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Interaction of acetylcholine with Kir6.1 channels heterologously expressed in human embryonic kidney cells

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Abstract

Kir6.1 subunit is one of the pore-forming components of K_{ATP} channel complex. The endogenous modulation of Kir6.1 subunit function has been largely unknown. Whether acetylcholine modulated the function of Kir6.1 subunit stably expressed in human embryonic kidney (HEK-293) cells was examined in the present study using the whole-cell patch-clamp technique. Acetylcholine from 1–100 μM concentration-dependently stimulated the heteologously expressed and PNU-37883A sensitive Kir6.1 channels ($p<0.05$). Co-expression of sulphonylurea receptor 1 subunit with Kir6.1 significantly inhibited the stimulatory effect of acetylcholine on K_{ATP} currents. Pretreatment of the transfected HEK-293 cells with atropine, α-bungarotoxin, mecamylamine, prazocine, propranolol, or dihydro-β-erythroidine hydrobromide did not alter the stimulatory effect of acetylcholine on Kir6.1 currents. When intracellular ATP was increased from 0.3 mM to 5 mM, acetylcholine at 10 μM still exhibited its stimulatory effect (-16.4 ± 2.3 to -25.5 ± 3.8 pA/pF, $n=8$, $p<0.05$). In conclusion, we have demonstrated an excitatory effect of acetylcholine on Kir6.1 channels, which is mediated neither by an acetylcholine receptor-dependent mechanism, nor by alteration in ATP metabolism.

Keywords: Acetylcholine; Neurotransmitter; K_{ATP} channel; Patch clamp; Smooth muscle cell; Stable transfection

1. Introduction

Acetylcholine is a major neurotransmitter found in parasympathetic nerve terminals and its effects on cardiac function are generally inhibitory via activation of myocardial muscarinic receptors. There are two important types of parasympathetic (acetylcholine) receptors. Nicotinic receptors are found mainly in skeletal muscle and ganglial cells. Muscarinic receptors have quite different structure from those of nicotinic receptors. Muscarinic receptors are encoded by a number of different genes; most important are the muscarinic M_1-receptor subtype, found mainly in autonomic ganglia and the central nervous system, M_2-subtype in the heart, and M_3-subtype in smooth muscle and secretory cells. All of these muscarinic receptors are blocked by atropine.

Stimulation of muscarinic receptors on the vascular endothelium has been shown to release nitric oxide, which produces vasodilatation and a resultant increase in blood flow. Vegh et al. (1992) has shown the involvement of the L-arginine/nitric oxide pathway in the antiarrhythmic effect of ischemic preconditioning in dogs. Acetylcholine mimics the effects of preconditioning to reduce infarct size in rabbits (Oldenburg et al., 2003). Balligand et al. (1995) have shown that an endogenous nitric oxide synthase pathway exists in isolated myocytes, which regulates the negative chronotropic effects of muscarinic receptor stimulation. Taken together, these results suggest a possible cardioprotective role of acetylcholine that stimulates cardiac muscarinic receptors and may involve nitric oxide release.

ATP-sensitive K^+ channels (K_{ATP} channels) play a crucial role in coupling cell's metabolic status to its membrane potential, thereby functioning as cellular metabolic sensors (Ashcroft and Ashcroft, 1990). K_{ATP} channels are regulated by intracellular ADP- and ATP-concentrations

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(Sun et al., 2004; Babenko et al., 1998) and plasma membrane phospholipid (Baukrowitz and Fakler, 2000). In addition, diazoxide activates the channels, and sulfonylureas like tolbutamide and glibenclamide inhibit channel activity (Kozlowski et al., 1989). Based on their central position in metabolic and signaling pathways, potassium channels are increasingly recognized as potential targets for pharmacological intervention (Sun et al., 2004). One of the best examples of this strategy is the successful therapy with sulfonylureas acting on K_{ATP} channels in pancreatic beta-cells, increasing their insulin output, and improving the impaired regulation of glucose metabolism in diabetic patients (Inagaki and Seino, 1998).

K_{ATP} channels are composed of a pore forming inwardly rectifying K^+ channel (Kir6.x) tetramer and a regulatory sulphonylurea receptor (SUR) tetramer that confers sulphonylurea sensitivity. Different combinations of Kir6.x and SUR yield the tissue-specific K_{ATP} channel subtypes with different electrophysiological and pharmacological features. Kir6.1 subunit has been recognized as the main pore-forming subunit of K_{ATP} channel complex in vascular smooth muscle cells (SMCs) (Fujita and Kurachi, 2000). Recently, we have identified and cloned Kir6.1 subunit from rat mesenteric artery SMCs and confirmed its presence in other vascular SMCs, including those from rat tail artery (Cao et al., 2002). The pore-forming Kir6.1 subunit shares 72% amino acid identity to Kir6.2. It has a minimal structure with only a "pore" region and two membrane-spanning segments. Kir6.1 is strongly expressed in heart, ovary, and adrenal gland and moderately expressed in vascular SMCs, skeletal muscle, lung, brain, stomach, colon, testis, and thyroid gland. Unlike Kir6.2, Kir6.1 is capable of independent expression in HEK-293 cell (Ammala et al., 1996), a fact that may be exploited to examine acetylcholine modulation of Kir6.1 subunit. Although the important roles of K_{ATP} channels in the regulation of cellular functions have been known, few pharmacological agents have been shown to act selectively on Kir6.1 subunit. There was also no electrophysiological or pharmacological study reporting the effects of acetylcholine on the activation of K_{ATP} channels. The first goal of the present study was to examine the effect of acetylcholine on cloned Kir6.1 subunit from rat mesenteric artery SMCs, which was heterologously expressed in HEK-293 cells. Our second goal was to investigate whether the effect of acetylcholine was mediated by the traditional pathways of muscarinic receptor stimulation.

2. Materials and methods

2.1. Stable transfection of HEK-293 cells with Kir6.1 gene or co-transfection with Kir6.1 and SUR1 genes

HEK-293 cells (American Type Culture Collection, Rockville, MD) were cultured in 35 mm Petri dishes as described before (Cao et al., 2002). Briefly, the pCR2.1-rvKir6.1 cDNA clone (GenBank # AB043637) was cleaved with appropriate restriction endonucleases to get the cloned rvKir6.1 subunit gene insert with proper restriction enzyme cleavage sites at both ends. It was then subcloned into a modified mammalian expression HA3 vector (King et al., 2000). The construct was linearized with Eam 1105I restriction endonuclease (MBI Fermentas). Linearized construct was mixed with a FuGENE™ 6 transfection reagent (Roche) in a ratio of 1 (μg):3 (μl) in 100 μl of fetal bovine serum-free Roswell Park Memorial Institute (RPMI)-1640 medium (Moore et al., 1999). After 45-min incubation at room temperature, the mixture was added to HEK-293 cell in 2 ml fetal bovine serum-free RPMI-1640 medium (cell density: 8×10^4/35 mm dish). Geneticine (G418) (GIBCO) selection was performed at concentration of 500 μg ml^{-1}. Mock transfection (vector only transfection) was also performed. Non-transfected HEK-293 cells were included as negative control for antibiotic selection. After 4 weeks of antibiotic selection culture, survived transfected cells were picked individually into 24-well culture plates for proliferation. When reaching >90% confluent in 90 mm culture dishes, the cells were harvested and stored for co-transfection with SUR1 subunit and for electrophysiological study. SUR1 (Genbank accession # AB052294) was inserted into a pcDNA3/Zeocin vector (Invitrogen). Co-expression of Kir6.1 with SUR1 subunits was performed using dual selection method (Cao et al., 2002). Briefly, pcDNA-SUR1 cDNA was heterologously expressed in Kir6.1 permanently expressing HEK-293 cells. HEK-293 cells expression only Kir6.1 subuint served as the negative control for the dual antibiotic selection, which used 500 mg/ml geneticin and 0.15 mg/ml Zeocin. The co-expression of SUR1 and Kir6.1 subunits was confirmed with Western blot analysis.

2.2. Electrophysiological recording of the whole-cell K^+ channel currents

The whole-cell patch-clamp technique was used to measure membrane currents. The Petri dish with the cultured cells was mounted on the stage of an inverted phase-contrast microscope. Pipettes with tip resistance of 2–4 MΩ were pulled from microhematocrit capillary tubes (Fisher, Nepan, ON). Membrane currents of HEK-293 cells were recorded using an Axopatch-200B patch clamp amplifier. The output signals were filtered at 1 kHz with an 8-pole filter, and stored on the hard disk of a computer for off-line analysis. Data acquisition and analysis were performed using pClamp software (version 7, Axon instruments). At the beginning of each experiment, junctional potential between pipette solution and bath solution was adjusted to zero (Hanna and Wang, 2002; Lu et al., 2002).

The pipette solution contained (mM): 132 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, and 5 Hepes. Na_2ATP 0.3 mM was included in the pipette solution immediately before the

experiments. The bath solution contained (mM): 40 KCl, 100 NaCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, and 5 Hepes. Holding potential was set at −20 mV. Together with the elevated KCl concentration in the bath solution, this holding potential will help amplify the inward currents carried by Kir6.1 subunit and largely inactivate the outward voltage-dependent K^+ channels. *I–V* curves were constructed using the sustained current amplitude at the end of 800 ms test pulses. If the current magnitude did not change from the third to the fifth minute after the rupture of the cell membrane, the rate of "run-down" of K_{ATP} current was usually negligible for 20 min or longer. Cells that had stable K_{ATP} currents from the third to fifth minute after penetration of the membrane were used. Cells that had a fast decline of K_{ATP} currents within this period were discarded. Experiments were conducted at room temperature (20–22 °C). A series of voltage steps from −150 to +80 mV were applied to determine the current–voltage relationship. Drugs were applied once the whole-cell currents had reached a stable level (i.e. >5 min after obtaining the whole-cell configuration). A stock solution of acetylcholine was prepared in distilled water. A home-made multi-reservoir perfusion system was used, including manually controlled switch valves, gravity-driven inflow and vacuum-driven outflow connections. The cells were superfused continuously with the freshly made bath solutions containing the desired concentrations of tested drugs at a rate of about 2 ml/min. A complete solution change in the recording chamber was accomplished within 30 s.

2.3. Chemicals and data analysis

Acetylcholine, dihydro-β-erythroidine hydrobromide, α-bungarotoxin, atropine, mecamylamine, prazocine, propranolol, 1,1-dimethyl-4-penylpiperazinum iodide (DMPP), and all other chemicals were purchased from Sigma. PNU-37883A was a generous gift from Pfizer Canada. Data were expressed as means ± S.E. The data were considered statistically different at the level of $p < 0.05$. Student's paired *t*-test was used to compare the data in the groups studied before and after the drug application. Unpaired *t*-test was used to compare data where two groups of cells needed to be compared.

3. Results

3.1. Effect of acetylcholine on Kir6.1 channels

HEK-293 cells possessed an endogenous background K^+ channel current with current density of −4.0 ± 1.1 pA/pF at −150 mV ($n = 9$). The amplitude of this current did not change significantly during 15 min of dialysis with an intracellular solution containing 0.3 mM ATP. In cells transfected with Kir6.1 cDNA, the whole-cell current had a significantly greater current density (−23.9 ± 1.6 pA/pF at −150 mV, $n = 6$) and this current did not rundown after 15 min of dialysis with 0.3 mM intracellular ATP (−24.1 ± 1.8 pA/pF). Dialysis of HEK-293 cells expressing Kir6.1 currents with 5 mM ATP in the pipette solution caused a decrease in the currents although not significantly (−22.4 ± 4.7 vs. −16.4 ± 2.3 pA/pF, $n = 8–5$ for each group, $p > 0.05$). To identify the expressed current as Kir6.1, we used anti-Kir6.1 antibody (Kir6.1 Ab) that was prepared in our laboratory (Sun et al., 2004). The electrophysiological data using Kir6.1 Ab demonstrated that K_{ATP} channels in HEK-293 cells transfected with Kir6.1 cDNA were significantly inhibited by anti-Kir6.1 antibody (Fig. 1A). The inhibitory effect of anti-Kir6.1 antibody was specific since the recorded K_{ATP} currents did not change their amplitude over the same time frame in the absence of the antibody in the pipette solution or with the inclusion of control serum. Within 10 min of application, the inhibition of K_{ATP} currents by the antibody became stable. Inward rectifier K^+ (Kir) channels are very sensitive to inhibition by extracellular Ba^{2+} at 0.1–0.5 mM (Nelson and Quayle, 1995). The significant inhibition of the recorded K^+ currents in the transfected HEK-293 cells by 0.5 mM Ba^{2+} in our study

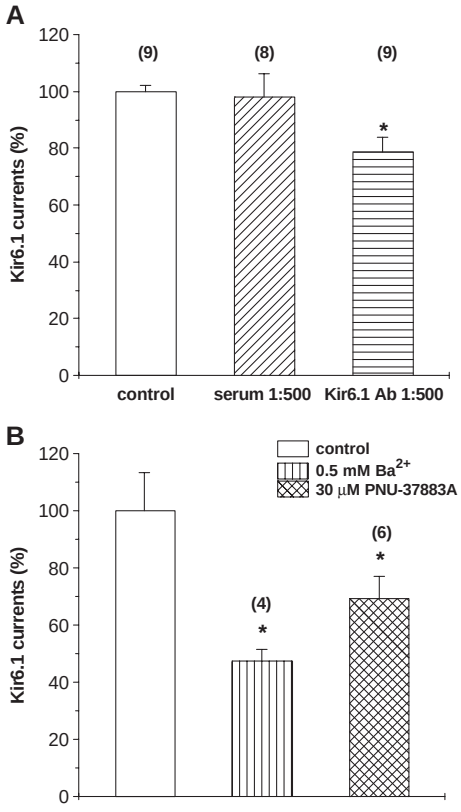

Fig. 1. Effect of different blockers on Kir6.1 currents. A. Effect of anti-Kir6.1 antibody (Kir6.1 Ab) on Kir6.1 channels in stably transfected HEK-293 cells. Test potential, −150 mV; Holding potential, −20 mV. Kir6.1 currents recorded with control serum or Kir6.1 Ab in the pipette solution. B. Effects of 0.5 mM Ba^{2+} and 30 μM PNU-37883A on Kir6.1 channels. Test potential −150 mV; Holding potential −20 mV. *$p < 0.05$ vs. control. Numbers of cells for each experiment are denoted.

indicates the existence of Kir channels, of which Kir6.1 channel being one.

PNU-37883A, a morpholinoguanidine, is a novel non-sulphonylurea K_{ATP} channel inhibitor that has selectivity for the vascular K_{ATP} channel (Kovalev et al., 2004). We examined the actions of PNU-37883A on currents generated by Kir6.1 subunit. Cells were bathed in 40 mM K^+ solution and currents were elicited by voltage steps from −150 to +50 mV at a holding potential of −20 mV. Application of 30 μM PNU-37883A markedly inhibited Kir6.1 currents (−37.4±2.8 vs. −25.9±2.9 pA/pF, $n=6$, $p<0.05$) (Fig. 1B). The effects of PNU-37883A were reversible. The identity of heterologously expressed Kir6.1 channel was further confirmed by Western blot analysis. In our previous paper (Sun et al., 2004) using Western blot experiment we detected a single 41 kDa band of Kir6.1 protein in HEK-293 cells which were permanently transfected with Kir6.1. After the antibody was pre-absorbed with Kir6.1 bacterial fusion protein prior to Western blot experiment, positive bands in HEK-293 cells disappeared.

In the presence of 0.3 mM ATP in the pipette solution, acetylcholine (10 μM) increased Kir6.1 current density from −22.4±4.7 to −36.9±5 pA/pF at −150 mV ($n=5$, $p<0.05$). This effect was partially reversed after washing out acetylcholine and the current density returned to −30±4 pA/pF. The effect of acetylcholine became even greater when the acetylcholine concentration was decreased to 1 μM ($n=8$, $p<0.05$) (Fig. 2A, B). At −150 mV, acetylcholine at 1 μM increased Kir6.1 currents by 95±33.8% (from −19±2.5 to −31.7±2.1 pA/pF, $n=8$, $p<0.05$). At −50 mV acetylcholine (1 μM) increased the Kir6.1 currents from −4.6±1.13 pA/pF to −9.2±2.0 pA/pF ($n=8$, $p<05$). After washout of acetylcholine, the current density became −6.2±0.9 pA/pF. The stimulatory effect of acetylcholine at 1, 10, 100 μM was concentration dependent as shown in Fig. 2C.

Co-expression of SUR1 with Kir6.1 yielded a K_{ATP} current with a density of −29.5±2.7 pA/pF ($n=4$), which was not significantly different from the current density of Kir6.1 subunit alone of −21.3±2.1 pA/pF ($n=19$, −150 mV, $p>0.05$). The current density of Kir6.1/SUR1 was increased by 31.9±13.9% in the presence of 1 μM acetylcholine (−38.9±4.1 pA/pF, −150 mV, $p<0.05$ vs. the basal level), which was significantly smaller than the

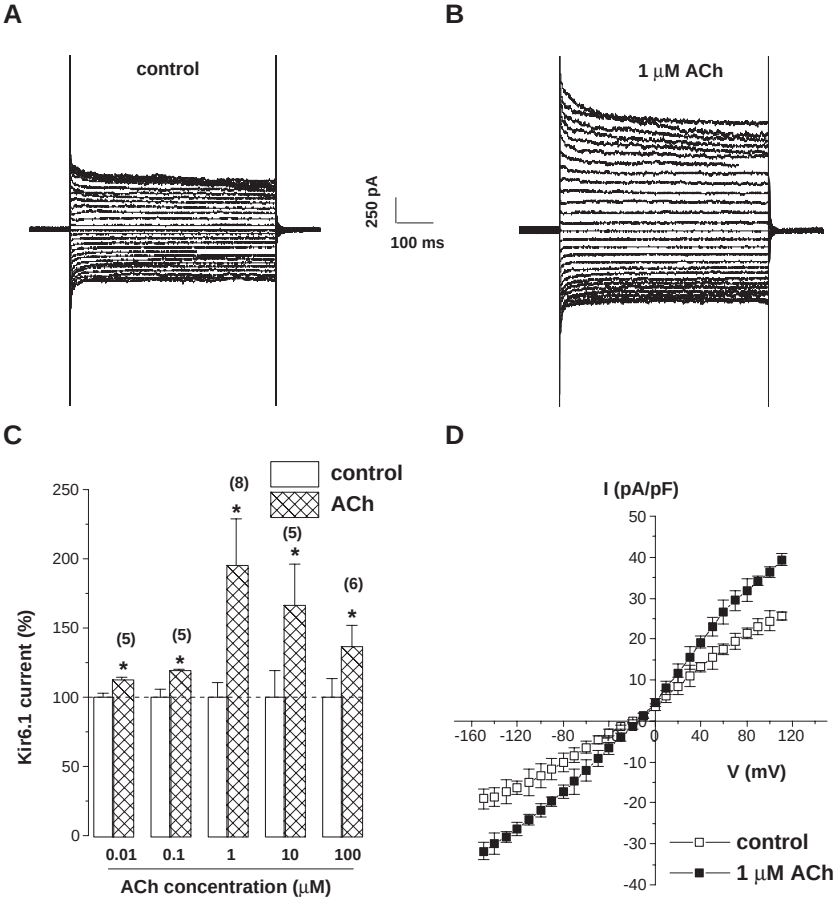

Fig. 2. Effect of acetylcholine on heterologously expressed Kir6.1 channels in HEK-293 cells. Kir6.1 currents recorded in the absence (A) and then presence (B) of 1 μM acetylcholine. Currents were evoked by voltage steps from −150 to +80 mV with a holding potential of −20 mV. C. Normalized changes in mean Kir6.1 currents (at −150 mV) induced by different concentrations of acetylcholine. *$p<0.05$ vs. control. Numbers of cells for each experiment are denoted. D. $I-V$ relationships of Kir6.1 channels before and after the application of 1 μM acetylcholine. Holding potential, −20 mV; $n=8$.

effect of acetylcholine at the same concentration on Kir6.1 alone ($p<0.05$). In non-transfected HEK-293 cells acetylcholine had no effect on the background currents (−4.0±1.1 vs. −4.0±1.4 pA/pF, $n=5$, $p>0.05$).

3.2. Effect of intracellular ATP concentrations on Kir6.1 channels

To determine whether acetylcholine stimulatory effect was affected by intracellular ATP concentration, we increased the ATP concentration in the pipette solution to 5 mM. Kir6.1 currents were smaller, although not significantly, with 5 mM ATP in the pipette solution in comparison with the currents with 0.3 mM ATP (−16.4±2.3 vs. −22.4±4.7 pA/pF, $n=8-5$, $p>0.05$). Acetylcholine at 10 μM still exhibited its stimulatory effect on Kir6.1 channels (−16.4±2.3 to −25.5±3.8 pA/pF, $n=8$, $p<0.05$).

3.3. Modification of acetylcholine effect on Kir6.1 channels by different membrane receptors

A general problem in identifying muscarinic receptor subtypes present in a tissue is the lack of subtype selective muscarinic antagonists. Thus, it is necessary to use a combination of various antagonists. DMPP, a non-specific nicotine acetylcholine receptor agonist, also did not affect Kir6.1 currents at concentrations of 100–500 μM. For example, after the application of 100 μM DMPP the inward currents was −14.4±2.3 pA/pF at −150 mV, which was not different from the control (−16.4±1.7 pA/pF, $n=4$, $p>0.05$). Atropine, a nonselective antagonist of muscarinic receptors, at 2 μM did not alter the stimulation of Kir6.1 channels by 10 μM acetylcholine (−22.1±4.3 vs. −36.6±3.1 pA/pF, $n=4$) (Fig. 3A, B). The stimulatory effect of 10 μM acetylcholine was partially reversible (−30.5±2.1 pA/pF). To exclude the possible involvement of other muscarinic receptors, we increased the concentration of atropine to 20 μM and 10 μM acetylcholine still stimulated Kir6.1 currents (−17.4±4.2 vs. −31.5±4.7 pA/pF, $n=4$) (Fig. 3C, D). To evaluate the involvement of other receptors in the effects of acetylcholine, we investigated the effect of acetylcholine in the presence of 100 μM mecamylamine (nicotine acetylcholine receptors antagonist), 2 μM prazocine (α1 adrenoceptor antagonist), and 1 μM propranolol (β-adrenoceptor inhibitor) in the bath solution (Wang and Wang, 2000). These different blockers had no influence

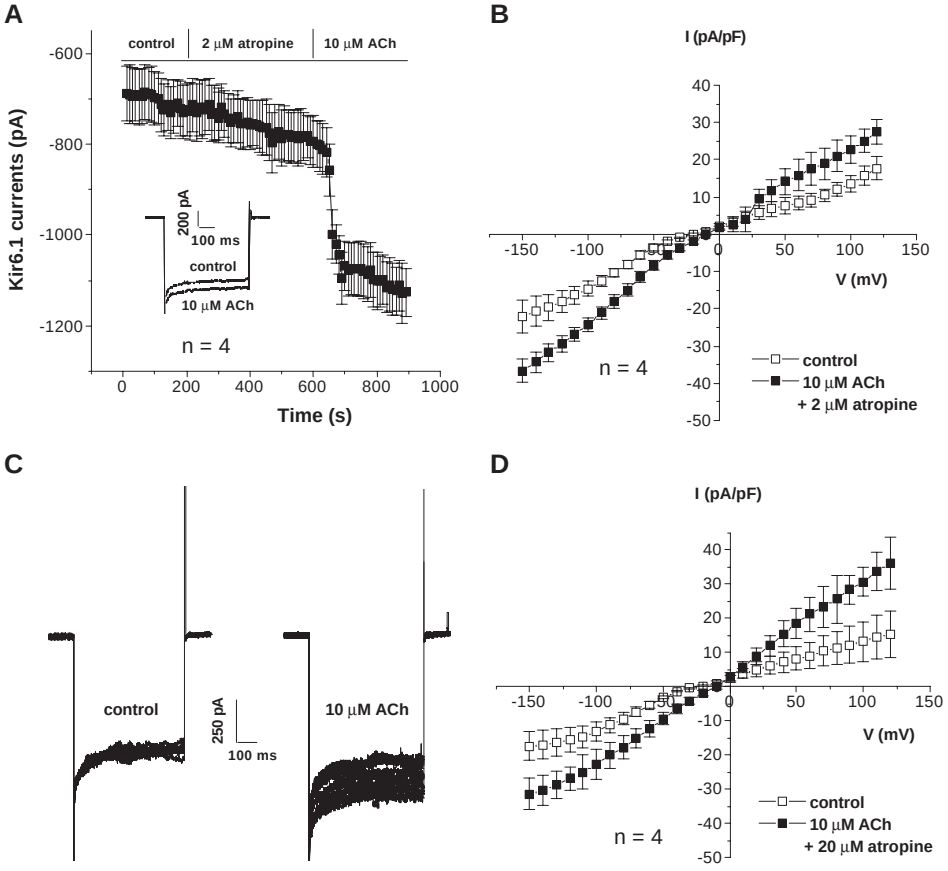

Fig. 3. The stimulatory effects of acetylcholine on Kir6.1 channels in stably transfected HEK-293 cells. A. Time-dependent stimulation of Kir6.1 channels by 10 μM acetylcholine in the presence of 2 μM atropine ($n=4$; test potential, −150 mV). B. $I-V$ relationship of Kir6.1 channels ($n=4$) with 2 μM atropine in the bath solution. Holding potential, −20 mV. C. The original recording of Kir6.1 currents before and after the application of 10 μM acetylcholine in the presence of 20 μM atropine in the bath solution. D. $I-V$ relationship of Kir6.1 channels at control and after application of 10 μM acetylcholine with 20 μM atropine in the bath solution.

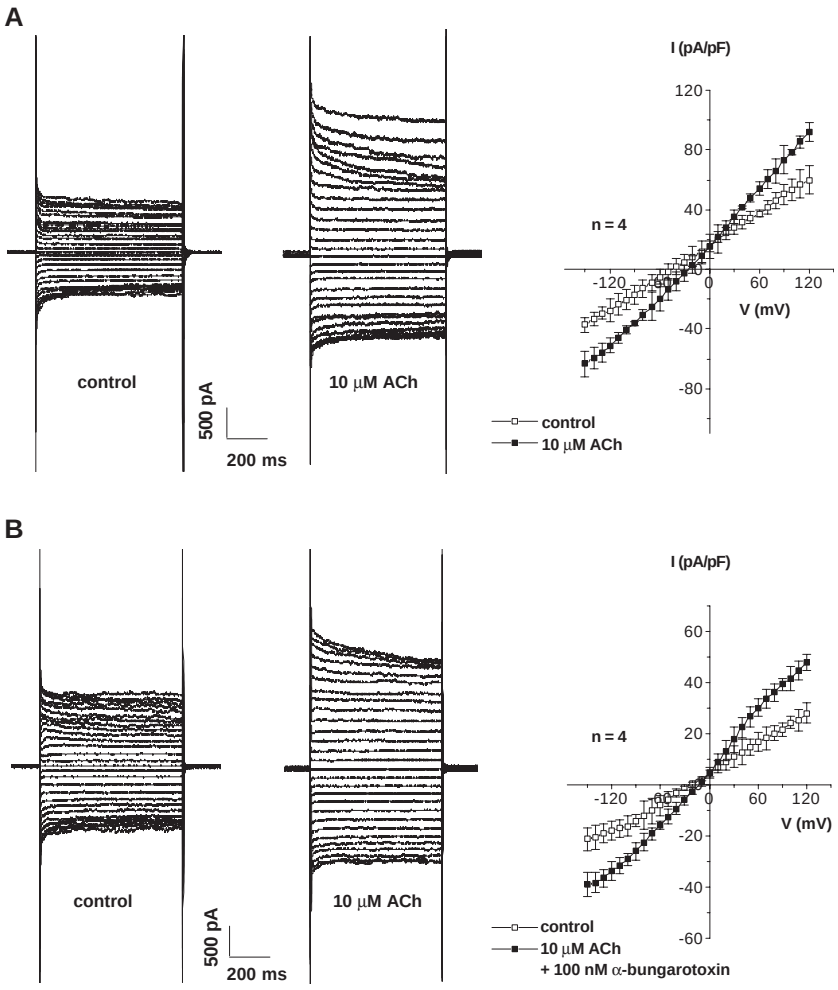

Fig. 4. The effects of acetylcholine on Kir6.1 channels in the presence of different membrane receptor antagonists. A. The original recording of Kir6.1 currents and the $I-V$ relationship of Kir6.1 channels before and after application of 10 μM acetylcholine in the presence of mecamylamine (100 μM), prazocine (2 μM), and propranolol (1 μM). *$p<0.05$ vs. control. B. The original recording of Kir6.1 currents and the $I-V$ relationship of Kir6.1 channels before and after application of 10 μM acetylcholine in the presence of 100 nM α-bungarotoxin. Holding potential, −20 mV.

on the stimulatory effect of acetylcholine on Kir6.1 channels (Fig. 4A). α-bungarotoxin, a specific α1 (Sciamanna et al., 1997) and α7 (Ji et al., 2002) nicotine acetylcholine receptors antagonist did not block the stimulatory effect of 10 μM acetylcholine (−21.2±4.4 vs. −39.0±4.9 pA/pF, $n=4$) (Fig. 4B). Dihydro-β-erythroidine is a competitive antagonist of the human α4−β2 nicotine acetylcholine receptors at micromolar concentrations (Tang et al., 1999). Pretreatment of HEK-293 cells with 10 μM Dihydro-β-erythroidine did not alter the stimulatory effect of 10 μM acetylcholine on Kir6.1 currents (−25.8±4.5 vs. −43.4±4.3 pA/pF, $n=5$) (Fig. 5).

4. Discussion

We have characterized the stimulatory effect of acetylcholine on Kir6.1 channels stably expressed in HEK-293 cells using the whole cell patch-clamp technique. Our major novel finding is that acetylcholine stimulates Kir6.1 subunits. This effect is not mediated by the acetylcholine receptor-dependent mechanisms for the stimulatory effect of acetylcholine was not altered by muscarinic or nicotinic receptor blockers. Four lines of evidence support that the expressed current in HEK-293 cells was conducted by Kir6.1 channels. First, K_{ATP} channels in HEK-293 cells transfected with Kir6.1 cDNA were significantly inhibited by anti-Kir6.1 antibody. The inhibitory effect of anti-Kir6.1 antibody was specific since the recorded K_{ATP} currents did not change their amplitude over the same time frame in the absence of the antibody in the pipette solution or with the inclusion of control serum. Subunit-specific anti-K_{ATP} channel antibodies are important tools for examining the molecular composition and structure–function relationship of native K_{ATP} channels in vascular SMCs. To date, in limited studies anti-Kir6.1 antibodies were produced using either synthetic peptides based on short sequences of Kir6.1 gene or Kir6.1-ST fusion proteins as antigen. None of these antibodies has been used to study the expression of Kir6.1 proteins in cardiovascular system. The antibody used in the

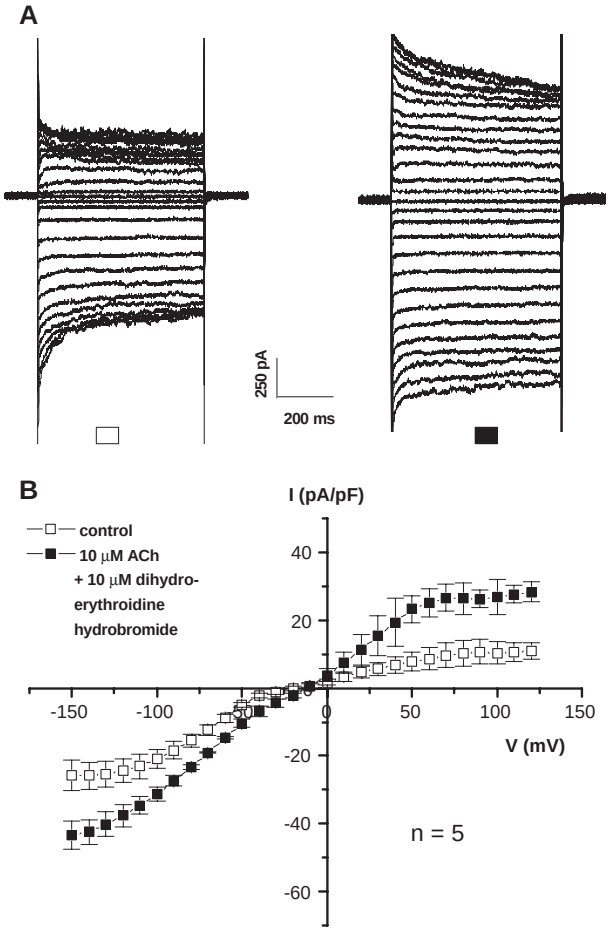

Fig. 5. The effects of acetylcholine on Kir6.1 channels in the presence of dihydro-β-erythroidine. A. The original recording of Kir6.1 currents. B. *I–V* relationship of Kir6.1 channels before and after the application of 10 μM acetylcholine with 10 μM dihydro-β-erythroidine hydrobromide in the bath solution.

present study targeted specifically on an in-framed 79 aa Kir6.1C fragment (L277–A355) (pGEX-Kir6.1C). Our previous study has demonstrated that this antibody is very specific and sensitive to Kir6.1 subunits (Sun et al., 2004). The partial blockade of Kir6.1 current by Kir6.1Ab indicates that the antigenic reaction of the specific epitope of Kir6.1 protein contributes only partially to the gating of the channel. Second, Kir channels are very sensitive to Ba^{2+} and in our experimental conditions Ba^{2+} at 0.5 mM inhibited Kir6.1 currents up to 52.5%. Third, PNU-37883A is reported to bind specifically to Kir6.1 subunit (Kovalev et al., 2004; Surah-Narwal et al., 1999). In our study PNU-37883A inhibited Kir6.1 channels (Fig. 1B). Fourth, in our previous paper (Sun et al., 2004) using Western blot experiment we detected a single 41 kDa band of Kir6.1 protein in HEK-293 cells which were permanently transfected with Kir6.1 cDNA. Kir6.1 subunits, unlike Kir6.2, are generally not sensitive to ATP. It did not come as a surprise that 5 mM ATP did not inhibit the current significantly. If there was no ATP in the pipette solution the K_{ATP} channel current will run down, demonstrated in other studies (Kamouchi and Kitamura, 1994) and in ours (data not shown).

It has been reported that atropine reduced the acetylcholine-induced membrane hyperpolarization of various smooth muscle preparations (Keef and Bowen, 1989; Yajima et al., 1999). In these studies, the effect of acetylcholine on membrane potential was mediated by acetylcholine receptors. However, in our study, transfected HEK-293 cells were used and no acetylcholine receptors have been detected in these cells. The lack of interaction of atropine and acetylcholine on Kir6.1 current provides another pharmacological evidence for the receptor-independent effect of acetylcholine on Kir6.1 subunit. Furthermore, Northern blot and Western blot analyses have shown the absence of nicotine acetylcholine receptors in native HEK-293 cells (Chavez-Noriega et al., 2000).

It is interesting to notice that co-expression of SUR1 subunit with Kir6.1 subunit significantly reduced the stimulatory effect of acetylcholine on K_{ATP} channel currents. Under physiological conditions, SUR subunits are intrinsically co-expressed with Kir6.1 subunits in different types of cells. The presence of SUR subunits, therefore, exerts an inhibitory role for the activation of K_{ATP} channel complex by acetylcholine. Although the nature of this SUR-mediated inhibition of acetylcholine effect on Kir6.1 cannot be ascertained yet, an analogy may be drawn to the interaction of ATP with K_{ATP} channel complex since ATP can bind to both SUR and Kir subunits.

Muscarinic receptors display both excitatory and inhibitory effects on cholinergic neurotransmission by coupling to different intracellular signal transduction pathways. Whereas the binding of ligands to muscarinic receptors subtypes M_1, M_3 and M_5 activates protein kinase C (PKC) and the mitogen-activated protein (MAP) kinase path way, activation of M_2 and M_4 muscarinic receptors is known to inhibit adenylate cyclase (Teber et al., 2004). In our study the effects of acetylcholine on Kir6.1 channels were not altered by mecamylamine (a nicotine acetylcholine receptor antagonist), prazosin (an α1-adrenoceptor antagonist), propranolol (a β-adrenoceptor inhibiotor), atropine (a muscarinic acetylcholine receptors antagonists), and dihydro-β-erythroidine hydrobromide (a competitive antagonist of human α4–β2 nicotine acetylcholine receptors). These results indicate that acetylcholine stimulation of Kir6.1 channels is most likely the consequence of the interactions between acetylcholine molecules and Kir6.1 channel proteins. Electrostatic and hydrogen bonding interactions between ionized amino acids are known to be important for ion channel subunit interaction and protein folding. Acetylcholine is a positively charged molecule with quaternary group of the choline moiety as its positively charged tail (Sussman et al., 1991; Sarri et al., 2004). The pore forming region (H5) of Kir6.1 channel is also positively charged due to two arginine residues (Inagaki et al., 1995). Since acetylcholine molecule cannot pass cell

membrane, it is possible that the positively charged acetylcholine interacts with the positively charged H5 region of Kir6.1 protein, causing conformational changes of the subunit and channel activation. This speculation still waits for experimental confirmations.

There are three main conclusions from our study. First, acetylcholine stimulates Kir6.1 channels in a dose-dependent manner. Second, the excitatory effect of acetylcholine on Kir6.1 channels heterologously expressed in HEK-293 cells is not mediated by an acetylcholine receptor-dependent mechanism, as had been conventionally thought. It is worth noting that, however, in the cells where acetylcholine receptors are expressed the interaction of acetylcholine and Kir6.1 channels might exhibit different profiles. Third, the stimulatory effect of acetylcholine is a result of the interaction of acetylcholine with Kir6.1 channels proteins, rather than an altered ATP metabolism by acetylcholine. The latter conclusion was based on the following two lines of evidence. 1) Intracellular ATP concentration in these studies was clamped at a fixed level (e.g., 0.3 mM) by dialyzing cells with the pipette solution. 2) Intentionally varying ATP concentrations inside the cells (from 0.3 to 5 mM) did not change the excitatory effect of acetylcholine on K_{ATP} channels. The direct interaction of acetylcholine with K_{ATP} channels may represent an important endogenous mechanism in SMCs, heart, neurons, and other excitable cells to couple cellular metabolism to excitability. It should be mentioned that acetylcholine also acts on many other ion conductances in excitable cells. By demonstrating the role of acetylcholine as a novel opener of K_{ATP} channels we begin to understand how the interaction of acetylcholine with Kir6.1 subunits provides an integrated regulation of cellular function.

Acknowledgments

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