Effects of Sodium Azide, Barium Ion, \textit{d-}Amphetamine and Procaine on Inward Rectifying Potassium Channel 6.2 Expressed in \textit{Xenopus} Oocytes

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\textbf{Background/Purpose:} Inward rectifying potassium channel 6.2 (Kir6.2ΔC26 channel) is closely related to ATP-sensitive potassium channels. Whether sodium azide, barium ion, \textit{d-}amphetamine or procaine acts directly on the Kir6.2ΔC26 channel remains unclear. We studied the effects of these compounds on Kir6.2ΔC26 channel expressed in \textit{Xenopus} oocytes.

\textbf{Methods:} The coding sequence of a truncated form of mouse Kir6.2 (GenBank accession number NP_034732.1), Kir6.2\textsubscript{1–364} (i.e. Kir6.2ΔC26), was subcloned into the pET20b\textsuperscript{(+)} vector. Plasmid containing the correct T7 promoter-Kir6.2\textsubscript{1–364} cDNA fragment [Kir6.2/pET20b\textsuperscript{(+)}] was then subject to NotI digestion to generate the templates for \textit{in vitro} run-off transcriptions. The channel was expressed in \textit{Xenopus laevis} oocytes. Two-electrode voltage clamping was used to measure the effects of sodium azide, barium ion, \textit{d-}amphetamine and procaine on Kir6.2ΔC26 channel current.

\textbf{Results:} Sodium azide activated and barium ion and \textit{d-}amphetamine inhibited the Kir6.2ΔC26 channel. Procaine did not have any significant effect on the Kir6.2ΔC26 channel.

\textbf{Conclusion:} Kir6.2ΔC26 channel expressed in \textit{Xenopus} oocytes can be used as a pharmacological tool for the study of inward rectifying potassium channels. [\textit{J Formos Med Assoc} 2008;107(8):600–608]

\textbf{Key Words:} amphetamine, barium ion, inward rectifying potassium channel, Kir6.2, $K_{\text{ATP}}$, mRNA, procaine, sodium azide, \textit{Xenopus} oocyte

Inwardly rectifying potassium channels (Kir channels) control cell membrane $K^+$ fluxes and electrical signaling in diverse cell types.\textsuperscript{1} Inward rectifying potassium channel 6.2 (Kir6.2ΔC26 channel) is closely related to ATP-sensitive potassium channels ($K_{\text{ATP}}$ channels). Whether sodium azide, barium ion, \textit{d-}amphetamine or procaine acts directly on the Kir6.2ΔC26 channel remains unclear. The aim of the present study was to understand the effects of sodium azide, barium ion, \textit{d-}amphetamine and procaine on the Kir6.2ΔC26 channel expressed in \textit{Xenopus} oocytes. We found that sodium azide activated, while barium ion and \textit{d-}amphetamine repressed the mouse Kir channel (Kir6.2/pET20b+ cDNA) expressed in \textit{Xenopus} oocytes. Procaine, however, did not show any significant effect on the

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\textbf{Received:} October 11, 2007 \textbf{Revised:} March 3, 2008 \textbf{Accepted:} April 10, 2008

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Effects of sodium azide, barium ion, d-amphetamine and procaine on Kir6.2 channel. It is concluded that the Kir6.2ΔC26 channel expressed in Xenopus oocytes can be used as a pharmacological tool for the study of Kir channels.

Materials and Methods

Preparation of cRNA by in vitro run-off transcription

The coding sequence of a truncated form of mouse Kir6.2 (GenBank accession number NP_034732.1), Kir6.2_{1–364} (i.e. Kir6.2ΔC26), which lacks the C-terminal 26 amino acid residues of mouse Kir6.2, and shares 96% amino acid identity with human Kir6.2_{1–364}^2 was subcloned into the pET20b(+) vector (Novagen, Madison, WI, USA). The sequence of the selected plasmid was confirmed by DNA sequencing (Core Facility, National Taiwan University). Plasmid containing the correct T7 promoter-Kir6.2_{1–364} cDNA fragment [Kir6.2/pET20b(+)] was then subjected to NotI digestion to generate the templates for in vitro transcription. T7 RNA polymerase (Takara, Shiga, Japan)-based in vitro transcription was conducted following standard procedures. The resulting RNA transcript was phenol/chloroform extracted, ethanol precipitated, and resuspended at an appropriate concentration.

Oocyte collection and preparation

Adult female Xenopus laevis (African Xenopus Facility, Knysna, South Africa) were anesthetized by immersion in tricaine (1.5 g/L). Ovarian follicles were removed from Xenopus frogs, cut into small pieces, and incubated in ND96 solution containing: 96 mM NaCl, 2 mM KCl, 1.8 mM MgCl₂, 1.8 mM CaCl₂, and 5 mM HEPES (pH 7.4). To remove follicular membrane, Xenopus oocytes were incubated in Ca^{2+}-free ND96 containing collagenase (2 mg/mL) on an orbital shaker (200 rpm) for 60–90 minutes at room temperature. After several washes with Ca^{2+}- and collagenase-free ND96, oocytes were transferred to ND96. Stage V–VI Xenopus oocytes were selected and stored at 18°C in ND96 supplemented with 50 mg/mL gentamicin.

Electrophysiologic recording and analysis

Oocytes were injected with 41.6 nL (3–5 ng/nL) Kir6.2Δ36 cRNA and incubated at 18°C in ND96 solution with gentamicin (50 ng/mL). Whole-cell currents were studied in the oocytes 2–5 days after injection. Two-electrode voltage clamping was performed using an amplifier (Gene Clamp 500; Axon Instruments, Molecular Devices, Sunnyvale, CA, USA) at room temperature (−24°C). The extracellular solution contained: 90 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂ and 5 mM HEPES (pH 7.4 with KOH). The microelectrodes were filled with 3.0 M KCl, and the recording and current electrode had a resistance of 1.0–2.0 and 0.3–0.6 MΩ, respectively. The currents were elicited by 500-ms command steps from holding potentials of −10 mV to stepping potentials (i.e. from −120 to +30 mV) at intervals of 10 mV. The steady-state currents were measured at 500 ms after changing from holding to test potentials. Niflumic acid (250 μM) was added to the chamber to avoid interference with calcium-activated chloride currents. All potentials and currents were recorded on tape via a digitalizing unit (Digidata 1200; Axon Instruments, Molecular Devices) and analyzed using a pCLAMP system (Axon Instruments, Molecular Devices).

The amplitude and currents after various treatments were compared with controls by means of the Student’s paired t test. Differences were considered significant at p < 0.05. To measure the extent of drug inhibition of ion currents, the Hill equation was used: \[ \frac{I}{I_C} = \frac{1}{1 + \left(\frac{x}{K_i}\right)^N} \], where I is the measured steady-state current with drug application, \( I_C \) is the measured steady-state current without drug application, x is the drug concentration, Ki is the drug concentration which inhibited half-measured steady-state current, and N is the Hill coefficient.

Results

Sequence of recombinant DNA of Kir6.2

To confirm the accuracy of recombinant DNA with Kir6.2/pET20b(+), DNA sequencing was...
performed in the Core Facility of National Taiwan University. The sequence of Kir6.2/pET20b(+) was checked against the GenBank database by BLAST from the National Center for Biotechnology Information (NCBI). The results showed that the sequence of Kir6.2/pET20b(+) did indeed belong to the coding sequence of \textit{Mus musculus} Kir channel, subfamily J, member 11 (GenBank accession number: NM_010602.2). The information is from the National Center for Biotechnology Information website.)

**Figure 1.** Kir6.2ΔC26 DNA sequence contains 1092 base pairs, which was confirmed by DNA sequencing (Core Facility, National Taiwan University). The coding sequence of a truncated form of Kir6.2 was from Kir channel, subfamily J, member 11 (\textit{Mus musculus}). (GenBank accession number: NM_010602.2. The information is from the National Center for Biotechnology Information website.)

Effect of sodium azide on Kir6.2ΔC26

Two-electrode voltage-clamping with 500-ms long-stepping pulses was used to measure the steady-state currents of Kir6.2ΔC26 channel in \textit{Xenopus} oocytes. Currents were obtained by stepping from a holding potential of 0 mV to testing potentials of −120 to +50 mV at intervals of 10 mV. As shown in Figure 3, the steady-state currents were measured at 500 ms after voltage stepping, and the steady-state current-voltage (I–V) relationships are shown in Figure 3E.

Before applying sodium azide, the amplitude of steady-state current was 581.6 ± 22.0 nA at −100 mV. After applying 3 mM sodium azide for 20 minutes, the amplitude of measured steady-state current was 876.0 ± 15.3 nA at −100 mV. The Kir6.2ΔC26 potassium current was significantly increased ($n = 3, p < 0.05$) by sodium azide. The effect of sodium azide on the Kir6.2ΔC26 channel was reversible. After 60 minutes of continuous washing, the effect of sodium azide on Kir6.2ΔC26 potassium current was significantly recovered to control level (Figure 3C). It appeared that sodium azide reversibly increased the ionic currents of the Kir6.2ΔC26 channel expressed in \textit{Xenopus} oocytes.

**Effect of barium ions on Kir6.2ΔC26**

The effects of barium ions on the Kir6.2ΔC26 channel expressed in \textit{Xenopus} oocytes were tested. Five-hundred-millisecond long-stepping pulses...
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were used to measure the steady-state currents of Kir6.2ΔC26 channel in Xenopus oocytes. Currents were obtained by stepping from a holding potential of −10 mV to testing potentials of −120 to +50 mV at intervals of 10 mV. The effects of barium ions on the steady-state currents and the steady-state I–V relationships are shown in Figure 4. The mean whole-cell current is shown in Figure 5. The steady state current after 30 minutes of BaCl2 treatment is shown in Figure 5A. Holding at −120 mV, the Kir6.2ΔC26 potassium current was significantly decreased (n = 3, p < 0.05) by BaCl2. The concentration of BaCl2 which inhibited half the Kir6.2ΔC26 potassium current (IC50) was 224.2 μM.

**Effect of procaine on Kir6.2ΔC26**

The effect of procaine on the Kir6.2ΔC26 channel expressed in Xenopus oocytes is shown in Figure 6. Currents were obtained by stepping from a holding potential of −10 mV to testing potentials of −120 to +100 mV at intervals of 10 mV. Effects of various concentrations of procaine (1, 3, 10, 30 mM) on Kir6.2ΔC26 channels expressed in Xenopus oocytes are shown in Figure 6B–E. The steady-state currents were measured at 500 ms after voltage stepping and the steady-state I–V relationships are shown in Figure 6G. The amplitude of the steady-state current in controls and 30 minutes after treatment with 1, 3, 10 or 30 mM procaine was 854.6 ± 0.9 nA and 827.3 ± 27.0, 831.7 ± 2.0 and 748.0 ± 32.0 nA at −120 mV, respectively. The Kir6.2ΔC26 potassium current was not significantly decreased (n = 3, p > 0.05) by procaine. It appeared that procaine had no significant effect on Kir6.2ΔC26 potassium current expressed in Xenopus oocytes.
Figure 4. Effects of BaCl2 on Kir6.2ΔC26 channels in Xenopus laevis oocytes. Kir6.2ΔC26 channels were elicited by a 500-ms duration command step from a holding potential of −10 mV to test potentials of −120 to +50 mV, at intervals of 10 mV. (A) Controls, Kir6.2ΔC26 channels in 90 mM K+ solution. (B) Kir6.2ΔC26 channels recorded 30 minutes after incubation with 100 μM BaCl2. (C) Kir6.2ΔC26 channels recorded 30 minutes after incubation with 300 μM BaCl2. (D) Kir6.2ΔC26 channels recorded 30 minutes after incubation with 1 mM BaCl2. (E) Voltage step commands. (F) I–V relationships of Kir6.2ΔC26 channels in Xenopus oocytes before (●), and after BaCl2 at 100 μM (●●), 300 μM (●●●) or 1 mM (●●●).
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**Effect of \textit{d}-amphetamine on Kir6.2\text{Δ}C26**

Two-electrode voltage clamping was used to test whether \textit{d}-amphetamine affected Kir6.2\text{Δ}C26 channels expressed in oocytes. Five-hundred-millisecond long-stepping pulses were used to measure the steady-state currents of Kir6.2\text{Δ}C26 channels in \textit{Xenopus} oocytes. Currents were obtained by stepping from a holding potential of 0 mV to testing potentials of -120 to +50 mV at intervals of 10 mV. The steady-state currents were measured at 500 ms after voltage stepping and the steady-state I–V relationships are shown in Figure 7J. The effect of \textit{d}-amphetamine (300 μM, or 1, 5, 63 or 80 mM) for 30 minutes on Kir6.2\text{Δ}C26 steady-state current is shown in Figure 8A. The Kir6.2\text{Δ}C26 potassium current was significantly decreased (n = 3, p < 0.05) by \textit{d}-amphetamine (5, 63 or 80 mM) at -120 mV, but not by 300 μM or 1 mM \textit{d}-amphetamine. The concentration of \textit{d}-amphetamine which inhibited half the Kir6.2\text{Δ}C26 potassium current (IC\textsubscript{50}) was 83.89 mM, as shown in Figure 8B. After washing with normal saline for 60 minutes, the effect of \textit{d}-amphetamine on Kir6.2\text{Δ}C26 potassium current was significantly recovered to control level (Figure 7H). It appeared that \textit{d}-amphetamine reversibly decreased Kir6.2\text{Δ}C26 potassium current expressed in \textit{Xenopus} oocytes.

**Discussion**

The \textit{K\textsubscript{ATP}} channel plays an important role in physiologic and pathophysiologic functions such as insulin secretion and epilepsy. The \textit{K\textsubscript{ATP}} channel senses metabolic changes in pancreatic β-cells, thereby coupling metabolism to electrical activity and ultimately to insulin secretion.\textsuperscript{6} \textit{K\textsubscript{ATP}} channels play an important role in the neuronal excitability and epileptogenesis.\textsuperscript{7}

In the present study, we expressed mouse Kir with a vector (Kir6.2/pET20b+ cDNA) which lacked the C-terminal 26 amino acid residues in \textit{X. laevis} oocytes. The Kir6.2 sequence of the selected plasmids was confirmed by DNA sequencing (Core Facility, National Taiwan University). The carboxy-truncated Kir6.2 (Kir6.2\text{Δ}C26) has been shown to function independently of sulfonylurea receptor, and is therefore an ideal
tool for studying the intrinsic characteristics of Kir6.2.8,9

Sodium azide is a metabolic inhibitor that inhibits cytochrome c oxidase and decreases the cellular concentration of ATP. Barium ions are a KATP channel inhibitor, which bind to the external mouth of the KATP channel and decrease the channel current. In the present study, we found that sodium azide activated, while extracellular application of BaCl2 inhibited, the Kir6.2/H9004C26 channel.

Figure 7. Effects of d-amphetamine on Kir6.2ΔC26 channels in Xenopus oocytes. Kir6.2ΔC26 channels were elicited by a 500-ms duration command step from a holding potential of 0 mV to test potentials of −120 to +50 mV, at intervals of 10 mV. (A) Controls, Kir6.2ΔC26 channels in 90 mM K+ solution. (B–G) Kir6.2ΔC26 channels recorded 30 minutes after incubation with d-amphetamine 300 μM, or 1, 5, 30, 63 or 80 mM, respectively. (H) 60 minutes after washing the preparation from G with 90 mM K+ solution. (I) Voltage step commands. (J) I–V relationships of Kir6.2ΔC26 channels in Xenopus oocytes before (●), and after application of d-amphetamine at 5 mM (○), 30 mM (●), 63 mM (○) or 80 mM (●), and after washing (▲).

Figure 8. Effects of d-amphetamine on the amplitude of Kir6.2ΔC26 channel currents in Xenopus laevis oocytes. (A) Mean whole-cell currents recorded at −120 mV before (control) and 30 minutes after application of d-amphetamine 300 μM, or 1, 5, 30, 63 or 80 mM, respectively. Data are expressed as mean ± SEM. *p < 0.05 vs. control. (B) Relationship between d-amphetamine concentration and whole-cell Kir6.2ΔC26 channel current (I), expressed as a fraction of that in the absence of the drug (Ic). Data are from two experiments and are expressed as mean ± SEM. The solid line is a fit to the Hill equation with a Ki of 83.9 mM.
potentials on central snail neurons, and the effect is associated with adenyl cyclase and phospholipase activity in the neurons, respectively.\(^{10,11}\) The bursts of potential elicited by procaine are not blocked by administration of: (1) prazosin, propranolol, atropine and \(d\)-tubocurarine; (2) calcium-free solution; (3) ryanodine; or (4) pretreatment with KT-5720 or chelerythrine. The bursts of potential elicited by procaine are blocked by adding 10 \(\mu\)M U73122, and they are decreased if physiologic sodium ions are replaced with lithium ions, or incubated with 3.5 mM neomycin or 30 mM magnesium solution. Pretreatment with 10 \(\mu\)M U73122 blocks initiation of bursts of potential. Ruthenium red (100 \(\mu\)M) or caffeine (10 mM) facilitates the procaine-elicited bursts of potential. This effect is not directly related to: (1) extracellular calcium ion fluxes; (2) ryanodine-sensitive calcium channels in the neurons; or (3) protein kinase C- or protein kinase A-related messenger systems. The procaine-elicited bursts of potential are associated with phospholipase activity and calcium mobilization in the neurons.\(^{10,11}\) In the present study, we also found that procaine did not alter the I–V relationship of the Kir6.2ΔC26 channels expressed in oocytes. This suggests that procaine does not directly affect the Kir6.2ΔC26 channel.

\(d\)-Amphetamine elicited bursts of potential in the central snail neuron, and the effects were closely related to intracellular second messengers, e.g. protein kinase A, phosphodiesterase and phosphatase activity.\(^{11-17}\) In the present study, we also found that \(d\)-amphetamine decreased the I–V relationship of the Kir6.2ΔC26 channel. This suggests that \(d\)-amphetamine directly affects the Kir6.2ΔC26 channel.

\(K_{\text{ATP}}\) channels control electrical signaling in diverse cell types by coupling cellular metabolism to potassium movement across cell membranes. The \(K_{\text{ATP}}\) channel and phosphatase blocker 2,3-butanedione monoxime elicited bursts of potentials on the RP4 central neurons of giant African snails. Glibenclamide and 5-hydroxydecanoic acid are also \(K_{\text{ATP}}\) channel blockers. Extracellular application of 300 mM glibenclamide or intracellular injection of 5-hydroxydecanoic acid also elicited bursts of potential in RP4 neurons.\(^{18}\)

\(K_{\text{ATP}}\) channels are heteromultimer complexes of subunits from members of the Kir channel and ATP-binding cassette protein superfamilies. \(K_{\text{ATP}}\) channels couple the metabolic state to membrane excitability, are distributed widely, and participate in a variety of physiologic functions.\(^{19}\) Under normoxic conditions, these channels are closed, but they become active when intracellular ATP level falls. This leads to a shortening of the action potential duration, which renders the heart susceptible to life-threatening arrhythmia.\(^{20}\) In the central nervous system, the substantia nigra pars reticulata, the brain area with the highest expression of \(K_{\text{ATP}}\) channels, plays a pivotal role in suppressing the propagation of generalized seizures by its silence.\(^{21,22}\) Mice that lack the Kir6.2 channel subunit are extremely susceptible to generalized seizures after brief hypoxia.\(^{22}\) The opening of \(K_{\text{ATP}}\) channels is responsible for reducing the action potential frequency for neuronal protection, which might be a negative feedback mechanism. It has been suggested that the epileptogenic properties of pentylentetrazol may be mainly due to the activation of \(K_{\text{ATP}}\) channels.\(^{23}\) In the present study, we found that \(d\)-amphetamine inhibited Kir6.2ΔC26 potassium current. It is concluded that Kir6.2ΔC26 (Kir6.2/pET20b+ cDNA) channels expressed in Xenopus oocytes are sensitive to sodium azide, barium ions and \(d\)-amphetamine, while they are not affected by procaine treatment. Kir channels play an important role in the epileptogenic process. The inhibitory effect of amphetamine on \(K_{\text{ATP}}\) channels may contribute to induction of neuronal bursting, although this needs further investigation. It is suggested that Kir6.2ΔC26 channels expressed in Xenopus oocytes can be used as a pharmacological tool to study Kir channels.

**Acknowledgments**

We are grateful to Dr R.L. Walsh (Research Technology Branch, National Institute of Drug Abuse,
USA) for the generous supply of d-amphetamine. This work was supported by grant number NSC 96-2320-B-002-063 from the National Science Council, Taipei, Taiwan.

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