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Inhibitory effect of protopine on K_{ATP} channel subunits expressed in HEK-293 cells

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Abstract

Protopine is an isoquinoline alkaloid purified from Corydalis tubers and other families of medicinal plants. The purpose of the present study was to investigate the effects of protopine on K_{ATP} channels and big conductance (BK_{Ca}) channels. Protopine concentration-dependently inhibited K_{ATP} channel currents in human embryonic kidney cells (HEK-293) which were cotransfected with Kir6.1 and sulfonylurea receptor 1 (SUR1) subunits, but not that with Kir6.1 cDNA transfection alone. At 25 μM, protopine reversibly decreased Kir6.1/SUR1 currents densities from −17.4±3 to −13.2±2.4 pA/pF at −60 mV (n=5, P<0.05). The heterologously expressed mSlo-encoded BK_{Ca} channel currents in HEK-293 cells were not affected by protopine (25 μM), although iberiotoxin (100 nM) significantly inhibited the expressed BK_{Ca} currents (n=5, P<0.05). In summary, protopine selectively inhibited K_{ATP} channels by targeting on SUR1 subunit. This discovery may help design specific agents to selectively modulate the function of Kir6.1/SUR1 channel complex and facilitate the understanding of the structure–function relationship of specific subtype of K_{ATP} channels.

Keywords: Protopine; K_{ATP} channel; Sulphonylurea receptor; Heterologous expression; BK_{Ca} channel

1. Introduction

The cardiovascular effects of medicinal plants of Papaveraceae, Fumariaceae, and other families (Ko et al., 1992; Deng et al., 2001b; Dostal et al., 2001) have been shown, including antiarrhythmia in many animal models (Burtsev et al., 1978; Lu et al., 1992), antihypertension in dogs (Wang et al., 1986), and antithrombotic and antiinflammatory effects in human (Saeed et al., 1997). Protopine (tetrahydro-5-methyl bis-[1, 3] benzdioxide-[4, 5-C:5′, 6]-azecin-13(5*H*)-one) is an isoquinoline alkaloid and common in the aforementioned medicinal plants (Ko et al., 1992; Deng et al., 2001b; Dostal et al., 2001). Protopine inhibits the contractility of isolated cardiac papillary muscles and the proliferation of vascular smooth muscle cells induced by endothelin (Deng et al., 2001a). It also shortens action potential duration and prolongs the effective refractory period in guinea pig cardiac papillary muscles (Jiang and Song, 1995; Teng et al., 1989). The protective effect on rat heart from ischemia–reperfusion damage (Song et al., 2000b) and the relaxation of rat thoracic aorta induced by protopine have been related to the inhibition of Ca^{2+} influx through both voltage- and receptor-operated Ca^{2+} channels (Ko et al., 1992).

K^+ channels play an essential role in modulating membrane excitability. At least four main classes of K^+ channel currents have been identified in excitable cells based on their biophysical and pharmacological properties: voltage-dependent outward K_V channel, ATP-sensitive K_{ATP} channel, Ca^{2+}-activated K_{Ca} channel, and inward rectifier K_{IR} channel. The opening of K^+ channels under physiological conditions results in K^+ efflux and membrane hyperpolarization. In vascular smooth muscle cells, this hyperpolarization would lead to vasorelaxation (Lu et al., 2001). In heart, different types of K^+ channels control the duration of action potentials, resting membrane potentials, automaticity, and refractoriness

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(Nerbonne et al., 2001). Different types of K^+ channels are also the targets of neurotransmitters, gasotransmitters, intracellular mediators, and exogenous drugs. Song et al. (2000a) reported that protopine non-specifically inhibited cardiac cation channels, including I_K and I_{K1}. The effects of protopine on K_{ATP} and BK_{Ca} channels, however, have not been reported to date. In the present study, different subunits of K_{ATP} channel complex and big conductance K_{Ca} channel were expressed in human embryonic kidney cells (HEK-293). The pharmacological sensitivities of these expressed K^+ channels to protopine were determined. Through this study, a clearer pharmacological profile of protopine on different types of K^+ channels is obtained.

2. Materials and methods

2.1. Culture of HEK-293 cells

HEK-293 cells (CRL# 1573, Batch# 203970) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and cultured at 37 °C in a humidified atmosphere containing 5% CO_2/95% air. Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Sigma) was used with supplementation of 10% fetal bovine serum, penicillin 100 U/ml, Fungizone 625 μg/ml (Gibco BRL), and 2 mM glutamine. The culture medium was changed every 3 days. HEK-293 cells used in the present study were passages 33 to 37. Kir6.1 (Genbank accession No. AB043637) and SUR1 (Genbank accession No. AB052294) coding cDNAs were co-transferred into HEK-293 cells using a dual transfection technique. In short, HEK-293 cells were stably transfected with Kir6.1-pcDNA3.1/Hygro plasmid using hygromycin (50 μg/ml) as the selective antibiotics. Hygromycin-resistant colonies were isolated and then transfected with SUR1-pcDNA3.1/Zeo plasmid using Zeocin (50 μg/ml) as the selective reagent. The hygromycin- and Zeocin-resistant cell colonies were cloned and the coexpression of Kir6.1 and SUR1 subunits were confirmed with both Western blotting and the patch-clamp techniques as we described previously (Cao et al., 2002). BK_{Ca},α subunit mSlo cDNA (Genbank accession No. U09383), provided by Dr. Ganetzky (University of Wisconsin, Madison, Wisconsin, USA), was expressed transiently in HEK-293 cells using a Fugene6

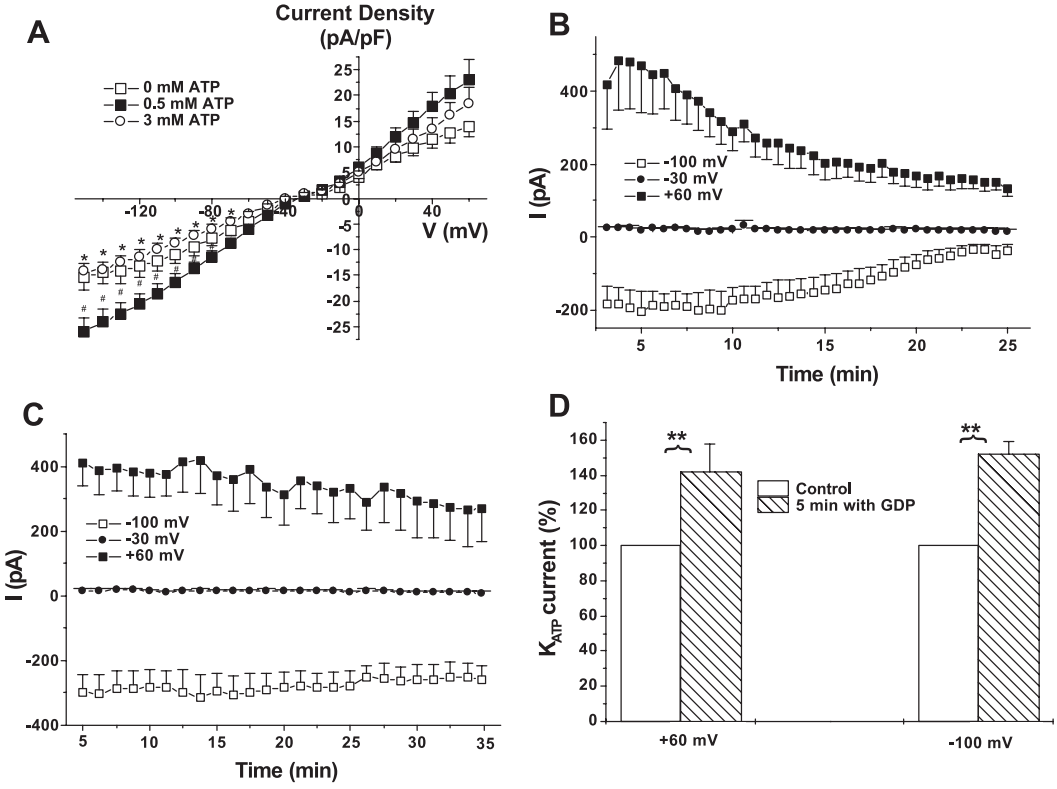

Fig. 1. Characterization of the heterologously expressed K_{ATP} channels encoded by Kir6.1/SUR1 cDNA. (A) ATP sensitivity of the expressed Kir6.1/SUR1 channels. K_{ATP} channel current was recorded during 600 ms depolarizing pulse from −150 to +50 mV (holding potential, −30 mV) with 10-mV increments. Different concentrations of ATP, 0 (□; n=13), 0.5 (■; n=12), and 3 mM (○; n=6) were included in the pipette solution (#,*P<0.05 vs. 0.5 mM ATP, respectively). (B) Rundown of Kir6.1/SUR1 channel currents in the presence of 0 mM ATP in the pipette solution. (C) Rundown of Kir6.1/SUR1 channel currents in the presence of 0.5 mM ATP in the pipette solution. Kir6.1/SUR1 currents shown in panels B and C were recorded using depolarization ramps from −100 to +60 mV (duration, 1232 ms; holding potential, −30 mV). Selective data points were extracted from the average ramp traces at membrane potential of −100 (□), −30 (●), and +60 mV (■). n=4 for each data points. (D) Inclusion of 1 mM GDP in the pipette solution amplifies both of K_{ATP} inward at −100 mV and outward currents at +60 mV. Data were the summary of 9 cells. Selective data points were extracted from the average ramp traces at membrane potential of −100 and +60 mV (**P<0.01).

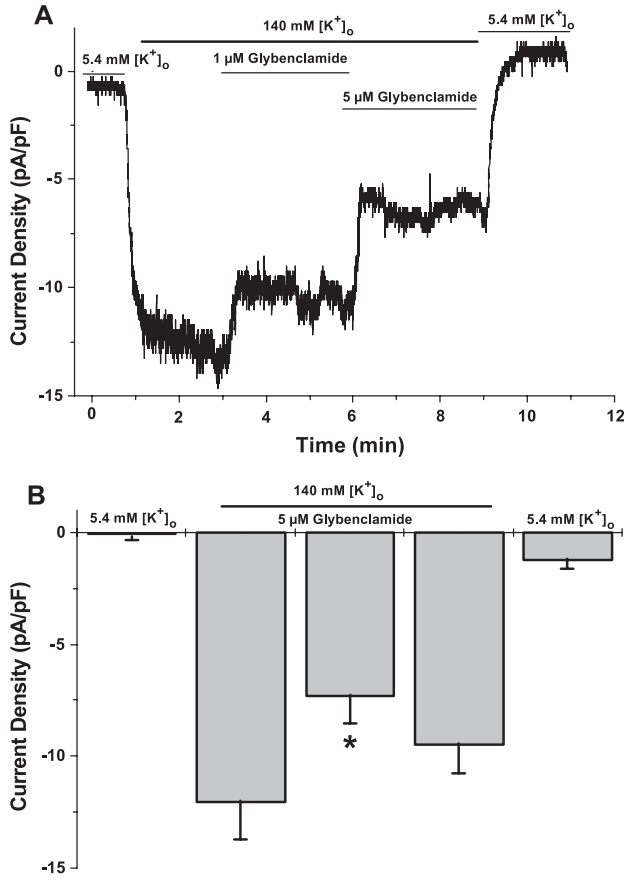

Fig. 2. The inhibitory effect of glybenclamide on the whole-cell Kir6.1/SUR1 current in cotransfected HEK-293 cells. K_{ATP} channel current was recorded at a constant membrane potential of −60 mV with different K^+ concentrations in the bath solution. (A) Representative original records of the effect of glybenclamide on K_{ATP} currents. (B) Summary of the effect of glybenclamide on K_{ATP} currents (n=7, *P<0.001).

transfection reagent (Roche). About 1 μg of BK_{Ca},α subunit cDNA, inserted into a green florescent protein (GFP) expressed vector pIRES2-EGFP, was used for each transfection as we described previously (Wu et al., 2002).

2.2. The patch-clamp experiments

K^+ channel currents were measured using the standard whole-cell configuration of the patch-clamp technique as described previously (Tang et al., 1999). Briefly, 35-mm petri dishes with the attached cells were mounted on the stage of an inverted phase-contrast microscope (Olympus IX70). A homemade superfusion chamber was inserted into the petri dish, and, in all experiments, the cells inside the superfusion chamber (volume of 1 ml) were superfused continuously at a flow rate of 3–5 ml/min. The time required for a complete solution change from the onset of a drug application was estimated at 10–15 s, as described before (Wu et al., 1996). Pipettes were pulled from soft microhematocrit capillary tubes (Fisher, Nepean, ON) with tip resistance of 2–4 MΩ when filled with pipette solution. Currents were recorded with an Axopatch 200-B amplifier (Axon Instruments), controlled by a Digidata 1200 interface and a pCLAMP software (Version 6.02, Axon Instruments). Membrane currents were filtered at 1 kHz with a four-pole Bessel filter. At the beginning of each experiment, junction potential was electronically adjusted to zero (Wu et al., 1996). Leakage subtractions were not performed on the original current records. Current–voltage curves were constructed using the sustained current amplitude at the end of different depolarization steps (600 ms each). Membrane capacitance current was measured with a test pulse of 5 mV. Membrane capacitance was obtained as the product of the derived time constant and resistance. The whole-cell current density was obtained by dividing whole-cell K^+ current amplitude (pA) with membrane capacitance (pF). All experiments were conducted at room temperature (20–22 °C).

Unless otherwise specified, the same bath solution containing (mM) 135 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 10 HEPEs, and 5 glucose (pH adjusted to 7.3 with NaOH) was used to record K_{ATP} and BK_{Ca} channel currents (Wu et al., 2002). When the concentration of KCl was altered in some experiments, the concentration of NaCl of the bath solution was correspondingly changed to maintain the osmolality of the bath solution unaltered. For recording K_{ATP}

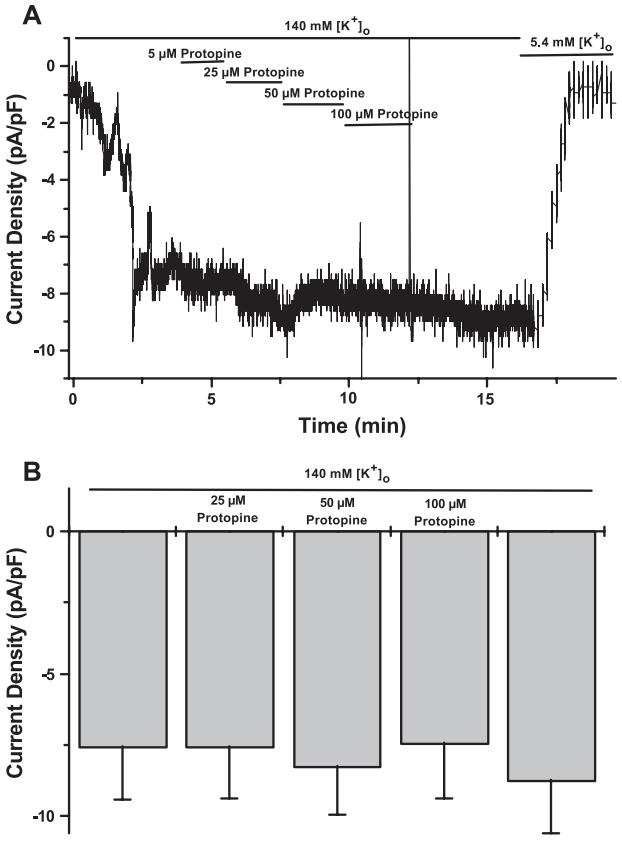

Fig. 3. The lack of effect of protopine on Kir6.1 current in HEK-293 cells transfected with Kir6.1 cDNA alone. K_{ATP} channel current was recorded at a constant membrane potential of −60 mV with different K^+ concentrations in the bath solution. (A) Representative original records of the effect of protopine on K_{ATP} currents. (B) Summary of the effect of protopine on K_{ATP} currents (n=7).

channel currents, test pulses were applied in a stepwise fashion with an interval of 10 s in 10-mV increments from −150 to +50 mV. Holding potential was set at −30 mV. The pipette solution contained (mM): 110 KCl, 30 KOH, 1 $MgCl_2$, 1 $CaCl_2$, 10 EGTA, 10 HEPES, and 0.3 Na_2-ATP (pH adjusted to 7.3 with KOH). For recording BK_{Ca} channel currents, test pulses were applied in a stepwise fashion with an interval of 10 s in 10-mV increments from −60 to +50 mV. Holding potential was set at −80 mV. The pipette solution contained (mM): 110 KCl, 30 KOH, 1 $MgCl_2$, 1 EGTA, 10 HEPES, and 0.3 Na_2-ATP (pH adjusted to 7.3 with KOH). $[Ca^{2+}]$ in the pipette solution was adjusted to 100 nM by adding adequate amount of $CaCl_2$. The osmolarity of recording solutions was always adjusted to 300 mosM.

2.3. Chemicals and data analyses

Protopine used in this study was purified from Corydalis tubers kindly provided as a gift by a pharmacy engineer Xiaochu Liu (Sichuan Guangsong Pharmaceutical, Guanghan, Sichuan, P.R. China), a white powder with a purity of over 99% (acetate salt). Stock solution (50 mM) of protopine was prepared in double distilled water, kept at +4.0 °C. Glybenclamide and iberiotoxin were purchased from Sigma. Data were expressed as mean±S.E.M. and analyzed using paired student *t*-test except Figs. 1A and 4B in which one-way analysis of variance between groups (ANOVA) analyses were implemented. Differences were considered statistically significant when $P<0.05$.

3. Results

3.1. Heterologously expressed K_{ATP} channels in HEK-293 cells

When transfected with GFP alone, HEK-293 cells showed a small inward current around 2.43±0.33 pA/pF

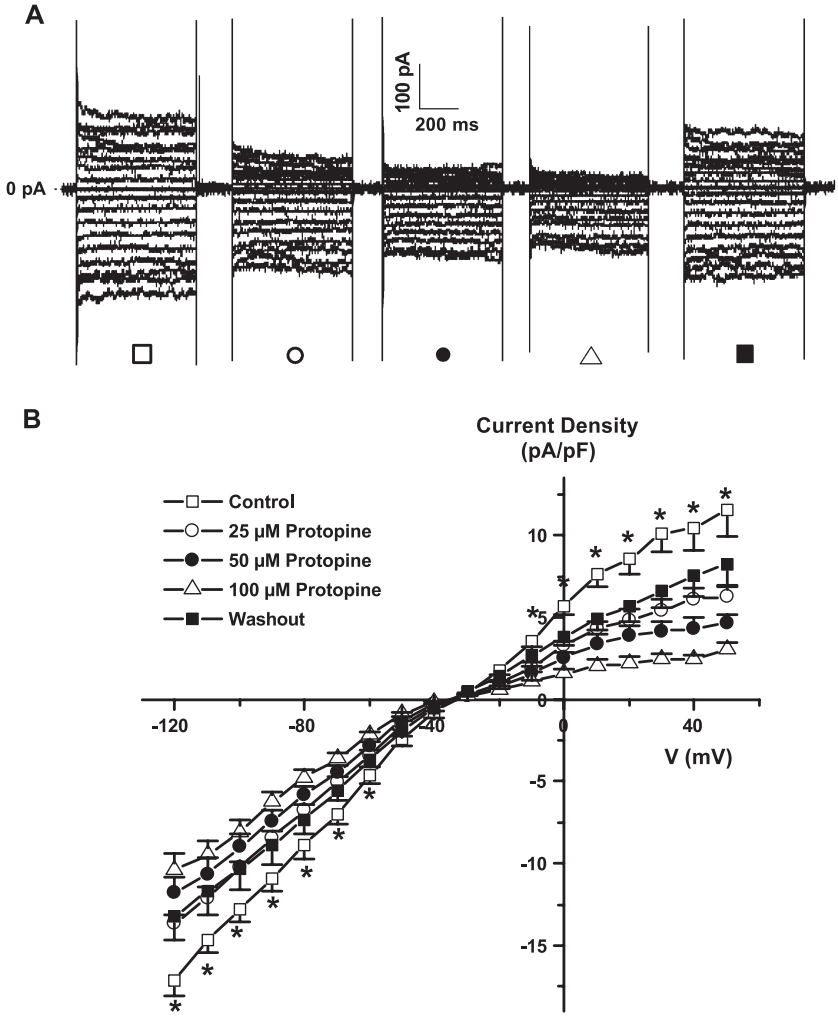

Fig. 4. The inhibition of the coexpressed Kir6.1/SUR1 channels by protopine in HEK-293 cells. K_{ATP} inward current was recorded with 40 mM KCl in the bath solution. (A) The original K_{ATP} current traces recorded during 600 ms depolarizing pulse (0.1 Hz) from −150 to +50 mV (holding potential −30 mV). (B) The concentration-dependent inhibitory effect of protopine on K_{ATP} channels. I–V relationship of K_{ATP} current in the absence (□; n=4), presence of protopine (25 (○), 50 (●), and 100 μM (△)), and after removal of protopine (■; n=4). *$P<0.05$ control group vs. all groups except washout group.

(n=6, data not shown) with 140 mM KCl in the bath solution and 0.5 mM ATP in the pipette solution (−60 mV). In the HEK-293 cells expressing Kir6.1 or Kir6.1/SUR1 channels, a large inward rectifying whole-cell current was recorded. The current densities were similar between the cells expressing Kir6.1 alone (11.83±1.12 pA/pF, n=10) and the cells coexpressing Kir6.1/SUR1 (13.16±0.89 pA/pF, n=16). In Kir6.1/SUR1-expressing cells, K_{ATP} channels exhibited the biggest current density with 0.5 mM ATP (n=12) in the pipette solution than that obtained with 0 (n=13) or 3 mM (n=6) ATP in the pipette solution (P<0.05; Fig. 1A). Dialyzing cells with 0.5 mM ATP most significantly delayed the rundown of the current in comparison with that without ATP in the pipette solution (n=4, P<0.05; Fig. 1B and C). Including 1 mM GDP in the pipette solution significantly amplified the expressed K_{ATP} channel currents (Fig. 1D). The inward Kir6.1/SUR1 current was significantly amplified when extracellular potassium concentration was increased from 5.4 to 140 mM. Glybenclamide significantly inhibited the expressed Kir6.1/SUR1 current (Fig. 2).

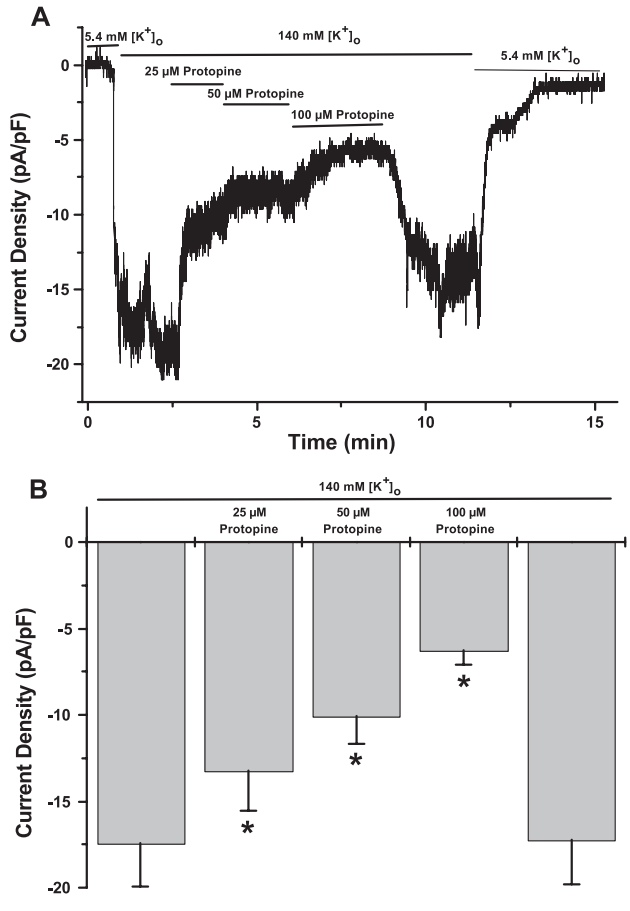

Fig. 5. The inhibitory effect of protopine on the whole-cell K_{ATP} current in the Kir6.1/SUR1 co-expressed HEK-293 cells. K_{ATP} channel current was recorded at a constant membrane potential of −60 mV with different K^+ concentrations in the bath solution. (A) Representative original records of the effect of protopine on K_{ATP} currents. (B) Summary of the effect of protopine on K_{ATP} currents. n=4, *P<0.001.

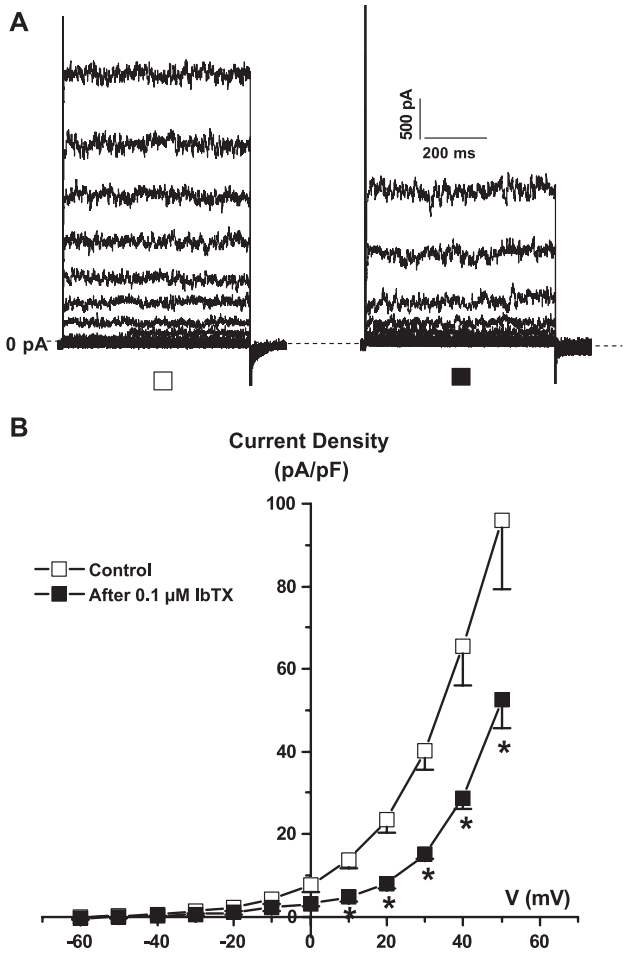

Fig. 6. The effect of iberiotoxin on the whole-cell BK_{Ca} current in HEK-293 cells transiently transfected with BK_{Ca},α subunit cDNA. Stepwise depolarizations with 600 ms duration were delivered from −60 to +50 mV in 10-mV increments from a holding potential of −80 mV at 0.1 Hz. (A) Representative original recordings of BK_{Ca} channel currents with different conditions. (B) Summary of the effect of iberiotoxin on BK_{Ca} channel currents (n=5).

3.2. Effect of protopine on heterologously expressed K_{ATP} channels

Protopine at different concentrations (5–100 μM) had no effect on the inward K_{ATP} channel current encoded by Kir6.1 cDNA alone (Fig. 3). The coexpressed Kir6.1/SUR1 channel was concentration-dependently inhibited by protopine. Removal of protopine from the bath solution partially recovered the Kir6.1/SUR1 channel from the inhibition (Fig. 4). To further facilitate the recording of the inward K_{ATP} channel currents, 140 mM KCl was used in the bath solution and membrane potential was constantly maintained at −60 mV. This increase in extracellular K^+ concentration significantly increased the inward Kir6.1/SUR1 current. Protopine concentration-dependently inhibited the amplified inward Kir6.1/SUR1 currents from −17.4±3 to −13.2±2.4 (25 μM protopine), −10.1±1.6 (50 μM protopine), and −6.2±0.9 pA/pF (100 μM protopine). This inhibitory effect was reversible. After washing protopine out of the bath

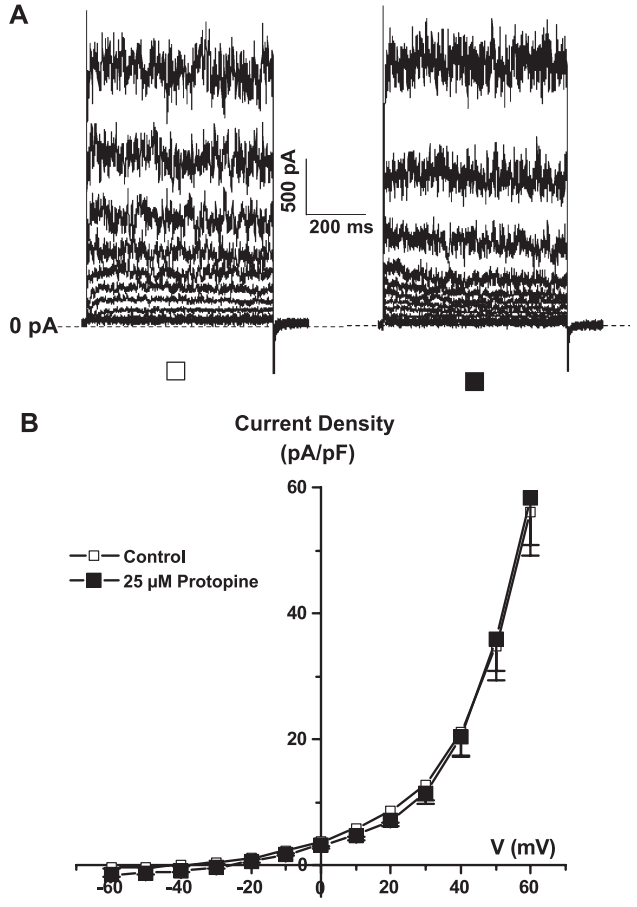

Fig. 7. The effect of protopine on the whole-cell BK_{Ca} current in HEK-293 cells transiently transfected with BK_{Ca},α subunit cDNA. Stepwise depolarizations with 600 ms duration were delivered from −60 to +50 mV in 10-mV increments from a holding potential of −80 mV at 0.1 Hz. (A) Representative original recordings of BK_{Ca} channel currents with different conditions. (B) Summary of the effect of protopine on BK_{Ca} channel currents (n=5).

solution, the density of Kir6.1/SUR1 current returned to the control level of −17.2±2.6 pA/pF (Fig. 5).

3.3. Effect of protopine on heterologously expressed BK_{Ca},α subunit

The expression of BK_{Ca},α subunit cDNA in HEK-293 cells yielded a big outward K^+ current in the presence of 100 nM cytoplasmic-free Ca^{2+}. Iberiotoxin (100 nM) significantly decreased BK_{Ca} channel currents from −96±16.6 to −52.6±7.1 pA/pF at +50 mV (n=5, P<0.05; Fig. 6). However, protopine (50 μM) had no effect on the expressed BK_{Ca} channel currents (Fig. 7).

4. Discussion

Protopine, a neutrally charged alkaloid, has a wide spectrum of actions on cardiovascular system. It inhibits the proliferation of cultured rabbit aortic vascular smooth muscle cells induced by endothelin (Deng et al., 2001a). In guinea pig cardiac papillary muscles, protopine altered the action potential behaviour by reducing action potential amplitude, overshoot, and maximum rate of depolarization. Consequently, in the presence of protopine, action potential duration was shortened and the effective refractory period prolonged and the contraction force of cardiac papillary muscles decreased (Jiang and Song, 1995; Teng et al., 1989). Protopine protects rat heart from ischemia–reperfusion damage (Song et al., 2000b) and suppressed norepinephrine-induced tonic contraction of rat thoracic aorta (Ko et al., 1992). Since $^{45}Ca^{2+}$ influx caused by either norepinephrine or K^+ was inhibited by protopine, it was suggested that the vasorelaxant effect of protopine could be mediated by an inhibited Ca^{2+} influx through both voltage- and receptor-operated Ca^{2+} channels (Ko et al., 1992). However, the failure of nifedipine (2–100 μM) to inhibit the protopine-induced vasorelaxation did not support an inhibitory effect of protopine on voltage-dependent Ca^{2+} channels (Ko et al., 1992). Song et al. (2000a) claimed that protopine might be a nonselective cation channel antagonist. It inhibited multiple cation channels, including $I_{Ca,L}$, I_K, I_{K1} as well as I_{Na}, in cardiac myocytes. The molecular identification of different native ion channels in myocytes is difficult and the effects of protopine on K_{ATP} and K_{Ca} channels had not been elucidated. Heterologous expression of the cloned ion channel subunits provide the most convincing means to specifically study the interaction of the selected pharmacological agents with the identified ion channel subunits. This approach helped us in the present study to pinpoint the interaction of protopine with K_{ATP} channels and K_{Ca} channels with molecular identity.

K_{ATP} channels play important roles in the physiology and pathophysiology of many tissues, including the response to cardiac and cerebral ischemia and the regulation of vascular smooth muscle tone. K_{ATP} channels couple cell metabolism to membrane excitability (Ashcroft, 1988). In vascular smooth muscle, opening of K_{ATP} channel causes membrane hyperpolarization, which increases K^+ efflux and leads to smooth muscle relaxation. The roles of K_{ATP} channels in pancreatic β-cells have been best characterized (Terzic et al., 1995). Glucose-induced insulin secretion is mediated by the closure of K_{ATP} channel in pancreatic β-cells (Cook et al., 1988). Generally, K_{ATP} channels are activated by low cytosolic ATP or elevated nucleotide diphosphate (NDP) levels. Potassium channel openers, including diazoxide, pinacidil, and levcromaklim, stimulate K_{ATP} channels and sulphonylurea agents such as glybenclamide and tolbutamide inhibit these channels. K_{ATP} channels are octameric complexes of four pore-forming Kir6.x subunits and four regulatory sulfonylurea subunits (Yokoshiki et al., 1998). Two isoforms of Kir (Kir6.1 and Kir6.2) and three of SUR (SUR1, SUR2A, SUR2B) have been identified. K_{ATP} channels are broadly distributed but quite tissue-specific in their expression patterns. In general, SUR2A and Kir6.2 form cardiac K_{ATP}

channels, SUR2B and Kir6.1 or Kir6.2 form vascular smooth muscle K_{ATP} channels. On the other hand, SUR1 and Kir6.2 form pancreatic β-cell K_{ATP} channels to mediate insulin release. SUR1 and Kir6.1 are believed to form mitochondrial K_{ATP} channels in cardiac myocytes and other cell types (Yokoshiki et al., 1998; Liu et al., 2001). SUR1 subunit also plays an important role in the nervous system for regulation of neuronal excitability. SUR1 and Kir6.2 are coexpressed in brain tissues including cholinergic basal forebrain neurons (Allen and Brown, 2003; Liss and Roeper, 2001). The coexpression of SUR1 with Kir6.1 has been shown in some glucose-receptive neurons within the rat ventromedial hypothalamus (Lee et al., 1999). We demonstrated that protopine had no effect on Kir6.1-encoded K_{ATP} channels whereas Kir6.1/SUR1 channels were significantly inhibited by protopine. It is thus rationalized that the target of protopine is SUR1 subunit, rather than Kir6.1 subunit.

BK_{Ca} channels are involved in excitation–contraction coupling in many excitable cells. BK_{Ca} channels are stimulated by increased intracellular Ca^{2+} concentration and by membrane depolarization. These channels are composed of two noncovalently linked subunits: the pore-forming α subunit and the accessory β subunit (Wu et al., 2002). Our study showed that protopine did not affect BK_{Ca} channel α subunit expressed in HEK-293 cells, although the expressed mSlo channels were inhibited by iberiotoxin.

In summary, protopine has no effect on Kir6.1 channel. The co-expression of SUR1 with Kir6.1 renders the Kir6.1/SUR1 complex sensitive to protopine. On the other hand, BK_{Ca} channels are not modulated by protopine. Our study provides evidence for the selective activation of SUR1 subunit of K_{ATP} channels. This study will help to derive a refined pharmacological profile of protopine and its derivatives, such as alprotopine. Not only the structure/function relationship of SUR1 can be better elucidated based on the specific effect of protopine, but clinical application of this alkaloid to certain cellular disorders related to the altered function and expression of SUR1 subunit also can be envisioned.

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