or abolished (70–100%) the overall CCh-sensitive and background current (Fig. 1B). However, Ni^{2+} turned out to be toxic to fish cardiac myocytes (most of the studied cells died 20–30 s after the start of Ni^{2+} application), which prevented its routine use as an NCX blocker. The noticed sensitivity of the CCh-induced current to standard blockers of I_{NCX} suggested that the recorded current represented parasympathetic stimulation of the outward I_{NCX}. Ion substitution experiments involving exclusion of Na^+ from the pipette solution or omission of Ca^{2+} from the extracellular saline solution did not, however, support this assumption. In the first series of experiments, 5 mmol l^{-1} Na_2ATP in the pipette solution was substituted by 5 mmol l^{-1} MgATP. The CCh-induced current was still present and not less than in the controls (Fig. 2A,B). In the second series of experiments, we used a Ca^{2+}-free external solution supplemented with 5 mmol l^{-1} EGTA to ensure practical absence of intracellular free Ca^{2+}. The application of this solution led to an expected decrease in the outward component of the background I_{NCX}, but subsequent addition of 10^{-5} mol l^{-1} CCh produced a large outward current (Fig. 2A,C). Substitution of Na^+ with Li^+ in the composition of the Ca^{2+}-free Tyrode, a well-known way to suppress I_{NCX} (Sanders et al., 2006), also failed to affect the CCh-induced current, although the basal I_{NCX} was reduced in a manner similar to the experiments with normal Ca^{2+}-free Tyrode (Fig. 2A,D). Therefore, the CCh-induced current is evidently not a component of the I_{NCX}.

In all experiments described above we used Cs^+-based pipette solution 1 (for composition, see Materials and methods). However, this solution contained 20 mmol l^{-1} BAPTA K^+ salt for intracellular Ca^{2+} buffering. Therefore, in the next series of experiments we used a modified pipette solution 1, where K_4BAPTA was substituted with

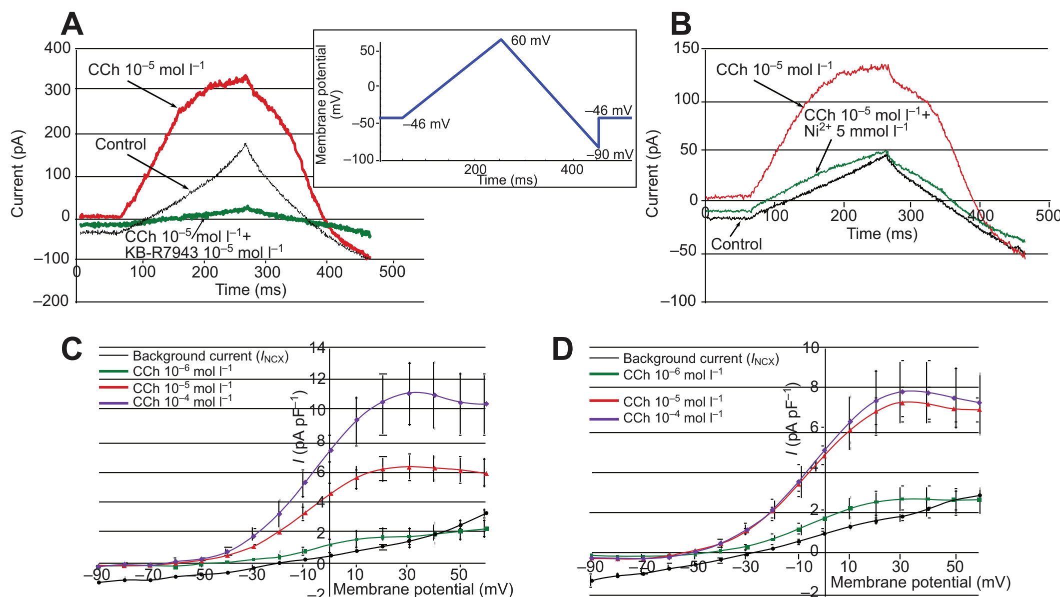

Fig. 1. Basal current and carbamylcholine (CCh)-induced current in fish atrial myocytes. (A,B) Original current–voltage recordings from two representative experiments. Black line shows the background sodium–calcium exchanger current (I_{NCX} or Control) after 5 min access to the whole-cell configuration in the absence of CCh; red and green lines indicate the current in the presence of 10^{-5} mol l^{-1} CCh and 10^{-5} mol l^{-1} CCh + 10^{-5} mol l^{-1} KB-R7943 (A) or 10^{-5} mol l^{-1} CCh + 5 mmol l^{-1} Ni^{2+} (B), respectively. The inset shows the voltage ramp used to elicit the current. Experiments were conducted using the external Cs^+-based Tyrode solution and pipette solution 1 (for composition, see Materials and methods). (C,D) Mean current–voltage curves of the background sodium–calcium exchange current (I_{NCX}) (C, n=25; D, n=10) and CCh-induced current recorded in the presence of 10^{-6} mol l^{-1} (C, n=25; D, n=14), 10^{-5} mol l^{-1} (C, n=40; D, n=16) and 10^{-4} mol l^{-1} (C, n=9; D, n=16) CCh in atrial cardiomyocytes from crucian carp (C) and rainbow trout (D). Current–voltage curves of the CCh-induced current were obtained after subtraction of the basal I_{NCX} current. Cells were obtained from five crucian carp and four rainbow trout.

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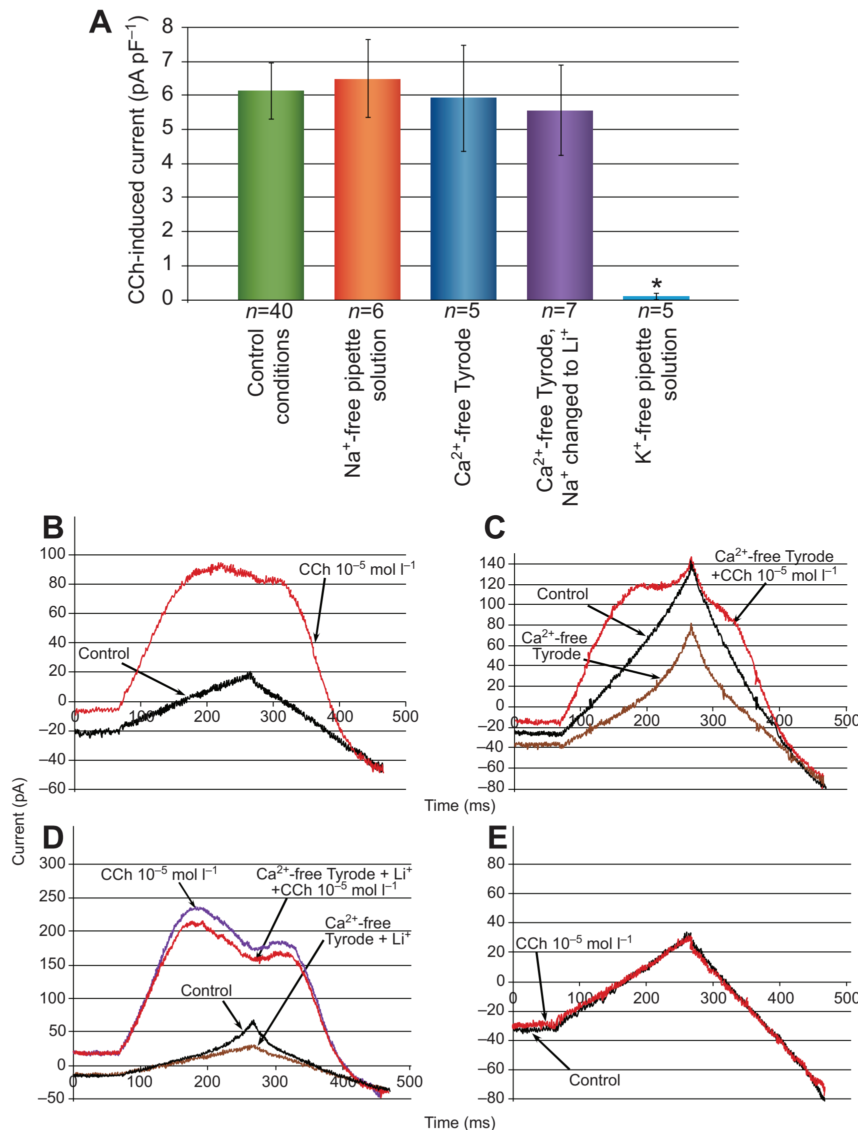

Fig. 2. Dependence of the CCh-induced current on intracellular Na^+ and K^+ and extracellular Ca^{2+} in crucian carp atrial myocytes. (A) Comparison of the CCh-induced current density at +20 mV in different experimental conditions. The results are means ± s.e.m. of five to 40 myocytes as indicated above the bars. An asterisk indicates a statistically significant difference (P<0.05, Mann–Whitney test) between the K^+-free conditions and all other experimental conditions. (B–E) Original traces of the basal I_{NCX} and the total current after CCh application recorded in different experimental conditions. (B) Na^+-free pipette solution 1 was used (Na_2ATP was substituted with MgATP). (C,D) Normal pipette solution 1 was used, but modified external Cs^+-based Tyrode solutions were applied during the recordings. (E) Pipette solution 1 with K_4BAPTA replaced with EGTA. In all experiments, the same voltage ramp pulse as shown in the inset in Fig. 1 was applied to elicit the current.

EGTA (free acid). Under these conditions, 10^{-5} mol l^{-1} CCh failed to induce any outward or inward current (Fig. 2A,E), clearly indicating that the studied current was carried by K^+ ions. Thus, although the CCh-induced current can be elicited using an NCX protocol (voltage ramp) and is blocked by Ni^{2+} and KB-R7943, it is independent of the NCX and is carried by K^+.

Effect of CCh is mainly mediated via M_2 muscarinic receptors

Using pharmacological blockers, we tried to find out which subtype(s) of cholinoreceptors might mediate the activation of the CCh-induced current. In atrial myocytes from both species, atropine (10^{-6} mol l^{-1}), which blocks muscarinic cholinoreceptors without subtype discrimination, completely abolished the outward current induced by 10^{-5} mol l^{-1} CCh (Fig. 3A,B). Atropine blocked only the CCh-induced current, without effect on the basal I_{NCX}. These findings strongly suggest that the induction of the outward current by CCh is mediated by muscarinic cholinoreceptors.

The major role of M_2 muscarinic receptors in the mediation of cardiac cholinergic response is widely recognized. However, the presence of physiologically relevant M_3 cholinoreceptors was confirmed during the last decade for mammalian myocardium (Hellgren et al., 2000; Kitazawa et al., 2009), and therefore cannot be neglected as a possible signaling pathway in fish cardiac myocytes. To this end, we have investigated effects of the selective M_3 blocker 4-DAMP (10^{-9} mol l^{-1}) and selective M_2 antagonist AF-DX 116 ($2{\times}10^{-7}$ mol l^{-1}) on the CCh (10^{-5} mol l^{-1})-induced current. The selected agonist concentrations have been shown in a previous patch-clamp study (Wang et al., 1999) to exert subtype-specific effects on muscarinic signaling, while higher concentrations may lead to non-selective binding of the blockers.

Application of AF-DX 116 suppressed the CCh-induced current by 84.3% (Fig. 3A,C). In contrast to the marked blocking effect of AF-DX 116, 4-DAMP produced only a slight reduction of the current by 16.6%, although this decrease was significant (P<0.05; Fig. 3A,D). These results suggest a predominant role of M_2 receptors in the mediation of the CCh effect. To confirm this assumption, we conducted a series of experiments with PTX.

Inhibition of the CCh-induced current by PTX

PTX is widely used as a selective inhibitor of G_i-protein-mediated signaling pathways. It irreversibly blocks the activity of α_i-subunit by ADP-ribosylation, which prevents the α_i-subunit from interacting with the receptor molecule. The routine way of PTX application is

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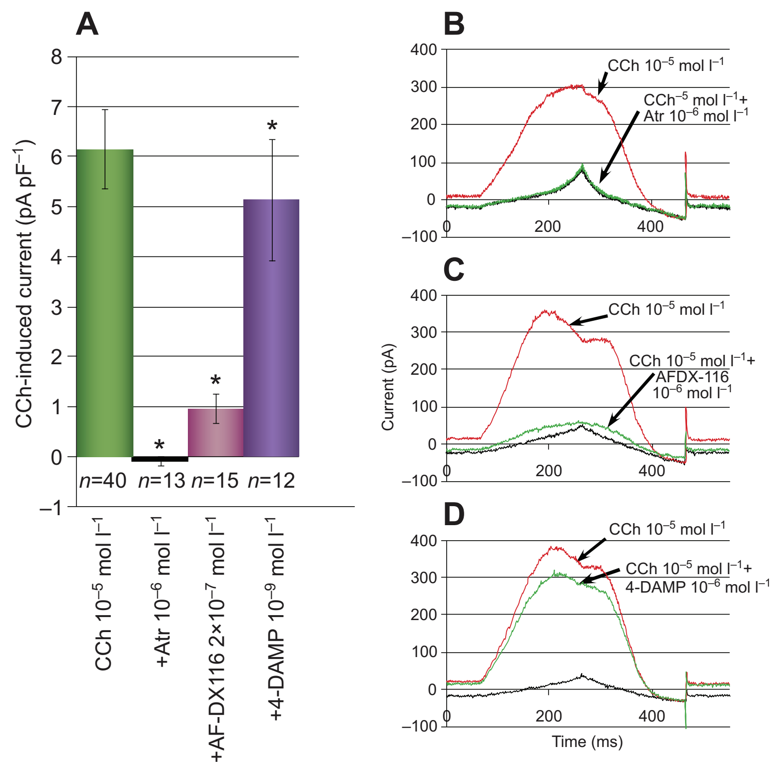

Fig. 3. Effect of cholinoreceptor antagonists on the CCh-induced current in crucian carp atrial myocytes. The current was first elicited with 10^{-5} mol l^{-1} CCh and then different blockers were applied in the continuous presence of CCh. (A) Mean (±s.e.m.) effects of atropine, AF-DX116 and 4-DAMP at +20 mV. An asterisk indicates a statistically significant difference (P<0.05, Mann–Whitney test) from the control (CCh alone). Original traces from representative experiments are shown in B–D. The same voltage ramp pulse as shown at the inset in Fig. 1 was used to elicit the current. Experiments were conducted using the external Cs$^+$-based Tyrode solution and pipette solution 1.

incubation of isolated myocytes in a medium containing the toxin. Several pilot experiments using the protocols of PTX application to mammalian and frog cardiac myocytes (2–4 h of incubation at room temperature in a medium containing up to 6 µg ml^{-1} PTX) failed to induce any decrease of the CCh-induced current in fish atrial myocytes. This negative result could be due to the absence of a membrane receptor for PTX or any other downstream step responsible for endocytosis of the toxin and its intracellular activation in fish cardiomyocytes. Therefore, we decided to add PTX directly to pipette solution 1 (1 µg ml^{-1}) before patching and wait for diffusion of PTX inside the cell. A similar method of PTX administration was previously successfully used in outer cells of guinea pig cochlea (Kakehata et al., 1993), although to our knowledge we have applied it in cardiomyocytes for the first time. To examine PTX action, we recorded the effect of 10^{-5} mol l^{-1} CCh on membrane current every 10 min during the 30 min internal perfusion of the cell with pipette solution containing PTX. Similar long-term control recordings were performed using a normal PTX-free pipette solution.

After the first minute of recording with pipette solution containing PTX, the cells demonstrated a normal strong increase of the outward current (6.03±1.95 pA pF^{-1}) in response to 10^{-5} mol l^{-1} CCh (Fig. 4). However, later on, the CCh-induced current started to gradually fade, so that after 30 min of internal perfusion it was only 0.3±0.33 pA pF^{-1} (P<0.05), i.e. 5% of the initial value. In control experiments without PTX in the pipette solution, the amplitude of CCh-induced current was not significantly changed during the 30 min internal perfusion (5.54±0.81 versus 6.2±1.83 pA pF^{-1}, respectively; P>0.05). Thus, PTX applied to the pipette solution effectively inhibits the response to CCh. Taken together with the data obtained using subtype-selective muscarinic blockers, these findings strongly suggest a crucial role of M_2 cholinoreceptors in mediation of the outward current induction by CCh.

Thus, the CCh-induced current is carried by K$^+$ ions and can be activated by stimulation of the cardiac muscarinic receptors. Because of the similarity of the CCh-induced current to the I_{KACh}, we decided to refer to it as I_{KACh2} and tested its possible identity with the classic I_{KACh}.

Distinguishing I_{KACh2} from other K$^+$ currents

We attempted to determine whether I_{KACh2} is a novel and separate current entity or just a CCh-sensitive component of the I_{KACh} or other known cardiac K$^+$ currents. The presence of Cs$^+$ in both external and intracellular solution and the addition of E-4031 to Cs$^+$-based Tyrode allowed to exclude the involvement of the delayed rectifier K$^+$ currents, which also have almost linear current–voltage dependence and are not coupled to muscarinic receptors.

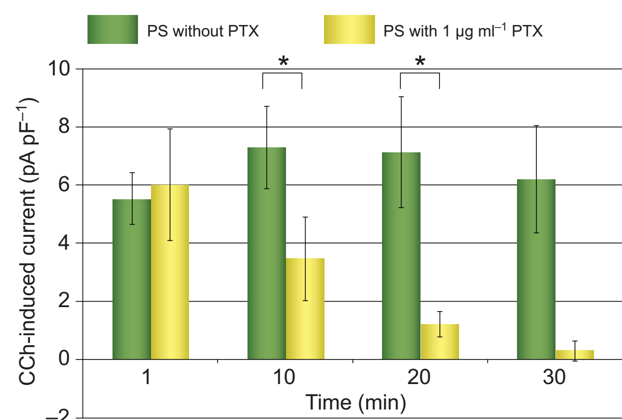

Fig. 4. Effect of intracellular perfusion with PTX on the density of CCh-induced current in atrial myocytes of the crucian carp. PTX was included in pipette solution (PS) 1 and the myocytes were perfused for 30 min with continuous recording of the current. An asterisk indicates a statistically significant difference (P<0.05, Mann–Whitney test) between two columns. The same voltage ramp pulse as shown at the inset in Fig. 1 was used to elicit the current. Experiments were conducted using the external Cs$^+$-based Tyrode solution. The number of experiments (n) was 8 and 6 cells for control and PTX experiments, respectively.

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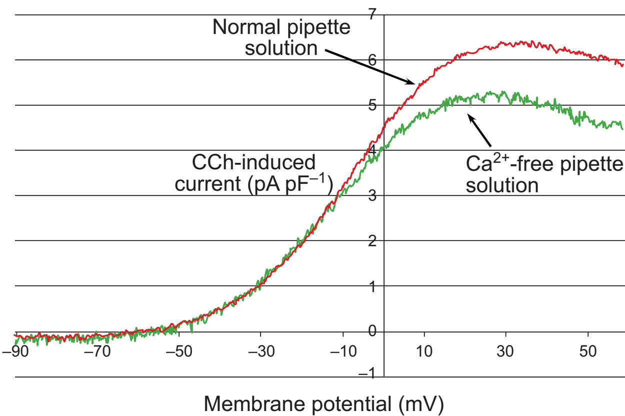

Fig. 5. Representative current–voltage curves of I_{KACh2} recorded in crucian carp atrial myocytes with the use of the standard pipette solution 1 and the same solution without $CaCl_2$. The intracellular calcium concentration was 105 nmol l^{-1} in control conditions and less than 1 nmol l^{-1} when using Ca^{2+}-free pipette solution. The same voltage ramp pulse as shown at the inset in Fig. 1 was used to elicit the current. Experiments were conducted using the external Cs^+-based Tyrode solution.

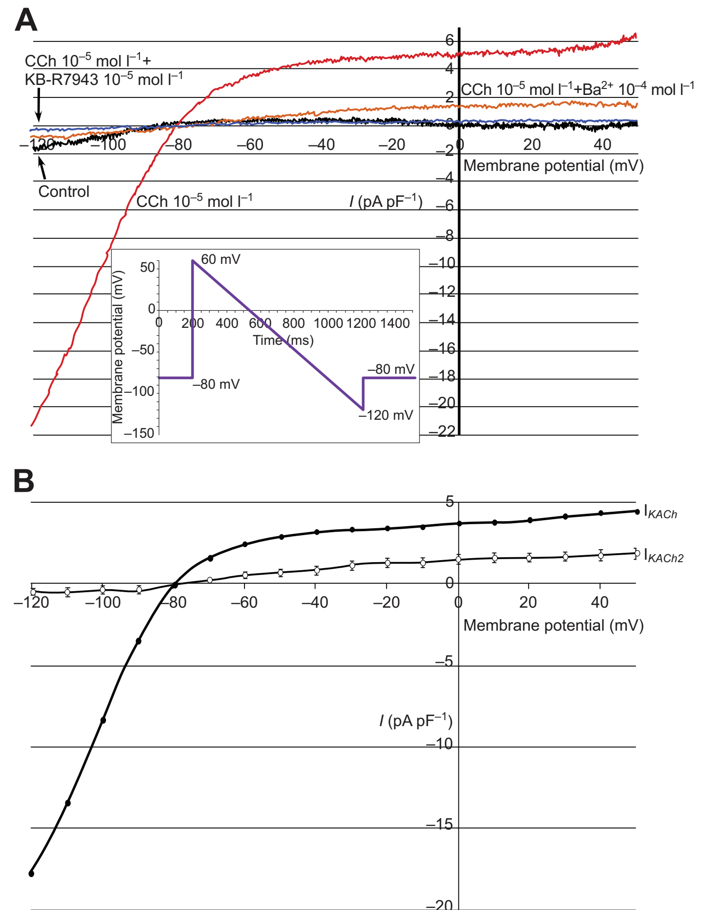

Fig. 6. Comparison of I_{KACh2} with the inward rectifiers in crucian carp atrial myocytes. (A) Original current–voltage curves of basal I_{Kir} (I_{K1}), total current after CCh application (I_{K1} + I_{KACh} + I_{KACh2}), the current recorded in the presence of CCh and Ba^{2+} (I_{KACh2}) and the leakage current obtained after addition of KB-R7943. The inset shows the voltage ramp pulse, which was used to elicit the current. Experiments were conducted using the external K^+-based Tyrode solution and pipette solution 2 (for composition, see Materials and methods). (B) Averaged current–voltage curves (n=11, 3 fish) of I_{KACh} and I_{KACh2}. The first curve was obtained by subtraction of basal I_{K1} + I_{KACh2} from the total current recorded under CCh.

In our experiments, we did not specifically block the putative Ca^{2+}-dependent K^+ current, which is carried by the small conductance Ca^{2+}-activated K^+ channels (SK_{Ca}) in mammalian atrial and ventricular myocytes (Xu et al., 2003). However, in murine and human cells, this current has a prominent inward component at the potentials below potassium equilibrium potential, while the outward current is substantial only at potentials more positive than −40 mV. Although Ca^{2+}-dependent K^+ current has never been registered in fish cardiomyocytes, in mammalian cells the current density strongly depends on intracellular Ca^{2+} content and is negligible at 10^{-8} mol l^{-1} intracellular Ca^{2+} (Xu et al., 2003). Experiments with Ca^{2+}-free pipette solution (BAPTA substituted with EGTA, no $CaCl_2$, free $[Ca^{2+}]_{in}$ <10^{-9} mol l^{-1}) were conducted in crucian carp atrial cells to prevent SK_{Ca} from contributing to the CCh-induced current. The I_{KACh2} at +20 mV was significantly less than in the control conditions (5.22±1.11 versus 6.16±0.83 pA pF^{-1}, respectively; P>0.05; Fig. 5), but still far from inhibited. Obviously, I_{KACh2} is different from SK_{Ca}, which would be completely blocked in the practical absence of intracellular free Ca^{2+}.

Finally, there was a possibility of incomplete inhibition of inward rectifiers (I_{Kir}) by 10^{-4} mol l^{-1} $BaCl_2$. Then I_{KACh2} could be just a part of the classic I_{KACh}. To test this assumption, we recorded I_{Kir} in the K^+-based Tyrode solution without $BaCl_2$. The density of the basal I_{K1} was very low, like it usually is in fish atrial cardiomyocytes (Fig. 6A). Application of 10^{-5} mol l^{-1} CCh produced a large increase in the inwardly rectifying current and, especially, the outward I_{Kir}. The density of recorded current was 4.87±0.42 pA pF^{-1} at +20 mV and −19.23±0.51 pA pF^{-1} at −120 mV. Subsequent addition of 10^{-4} mol l^{-1} $BaCl_2$ blocked the inward current by 97% (−0.58±0.06 pA pF^{-1} at −120 mV), while the outward current persisted (1.45±0.69 pA pF^{-1} at +20 mV). Increase in Ba^{2+} concentration up to 3×10^{-4} mol l^{-1} did not lead to further decrease of the outward current (1.45±0.69 pA pF^{-1} at +20 mV). On the contrary, addition of 10^{-5} mol l^{-1} KB-R7943 practically abolished the outward current (0.27±0.07 pA pF^{-1} at +20 mV). It seems that 10^{-4} mol l^{-1} Ba^{2+} completely blocks the inward rectifiers (both I_{K1} and I_{KACh}), while I_{KACh2} resists Ba^{2+}, but not KB-R7943.

The current–voltage curves for I_{KACh} and I_{KACh2} were obtained from these data (Fig. 6B). It is clear that the former current is a typical inward rectifier, very large at potentials below the E_K, while the latter has a tiny inward but a significant outward component. Thus, two currents not only differ in sensitivity to $BaCl_2$, but demonstrate principally different current–voltage relationships.

DISCUSSION

Muscarinic receptor stimulation plays a central role in the parasympathetic control of cardiac function by modulating heart rate (chronotropic effect), contractility (inotropic effect) and conduction velocity (dromotropic effect) (Brodde and Michel, 1999). These effects are particularly strong in pacemaker and atrial tissues. The M_2 muscarinic receptors prevail in the mammalian myocardium, although some M_3 receptors are also present and physiologically active (Pönicke et al., 2003; Wang et al., 2007). The even-numbered muscarinic receptors, including M_2, are coupled to G_i proteins and act via inhibition of adenylate cyclase and a subsequent decrease in cellular cAMP content (Dhein et al., 2001). However, M_2 and M_3 receptors can activate different effectors without the second messenger systems. The channels responsible for the inward rectifier I_{KACh} are directly stimulated by βγ-subunits of the G_i protein, while joint

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activity of βγ- and α_q-subunits is believed to be involved in the stimulation of the delayed rectifier type K^+ current, I_{KM3} (Wang et al., 1999). The molecular basis of the I_{KM3} current is still unresolved, but it was recently proposed to be carried by the same channels as the I_{KACh} (Navarro-Polanco et al., 2013). The delayed rectifier-like properties of the I_{KM3} could be explained by the increasing affinity of muscarinic receptors to their agonists at positive membrane potentials.

In the present study, we describe a novel K^+ current, induced by muscarinic stimulation, with a distinct current–voltage relationship and pharmacological properties from the known ACh-activated currents, I_{KACh} and I_{KM3}. Clear dependence of the current on intracellular and extracellular K^+ concentration and reversal of the current close to the Nernst potential of K^+ ions indicate that the current is carried by K^+ ions. Furthermore, the current is independent from intracellular Na^+ and external Ca^{2+}. Unlike the cardiac inward rectifiers, the new current cannot be blocked by 10^{-4} mol l^{-1} Ba^{2+}, which, unlike mammalian cells, is sufficient to abolish both I_{K1} and I_{KACh} in crucian carp myocytes (Hassinen et al., 2008). Different from I_{KACh} and I_{K1}, it is mainly an outward current with only a tiny inward component. Because of its inwardly rectifying properties at positive voltages and its activation by the intracellular signaling pathway of the M_2 receptors (similar to the I_{KACh}), we refer to it as I_{KACh2}.

Experiments using the subtype-selective muscarinic antagonists and PTX strongly suggest a dominant role of M_2 cholinoreceptors in CCh activation of I_{KACh2}, a property shared with the I_{KACh}. AF-DX 116, which at the concentration of 2×10^{-7} mol l^{-1} is considered to be relatively specific against M_2 cholinoreceptors (Doods et al., 1987; Giachetti et al., 1986), almost completely abolished the I_{KACh2}. In contrast, an M_3 cholinoreceptor blocker 4-DAMP (Doods et al., 1987), had only a weak inhibitory effect on the CCh-induced current. M_2 cholinoreceptors are coupled to the G_i proteins and convey their physiological effects via the α_i-subunit by decreasing cellular cAMP content or via direct coupling of the βγ-subunit to their target (channels of inward rectifier I_{KACh}) (Brodde and Michel, 1999). Our experiments on CCh were conducted in the absence of β-adrenergic stimulation, i.e. without activation of the cAMP-dependent pathway. Therefore, it could be argued that the α_i-subunit of the G protein and cAMP might not be involved in I_{KACh2} induction. It is, however, possible that there is a basal activation of adenylate cyclase in the absence of β-adrenergic stimulation, which is antagonized by a CCh-dependent mechanism. Then the increase in cAMP intracellular content caused by noradrenaline or other adenylate cyclase-stimulating compounds should suppress the I_{KACh2}. So, further research should shed light on the mechanism of I_{KACh2} activation.

The present study did not try to clarify the molecular basis of the I_{KACh2}. However, the M_2-dependent activation pathway and the inwardly rectifying properties of the current strongly suggest that it is closely related to the I_{KACh}, which in the mammalian heart is carried by Kir3.1 and Kir3.4 channels (Hibino et al., 2010). The molecular basis of ACh-activated inward rectifiers of the fish heart is not yet known, but because of the whole genome duplication in the teleost fishes (Jaillon et al., 2004), the diversity of this current system may be greater in fishes than mammals. For example, the rapid component of the cardiac delayed rectifier current is represented by at least two different ERG channels in the zebrafish heart (Langheinrich et al., 2003; Milan et al., 2003). Molecular cloning of the fish Kir3 channels and their expression in heterologous system is needed to resolve this issue.

Considering the biophysical similarities of I_{KAch2} and I_{KAch}, the physiological role of the I_{KAch2} is assumed to be comparable to that of the I_{KAch}, i.e. shortening of atrial action potential duration with consequent reduction of atrial contraction. However, activation of the I_{KACh2} requires relatively high (micromolar) agonist concentrations and therefore it is likely to appear only at a strong parasympathetic tone. In contrast to mammals, in fish and amphibians, strong cholinergic stimulation not only reduces AP duration, but gradually decreases AP amplitude in atrial myocardium until the full cessation of electrical activity (Abramochkin et al., 2010). This effect is putatively attributed to induction of a strong outward K^+ current, which overwhelms all inward currents and makes the depolarization impossible. The described I_{KACh2} may be involved in mediation of this cholinergic effect. Further research, including distribution of Kir3 channels in supraventricular tissues of the fish heart, could reveal possible physiological implications of this novel K^+ current.

Conclusions

In fish atrial cardiomyocytes, stimulation of the M_2-mediated second messenger pathway leads to induction of a novel type of K^+ current, the I_{KACh2}. Different from the I_{KACh}, this current has a very small inward component and is relatively insensitive to Ba^{2+} blocking. Together with I_{KACh}, I_{KACh2} may provide shortening of APs in fish atrial myocardium and particularly under a strong cholinergic influence. The similarity of results obtained from crucian carp (family Cyprinidae) and rainbow trout (family Salmonidae) atrial myocytes suggests that the described CCh-dependent activation of I_{KACh2} might be a common mechanism for different fish groups, and raises the question of whether this mechanism is also relevant for other vertebrates.

MATERIALS AND METHODS

Animals

Experiments were conducted on atrial myocytes of two fish species, crucian carp (*Carassius carassius* L., *n*=31) and rainbow trout (*Oncorhynchus mykiss* W., *n*=7). Fish were held separately in 500 l stainless steel aquaria with continuous flow of aerated (11 mg O_2 l^{-1}) groundwater at constant temperature of either 18°C (crucian carp) or 14°C (rainbow trout) and under a 12 h:12 h light:dark photoperiod. During the laboratory maintenance (>4 weeks), the fish were fed aquarium fish food five times a week. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85–23, revised 1996) and the experimental protocols were approved by the Animal Experiment Board in Finland (permission no. STH252A).

Isolated myocyte preparation

Fish were stunned with a blow to the head, the spine was cut immediately behind the head and the heart was rapidly excised. Atrial myocytes were isolated by retrograde perfusion of the heart with proteolytic enzymes (collagenase type 1A, 0.75 mg ml^{-1}; trypsin type IX, 0.5 mg ml^{-1}; fatty-acid-free BSA, 0.75 mg ml^{-1}) as described previously in detail (Vornanen, 1997). Isolated cells were stored for up to 8 h at 5°C in low-Na^+ solution containing (in mmol l^{-1}): NaCl 100, KCl 10, $KH_2PO_4\cdot2H_2O$ 1.2, $MgSO_4\cdot7H_2O$ 4, taurine 50, glucose 10 and Hepes 10 at a pH of 6.9.

Measurement of sarcolemmal ionic currents using the whole-cell patch-clamp method

Atrial myocytes were placed in the experimental chamber (RCP-10T, Dagan, Maryland, MI, USA, volume 150 µl) and superfused with an external saline solution. In most of the experiments, we used Cs^+-based Tyrode solution containing (in mmol l^{-1}): NaCl 150, CsCl 5.4, NaH_2PO_4 0.4, $MgSO_4$ 1.5, $CaCl_2$ 1.8, glucose 10 and Hepes 10 at pH 7.6 (adjusted with CsOH). In other experiments, we used a K^+-based Tyrode solution of the same content with the exception of CsCl and CsOH substitution for KCl and KOH, respectively. Tetrodotoxin (5×10^{-7} mol l^{-1}), nifedipine (10^{-5} mol l^{-1}),

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E-4031 (10^{-6} mol l^{-1}) and $BaCl_2$ (10^{-4} mol l^{-1}) were added to external solution to block Na^+ channels, L-type Ca^{2+} channels, K^+ channels of the fast delayed rectifier K^+ current (I_{Kr}) and K^+ channels of the inward rectifier K^+ current (I_{K1}), respectively. In experiments with I_{K1} and I_{KACh}, $BaCl_2$ was not added in advance to the K^+-based Tyrode. A constant flow of external solution (1.5–2 ml min^{-1}) in the experimental chamber was maintained throughout the experiment. Temperature of the saline solutions was regulated at 18°C using a Peltier device (TC-100, Dagan). Pipette solution 1, which was used in the majority of experiments, contained (in mmol l^{-1}): CsCl 140, $MgCl_2$ 1, $CaCl_2$ 9, BAPTA 20, Na_2ATP 5, Na_2GTP 0.03 and Hepes 10, adjusted to pH 7.2 with CsOH at 20°C. Under these conditions, free intracellular Ca^{2+} concentration is buffered close to the diastolic level (105 nmol l^{-1}; calculated using MaxChelator). Pipette solution 2 was used for recording of the inward rectifiers (I_{Kir}) and contained (in mmol l^{-1}): KCl 140, $MgCl_2$ 1, EGTA 5, MgATP 4, Na_2GTP 0.03 and Hepes 10 with pH adjusted to 7.2 with KOH.

The whole-cell voltage clamp recording of ionic currents was performed using an Axopatch 1-D amplifier (Axon Instruments, Foster City, CA, USA) and the pClamp 8.2 software package. Resistance of patch electrodes was 2–4 MΩ when filled with the pipette solution. Pipette capacitance, whole-cell capacitance and access resistance were routinely compensated. In most experiments, the current was elicited at 15 s intervals from the holding potential of −46.5 mV (the calculated reversal potential of NCX) by 1 s voltage ramp pulses (see Fig. 1A, inset). The current was measured during the hyperpolarizing phase of the ramp. For recording of the I_{Kir}, the common hyperpolarizing ramp (from +60 to −120 mV) protocol with 10 s intervals was used. The holding potential was −80 mV.

Drugs

Tetrodotoxin, E-4031, KB-R7943, AF-DX 116 {11-([2-[(diethylamino)methyl]-l-piperdinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-on, a selective M_2 cholinoreceptor blocker}, 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide, a selective M_3 receptor antagonist) and PTX, which irreversibly inhibits G_i signaling protein, were all purchased from Tocris (Bristol, UK). Collagenase, trypsin, nifedipine (a blocker of L-type Ca^{2+} channels) and carbamylcholine chloride (a muscarinic agonist) were purchased from Sigma (St Louis, MO, USA).

Statistics

All data in the text and figures except the original recordings are presented as means ± s.e.m. for *n* experiments. Effects of CCh on sarcolemmal ionic current relative to the respective basal value of the current were compared using the Wilcoxon test. The density of CCh-induced current in normal conditions and in the presence of muscarinic blockers, PTX, KB-R7943 and other agents was compared using the Mann–Whitney test. $P<0.05$ was adopted as the level of statistical significance.

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Competing interests
The authors declare no competing financial interests.

Author contributions
M.V. and D.A. designed the research and experimental design. All authors performed experiments and participated in writing and editing of the paper.

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