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Studies of the effect of ionomycin on the KCNQ4 channel expressed in *Xenopus* oocytes

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Abstract

The effect of ionomycin on the human KCNQ4 channels expressed in *Xenopus leavis* oocytes was investigated. KCNQ4 channels expressed in *Xenopus* oocytes were measured using two-electrode voltage clamp. The activation of KCNQ4 current had slow activation kinetics and low threshold (~−50 mV). The expressed current of KCNQ4 showed the half-maximal activation ($V_{1/2}$) was −17.8 mV and blocked almost completely by KCNQ4 channel blockers, linopirdine (300 μM) or bepridil (200 μM). The significant increase of KCNQ4 outward current induced by ionomycin (calcium salt) is about 1.7-fold of control current amplitude at +60 mV and shifted $V_{1/2}$ by approximately −8 mV (from −17.8 to −26.0 mV). This effect of ionomycin could be reversed by the further addition of BAPTA-AM (0.3 mM), a membrane–permeable calcium chelator. Furthermore, the increased effect of ionomycin on KCNQ4 current is abolished by pretreatment of linopirdine or bepridil. In contrast, direct cytoplasmic injection of calcium medium (up to 1 mM calcium, 50 nl) did not mimic the effect of ionomycin. In conclusion, the effect of ionomycin on enhancement of KCNQ4 current is independent of intracellular calcium mobilization and possibly acts on intramembrane hydrophobic site of KCNQ4 protein expressed in *Xenopus* oocytes.

Keywords: KCNQ4; Ionomycin; Calcium; BAPTA-AM; Channel

KCNQ K^+ channels play an important role in regulating the membrane potential and function of many cell types in numerous tissues of the body [1]. The family of KCNQ channels was recently included in the Kv nomenclature as Kv7.1 to Kv7.5 [2]. KCNQ4 current is a low-threshold, non-inactivating K^+ current, which is expressed in the outer hair cell of cochlea, brain, heart, and skeletal muscle. Mutations in KCNQ4 channel produce inherited syndrome of deafness [3]. The deficit of KCNQ4 function might result in a chronic potassium overload of outer hair cells, causing their slow degeneration. KCNQ4 produces a potassium current similar to the M-current in the *Xenopus* expression system [3]. It has been known heteromeric KCNQ2 plus KCNQ3 channels contribute to the native M-current [4]. Previous studies have shown that intracellular calcium can modulate M currents, however, the modulated effect by calcium is conflicting. The modulated effect of KCNQ2/KCNQ3 by calmodulin is suggested by a Ca^{2+}-dependent [5] and -independent interaction [6]. M currents in mammalian neuron are inhibited but in amphibian are enhanced by $[Ca^{2+}]_i$. Recently report demonstrated that bradykinin modulation of native M-type-current is highly sensitive to Ca^{2+} and this effect is mediated by calcium binding protein calmodulin [7]. The increase of intracellular calcium destabilizes the resting potential and may result from impaired KCNQ function in inner hair cells of mouse cochlea [8]. However, the modulated effect of calcium on KCNQ4 currents is still unclear. Recently, report using

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CHO cells as an expressed system to examine the properties and regulation of KCNQ4 channel showed that the intracellular calcium up to 100 nM increased the run-down of KCNQ4 current [9]. In the present study we use the ionomycin, a calcium ionophore, to elevate the concentration of intracellular calcium of expressed oocytes. The result showed ionomycin enhances the KCNQ4 current which is expressed in *Xenopus* oocytes. The enhanced effect is reversed by the application of BAPTA-AM, a fast calcium chelator. Surprisingly, the intracellular injection of calcium (0.01–1 mM) into the cytoplasm directly did not change the KCNQ4 currents. These data demonstrated that the effect of ionomycin acts on intramembrane site of KCNQ4 protein and without relation to cytoplasmic calcium concentration.

Materials and methods

Human KCNQ4 cRNA in vitro transcription. The KCNQ4-containing PTLN vector was the kind gift of Dr. Thomas Jentsch in the *Xenopus* oocyte expression vector pTLN. After linearization of the KCNQ4-containing PTLN vector with *Hpa*I, capped cRNA was transcribed in vitro using the mMessage mMachine kit (Ambion).

Transient expression in Xenopus oocytes. Female *Xenopus laevis* frogs were anaesthetized in 0.1% sodium bicarbonate solution containing 0.15% tricaine (ethyl 3-aminobenzoate, methanesulfonic acid salt, Sigma–Aldrich). The ovarian lobe was then surgically removed from the abdominal cavity through a small (about 1 cm) insertion and placed in modified Barth's solution (MBS: 90 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, and 15 mM HEPES–Tris, pH 7.6). The oocytes were defolliculated enzymatically by incubation in collagenase (2 mg/ml, type 1, Sigma) in sterile MBS for 2 h followed by five to six washes in MBS containing 0.1% BSA (Sigma). Digested oocytes (Stage V or VI) were then incubated and kept overnight at 18 °C before injection with 50 nl mRNA (approx. 10–15 ng) using a Nanoject microinjector (Drummond, USA). All experiments conformed to the guidelines of the Animal Care Committee of the Chung Shan Medical University. Injected and non-injected oocytes were kept in sterile MBS (with gentamycin 50 mg/L) at 18 °C for 2–4 days before electrophysiological measurements were performed. The MBS medium was changed once a day.

Electrophysiology. Current through expressed KCNQ4 channels was recorded using a two-electrode voltage-clamp amplifier (AxoClamp-2B, Axon Instrument Inc., Foster City, CA, USA). Electrodes were pulled from borosilicate glass capillaries on a vertical electrode puller (Model PP-830, Narishige Scientific Instrument Lab, Japan) and had tip resistances between 0.5 and 2.0 MΩ when filled with 3 M KCl. One of the electrodes was used as a voltage recording which was connected to an HS-2 × 1 L headstage, and the other electrode was used for current recording connected to an HS-2 × 10 MG headstage. During the experiments oocytes were placed in a small chamber (volume, 3 ml). KCNQ4 channels were activated by membrane depolarization and channel activity was measured in ND 96 solution consisting of (mM): 96 NaCl, 1 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 5 HEPES; pH was 7.4. For the intracytoplasmic injection of intracellular calcium or ionomycin medium, the third glass electrode was used. Intracellular calcium or ionomycin medium for intracytoplasmic injection consisted of the following compound respectively (mM): 96 KCl 1 $MgCl_2$, 1 $CaCl_2$, and 5 HEPES or 96 KCl 1 $MgCl_2$, 0.1 ionomycin, 5 HEPES, pH was 7.4. The condition of each single oocyte was controlled before measurements by recording membrane potentials. Only oocytes with membrane potentials below −30 mV were used for current recordings. A steady current level was always obtained before electrophysiological analysis or drug application. All experiments were carried out at room temperature (22–26 °C). Data were digitized at 5 kHz and stored using Digidata 1322A (Axon Instruments) and analysis were accomplished with the pClamp 9.0 software (Axon Instruments). To determine the current/voltage (*I*/*V*) relations, a step protocol was employed, whereby the oocytes were clamped at −80 mV for 3 s and depolarized at +60 mV with 20 mV increments to −100 mV. Tail current analysis for conductance–voltage (*G*–*V*) relations was measured at −30 mV for 1 s.

Calculations. Steady-state activation curves were fitted to a two-state Boltzmann functions as follows:

$$I_{\text{tail}}(V_{\text{m}}) = I_{\text{tail(max)}}/\{1 - \exp[(V_{1/2} - V_{\text{m}})/k]\},$$

where V_{m} is the membrane potential, $V_{1/2}$ is the half-activation potential, and k is the slope factor. $I_{\text{tail(max)}}$ is the maximal tail current. Current and membrane potential levels are stated as means ± SEM, and differences in the mean were tested with Student's t test or ANOVA and P value <0.05 was accepted as significant.

Chemicals. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ionomycin (calcium salt) and BAPTA-AM were dissolved as a stock solution in dimethyl sulphoxide (DMSO). Linopirdine was dissolved in 0.1 N HCl to give a 25 mM stock solution. Bepridil hydrochloride was dissolved in dimethyl sulphoxide (DMSO) to make the stock solution at a concentration of 50 mM.

Results

Characteristics of KCNQ4 current expressed in Xenopus oocytes

Human KCNQ4 current heterogeneously expressed in *Xenopus* oocytes produced a low- threshold non-inactivating current (Fig. 1A). The expressed oocytes were voltage clamped at −60 mV and stepped to potentials ranging from −80 to +60 mV, which produce slowly activated currents as shown in Fig. 1A (upper traces). After the 2 s duration command pulse, the oocytes were clamped at −30 mV for tail current analysis. Tail current at −30 mV showed that the mean half-activation voltage was -17.6 ± 0.7 mV and k was 1.9 ± 0.1 (Fig. 2B), which are similar to previous reported that $V_{1/2}$ was -15.8 ± 2.3 mV and k was 1.8 ± 0.2 [10]. The expressed K^+ currents were sensitive to linopirdine, a KCNQ4 K^+ channel blocker (Fig. 3A). Linopirdine was not blocked by the background current of water-injected or uninjected oocytes.

Ionomycin, a calcium ionophore increase the KCNQ4 current

Ionomycin, a calcium ionophore, can increase the KCNQ4 current. Fig. 1B shows that the slope of current–voltage (*I*–*V*) relationship of KCNQ4 increased by addition of ionomycin (1 μM). The maximal enhanced effect of ionomycin when step to +60 mV was 173 ± 7% of the KCNQ current. Fig. 2A illustrates the time course of the ionomycin-induced KCNQ4 current increase, the current amplitude was gradually increased and significantly at 5 min, then reached a maximum at 10 min exposure to ionomycin. We recorded tail current amplitudes at −30 mV and plotted the conductance–voltage (*G*–*V*) curves obtained before and after ionomycin stimulation. The activation curve was

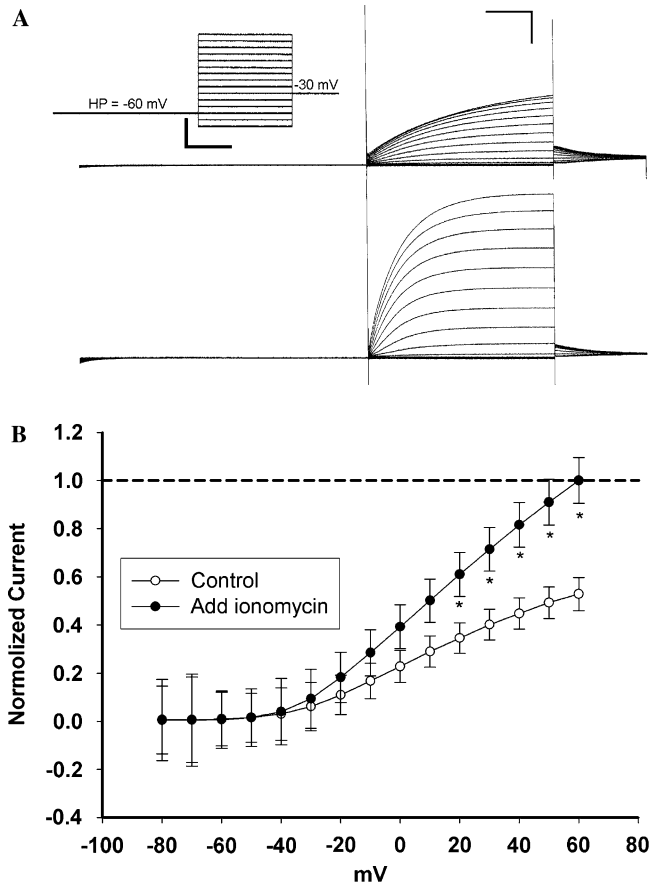

Fig. 1. Effect of ionomycin on transient expression of the human voltage-gated K^+ channel KCNQ4 in *Xenopus* oocytes. (A) Representative current traces recorded from *Xenopus* oocytes expressed with KCNQ4 (upper traces) and after a 10 min application of 1 μM ionomycin (lower traces) in response to a step protocol as follows: oocytes were clamped at −60 mV for 3-s and the channel activated by a 2-s command step from −100 to +60 mV in 10 mV increments, followed by a 1-s step to +30 mV. Left trace is the voltage step protocol. (B) Current/voltage (I/V) relationship for the KCNQ4 channels in the absence (open circles, $n = 15$) or presence (filled circles, $n = 6$) of ionomycin (1 μM). Current amplitudes are normalized to the mean maximum current amplitude (dotted-line) obtained at +60 mV after ionomycin-treatment. $^*P < 0.05$ as compared with the before ionomycin treatment. Calibrations in (A): right, 0.5 s and 1 μA; left, 1 s and 50 mV.

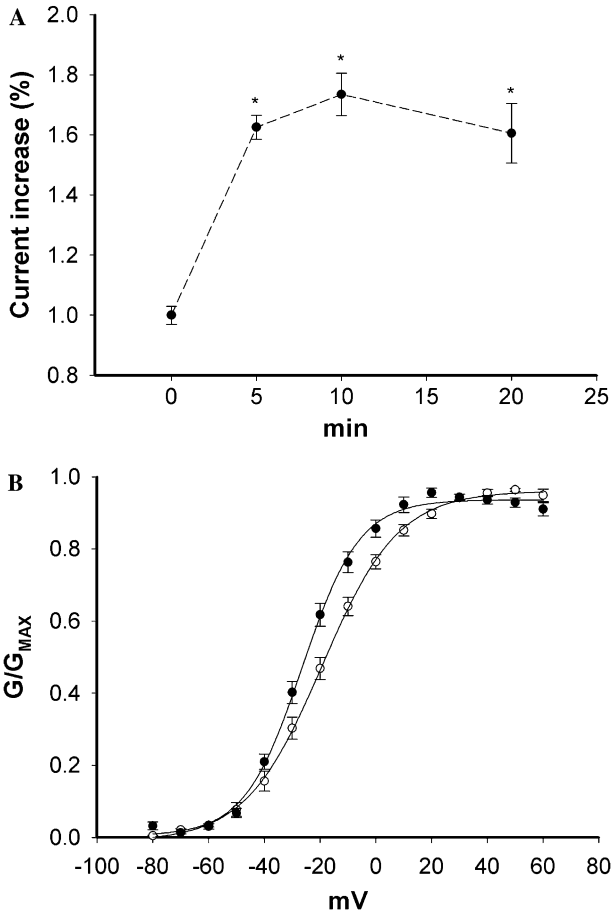

Fig. 2. The time course and conductance/voltage (G–V) relationship of ionomycin-induced activation of KCNQ4 currents. (A) On-line analysis showing that ionomycin enhances the KCNQ4 current reaching the maximal effect after 10 min ($n = 5$). The current amplitude was measured at the end of the 2-s voltage step at +60 mV. $^*P < 0.05$ as compared with the before ionomycin-treatment. (B) G–V curves for KCNQ4 channels before (open symbols) and after (filled symbols) ionomycin stimulation ($n = 5$). G–V curves were fitted using a two-state Boltzmann equation as described in Materials and methods.

then fitted by the Boltzmann equation (see Materials and methods). Ionomycin (1 μM) induced a G–V curve negatively shift from -17.6 ± 0.6 to -26.0 ± 0.8 mV (Fig. 2B). Ionomycin had no effect on the background current amplitudes of water (~50 nl)-injected oocytes (data no shown).

KCNQ4 blockers abolish the enhanced effect of ionomycin

Fig. 3 shows the normalized current–voltage relationship of KCNQ4 currents. KCNQ4 blockers, linopirdine (300 μM) and bepridil (200 μM), blocked outward currents at +60 mV by 80 ± 4% and 80 ± 5%, respectively (Fig. 3). Pretreatment of linopirdine (300 μM) for 10 min blocked the KCNQ4 K^+ current mostly, subsequent application of ionomycin (1 μM) had no effect on the KCNQ4 K^+ current (Fig. 3A). Another KCNQ4 current blocker, bepridil (200 μM), has a similar effect with linopirdine, preventing the enhanced effect of ionomycin on KCNQ4 current (Fig. 3B).

Effect of BAPTA-AM on the enhanced current induced by ionomycin

BAPTA-AM, a membrane–permeable calcium chelator, inhibits the enhanced component of KCNQ4 tail currents (at +30 mV) induced by ionomycin (Fig. 4A). The left-shifted G–V curve of KCNQ4 current induced by ionomycin was partially reversed by the further addition of BAPTA-AM (0.3 mM). The midpoint of the G–V curve was -26.0 ± 0.8 mV for ionomycin and -21.5 ± 0.9 mV for the further addition of BAPTA-AM (Fig. 4B).

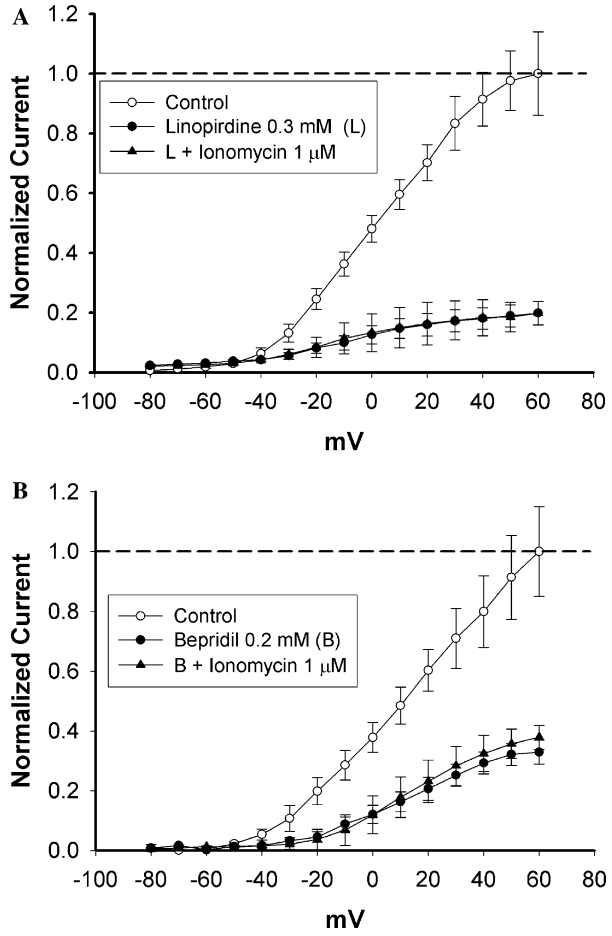

Fig. 3. KCNQ4 blocker, either linopirdine (0.3 mM), or bepridil (0.2 mM), blocked the increasing effect of KCNQ4 current induced by ionomycin. The oocytes were treated by KCNQ4 blocker, linopirdine [(A), $n = 5$ or bepridil (B), $n = 6$], for 10 min and subsequently addition of ionomycin for further 10 min (1 μM, filled triangle symbols). Both KCNQ4 blockers prevent the current increased by the further addition of ionomycin. Current amplitudes are normalized to the mean maximum current amplitude (dotted-line) obtained at +60 mV before drug treatment. No significant difference between KCNQ4 blocker treated (filled circle symbols) and the further addition of ionomycin (filled triangle symbols).

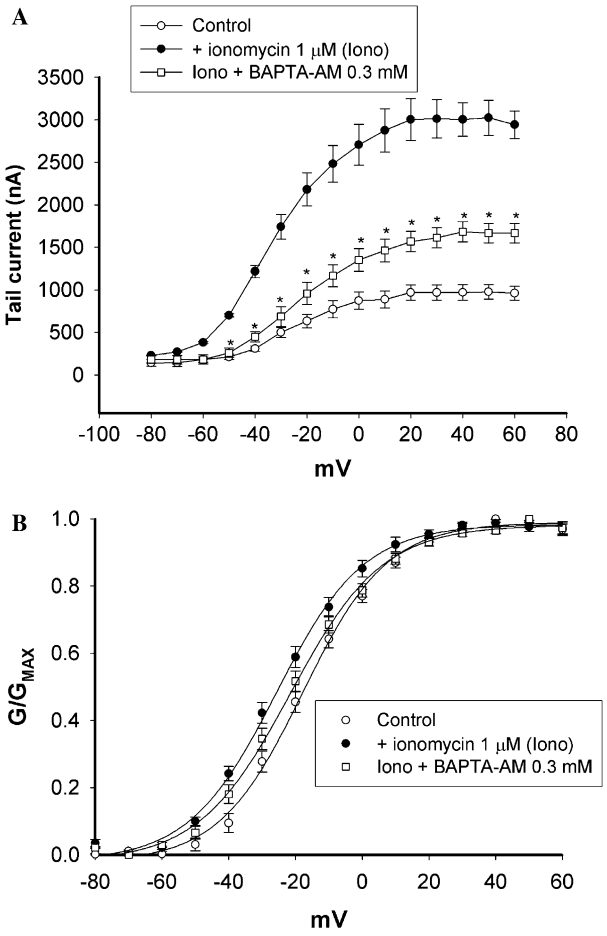

Fig. 4. Effect of BAPTA-AM on the enhanced KCNQ4 current induced by ionomycin. (A) The tail current–voltage relationship in control condition (before drug treatment: open circle) and after ionomycin (1 μM) treatment for 10 min (filled circle) and subsequently addition of BAPTA-AM (0.3 mM; $n = 5$) for further 10 min (open square). $^*P < 0.05$ as compared between ionomycin treatment (filled circle) and ionomycin plus BAPTA-AM (open square). (B) *G–V* curves were determined from the tail current analysis and fitted using a two-state Boltzmann equation.

Effect of intracellular injection of calcium or ionomycin medium on the KCNQ4 current

To test whether the intracellular calcium can modulate the KCNQ4 current directly, the method of intracytoplasmic injection of calcium (50 nl) medium to sub-membrane space of oocytes was used to increase intracellular calcium levels directly. We did not observe the significant modulatory effect on KCNQ4 current by the injection of calcium medium from 0.01 to 1 mM (50 nl). The current amplitude and *I–V* current were not changed by the injection of calcium buffer (1 mM) after 10 min (Fig. 5A). However, the increased effect of KCNQ4 current still can be observed after 10 min of intracytoplasmic injection of ionomycin medium. The current amplitude was increased by 120% at +40 mV (Fig. 5B), and midpoint of *G–V* current was negatively shifted by about 7 mV (from -18.3 ± 0.8 to -25.0 ± 0.7 mV, $n = 5$) after the injection of ionomycin (0.1 mM, 50 nl). The composition of intracellular calcium or ionomycin medium for intracytoplasmic injection is as described in Materials and methods.

Discussion

In this study, we have found that ionomycin was able to increase the KCNQ4 current and shift the midpoint of *G–V* current to left, these effects can reverse by the addition of BAPTA-AM, a membrane–permeable calcium chelator. Since the properties of KCNQ4 current did not change by the direct intracytoplasmic injection of calcium medium, the action site of ionomycin is probably in the intramembrane or a hydrophobic site within KCNQ4 protein without relation to cytoplasmic calcium level. Both ionomycin and BAPTA-AM are hydrophobic and membrane–permeable compounds, they can enter the hydrophobic site of KCNQ4 protein easily rather than calcium medium, a hydrophilic solution.

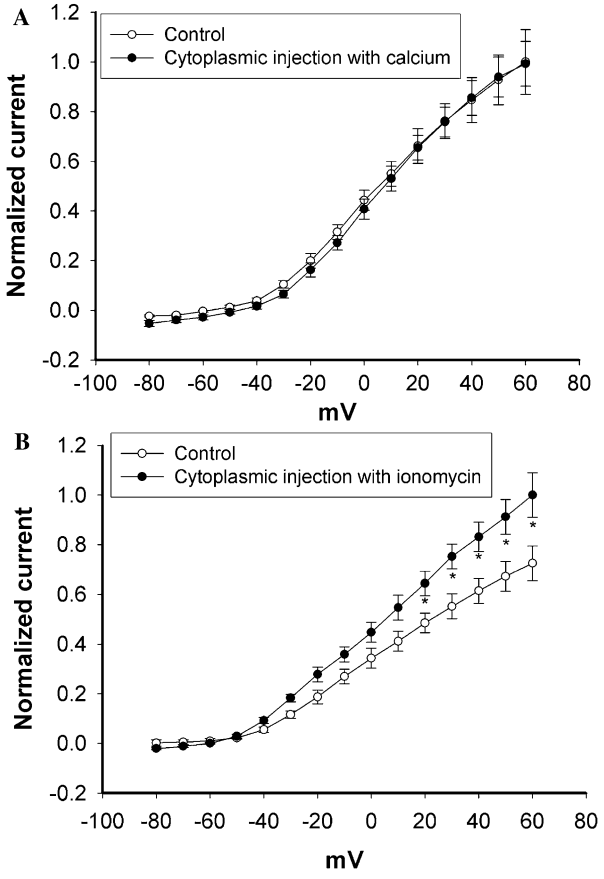

Fig. 5. Effects of cytoplasmic injection of intracellular calcium and ionomycin medium on KCNQ4 currents. KCNQ4 currents were recorded before (open circle symbols) and after 10 min cytoplasmic injection of 50 nl either intracellular calcium [1 mM, (A), filled circle; $n = 5$) or ionomycin medium (0.1 mM, (B), filled circle; $n = 5$] into *Xenopus* oocytes expressed with KCNQ4. Current amplitudes are normalized to the mean maximum current amplitude obtained at +60 mV after calcium (A) or ionomycin-injected (B). $^*P < 0.05$ as compared with before ionomycin injection as shown in (B).

The regulated role of calcium and calmodulin on M-like (KCNQ2 + KCNQ3) or native M current has been widely studied previously. M-channel activity in mammalian sympathetic neurons is tonically regulated by intracellular calcium [11], the channel activity increased during application of calcium-free solution and was rapidly reduced on applying calcium-containing medium. The IC_{50} of M channels by calcium in rat sympathetic neurons is approximal 100 nM [12]. M-type current highly sensitive to intracellular calcium and that calmodulin acts as their calcium senor [7]. The overexpression of calmodulin gives rise to the reduction of basal KCNQ2/3 currents in CHO cells or M current in SCG neurons, suggesting that overexpression of calmodulin creates more calcium bound calmodulin molecules able to interact with the channels, thus increasing tonic calmodulin action. Therefore, the increase of cytoplasmic calcium leads to a significant rundown of M-like current by calmodulin in CHO expression system. However, the sensitivity of KCNQ4 currents to calcium has not been extensively studied. In the *Xenopus* expression system, KCNQ4 produces a potassium current similar to the M-current, but recordings of KCNQ4 channels expressed in *Xenopus* oocytes suggest that there may also be incongruities such as a sensitivity to M-current blockers being too low and an activation threshold that is too positive [3].

In the present studies the human KCNQ4 channels expressed in *Xenopus* oocytes produced slow activated and deactivated currents similar to previous report [3]. The activation threshold is about −50 mV and half-activation at −17.8 mV which is close to other report at −15 mV [10] but slightly different from −10 mV [3]. KCNQ4 transfected in CHO cell the half-activation voltage is more negative than expressed in *Xenopus* oocytes ranging from −18 to −32 mV [9,13], these may be due to the different expression systems used. The recent report showed that the KCNQ4 channels are inhibited by the increase of intracellular calcium in CHO expression system [9]. Inhibition of calmodulin by W-7 prevented the calcium-dependent rapid run-down, it suggested the role of calcium-binding protein in the regulation of KCNQ4 channel. Surprisingly, our result showed that the KCNQ4 channels expressed in *Xenopus* oocytes were unaffected by the increase of intracellular calcium by using the direct intracytoplasmic injection of calcium solution (from 0.01 to 1 mM, 50 nl). Although the volume *Xenopus* laevis oocytes (about 900 nl for a diameter of 1.2 mm) are much larger and cytoplasmic velocity possibly higher than CHO cells, the direct injection of 50 nl, 1 mM calcium into oocytes should be sufficient to reach hundreds of nanomolar levels of calcium in the area of sub-plasma membrane after 10 min. Another unexpected finding in our study was that ionomycin significantly increases the KCNQ4 current amplitude and shifts the G–V curve to the left, the midpoint of voltage being −17.8 mV before and −26 mV after the application of ionomycin. Since these effects of ionomycin were abolished by pretreatment of the oocytes with KCNQ4 blocker, linopirdine or bepridil [14], the enhanced current by ionomycin is considered as a KCNQ4 component. Furthermore, the enhanced effect of ionomycin on KCNQ4 current can be reversed by the further addition of BAPTA-AM. Interestingly, previous studies showed that raised cytoplasmic Ca^{2+} produced an inhibition effect on expressed KCNQ4 current in CHO cells [9] and M currents in mammalian sympathetic neurons [11,12]. By contrast, recent studies showing that KCNQ1 and I_{KS} (KCNQ1/KCNE1) currents are stimulated by increases in intracellular Ca^{2+} and are markedly inhibited by calmodulin antagonists [15]. The calcium-induced increase in cardiac I_{KS} currents plays a significant role in shortening action potential duration [16] and this calcium-induced I_{KS} stimulation was sensitive to the calmodulin antagonist. Therefore, calcium probably exerts a dual effect on KCNQ channel by different modulatory pathway. As far as we know, until now at least, we are first finding that ionomycin can increase the activity of KCNQ4 current and the effect might not be relative to the level of intracellular calcium. In conclusion, ionomycin

can increase the current amplitude and reduce the activation threshold of KCNQ4 but the direct increase of intracellular calcium by intracellular injection did not change these properties of KCNQ4 current. The action mechanism of ionomycin on the KCNQ4 current has two possible pathways based on our experimental results. First, the action of ionomycin is to direct by bind to a specific site of KCNQ protein dependent on its specific structure without relation to the properties of calcium ionophore. Second, ionomycin penetrates into plasma membrane allowing calcium to bind to hydrophobic site of KCNQ4. BAPTA-AM, a membrane–permeable calcium chelator, therefore, can reverse the effect of ionomycin.

Acknowledgments

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