Amoxapine Inhibits the Delayed Rectifier Outward \( K^+ \) Current in Mouse Cortical Neurons via cAMP/Protein Kinase A Pathways

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Received July 30, 2009; accepted November 12, 2009

ABSTRACT

Ion channels are known to be modulated by antidepressant drugs, but the molecular mechanisms are not known. We have shown that the antidepressant drug amoxapine suppresses rectifier outward \( K^+ \) currents in mouse cortical neurons. At a concentration of 10 to 500 \( \mu M \), amoxapine reversibly inhibited \( I_{Kr} \) in a dose-dependent manner and modulated both steady-state activation and inactivation properties. The application of forskolin or dibutyryl cAMP mimicked the inhibitory effect of amoxapine on \( I_{Kr} \) and abolished further inhibition by amoxapine. \( N\)-[2-(p-Bromocinnamylamino)ethyl]-5-iso-quinolinesulphonamide (H-89), a protein kinase A (PKA) inhibitor, augmented \( I_{K} \) amplitudes and completely eliminated amoxapine inhibition of \( I_{Kr} \). Amoxapine was also found to significantly increase intracellular cAMP levels. The effects of amoxapine on \( I_{Kr} \) were abolished by preincubation with 5-hydroxytryptamine (5-HT) and the antagonists of 5-HT\(_2\) receptor. Moreover, intracellular application of guanosine 5’-[\( \gamma \)-thio]-triphosphate increased \( I_{K} \) amplitudes and prevented amoxapine-induced inhibition. The selective Kv2.1 subunit blocker Jingzhaotoxin-III reduced \( I_{K} \) amplitudes by 30% and also significantly abolished the inhibitory effect of amoxapine. Together these results suggest that amoxapine inhibits \( I_{K} \) in mouse cortical neurons by cAMP/PKA-dependent pathway associated with the 5-HT receptor, and suggest that the Kv2.1 \( \alpha \)-subunit may be the target for this inhibition.

Tricyclic antidepressants (TCAs), named after their three-ring molecular core structure, were the first successful antidepressants and have been widely used for the treatment of depression and other psychiatric disorders (Chen et al., 2004; Gillman, 2007). The therapeutic effects and side effects of these molecules. It is hypothesized that there is a specific imipramine binding site consisting of an aliphatic and aromatic site on the external side of the channel pore (Kuo, 1998). Moreover, imipramine and amitriptyline can inhibit G protein-activated inwardly rectifying K\(_v\) channels in astrocytes (Su et al., 2007). Imipramine and structurally related compounds can directly inhibit transient K\(_v\) currents in rat hippocampal neurons, and it was hypothesized that there is a specific imipramine binding site consisting of an aliphatic and aromatic site on the external side of the channel pore (Kuo, 1998). Moreover, imipramine and amitriptyline can inhibit G protein-activated inwardly rectifying K\(_v\) channels expressed in Xenopus oocytes (Takahashi et al., 2006). In addition, TCAs have been shown to modulate Ca\(_{2+}\)-activated K\(_v\) channels and Cl\(^-\) channels (Terstappen et al., 2001; Maertens et al., 2002). Moreover, TCAs are known to alleviate various pain syndromes by overlapping with the local anesthetic site in Na\(^+\) channels (Song et al., 2008). Although the effects on neurotransmitter transporters are held to underlie the activity of TCAs, it is possible that their affinity for ion channels may modulate the therapeutic effects and side effects of these molecules.

Amoxapine is a second-generation TCA previously introduced as an alternative to traditional TCAs because of its shorter onset of action and reduced cardiotoxicity. The molecule was found to exert antipsychotic effects in animal models.
els (Greenblatt et al., 1978) and was tested as a single-drug treatment for psychotic depression. In systematic double-blind trials, amoxapine was found to be of equal efficacy to that of an antidepressant and an antipsychotic (Anton and Burch, 1990). A recent study found that the antipsychotic activity of amoxapine was comparable with that of risperidone (Apiquian et al., 2005). Furthermore, because amoxapine is now off-patent and can be produced at generic prices, the drug could potentially rival other typical antipsychotics. Therefore, an understanding of the molecular and cellular mechanisms underlying the therapeutic effects and/or side effects of amoxapine is not of purely academic interest. Although some reports have suggested that amoxapine may also target 5-HT or D₂ receptors (Nasu et al., 2000), few studies have addressed the molecular mechanism that is responsible for the action of this molecule.

K⁺ channels are extremely diverse in structure and function and have been held to be among the most important signaling macromolecules both in neuronal and non-neuronal cells. In the central nervous system, K⁺ currents’ activity determines the shape, frequency, and duration of action potentials (Melishchuk et al., 1998). In addition, K⁺ channels play a major role in setting and maintaining the resting membrane potential, and the modification of this potential can alter neuronal excitability by activating Ca²⁺ channels and modulating neuronal transmitter release (Roeppe and Pongs, 1996). Indeed, blockade of K⁺ channels has been proposed as a potential therapy for diverse disorders, including myasthenia gravis, multiple sclerosis, Huntington’s chorea, and Alzheimer’s disease. The role of K⁺ channels is underscored by the accumulation of potassium ion that takes place during repetitive neuronal firing, and anticonvulsant drugs modulating K⁺ channel activity have potential as a means to control seizure activity (Liu et al., 2007). Although some TCaVs have previously been reported to target K⁺ channels, few investigations have addressed the possibility that amoxapine might modify the activity of voltage-gated K⁺ channels.

In the present study, we used patch-clamp techniques, immunocytochemistry, and cAMP assays to investigate the effects of amoxapine on the major K⁺ channels of mouse cortical neurons. We report that amoxapine inhibits the delayed rectifier potassium current (Iᵳ) in cortical neurons, and the inhibition is associated with the modulation of cAMP/protein kinase A (PKA)-dependent pathways and the 5-HT receptor activity.

Materials and Methods

**Mouse Cortex Neuron Culture.** Primary neuronal cultures were prepared from the cerebral neocortex of 15-day-old embryonic mice as originally described by di Porzio (Prochiantz et al., 1979) with several modifications. In brief, cerebral neocortices were dissected from embryonic Institute for Cancer Research (Swiss Hau- schka) mice. Dissociated cortical neurons were then plated onto poly-l-lysine-coated (10 μg/ml) 35-mm-diameter Petri dishes (Corning Life Sciences, Lowell, MA) at a density of 1 × 10⁶ cells/dish. Cultured cells were incubated at 37°C with 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 10% (v/v) heat-inactivated horse serum, and 1% antibiotic–antimycotic solution. After culturing for 24 h, cytosine 1-β-D-arabinofuranoside (5 μM) was applied to the culture medium to eliminate actively proliferating fibroblasts for 48 h. On day 4, cultured cells were washed and maintained in fresh medium. All the experiments were carried out with cortical neurons during 6 to 10 days in culture.

**Patch-Clamp Recordings.** Whole-cell currents of cortex neurons were recorded using a patch-clamp technique. Before Iᵳ recording, the culture medium was replaced with a bath solution containing 140 mM NaCl, 2.5 mM KCl, 10 mM HEPES, 1 mM MgCl₂, and 0.001 mM tetrodotoxin (pH adjusted to 7.4 using NaOH). Soft glass recording pipettes were filled with an internal solution containing 135 mM potassium glutamate, 10 mM KCl, 10 mM HEPES, 1 mM CaCl₂, 2 mM MgCl₂, and 10 mM EGTA (pH adjusted to 7.3 using KOH). The pipette resistance is 5 to 7 MΩ after filling with the internal solution. All the recordings were performed at room temperature (23–25°C).

**Data Acquisition and Analysis.** All the currents were recorded using an Axopatch 200B amplifier (Axon Instruments, Sunnyvale, CA) in voltage-clamp mode. A Pentium (Intel Corporation, Santa Clara, CA) computer connected to the recording equipment with a Digidata 1300 (MDS Analytical Technologies, Sunnyvale, CA) analog-to-digital interface. Currents were digitally sampled at 100 μs (10 kHz). The current signals were filtered by a 3-kHz, three-pole Bessel filter. Currents were corrected on-line for leak and residual capacitance transients by a P/4 protocol. Data acquisition and analysis were performed with pClamp 8.01 software (Axon Instruments) and/or Origin 6.1 (MicroCal, Northampton, MA). Statistical analysis was performed using the Student’s t test with nonpaired comparison or paired comparisons where it was relevant. Values were given as mean ± S.E.M., with n as the number of cell tested. P value <0.05 was used to denote the statistical difference between groups. When multiple comparisons were made, data were analyzed by a one-way analysis of variance (ANOVA) test.

**cAMP Assay.** cAMP levels were measured as described previously (Clark et al., 2004). In brief, 1 × 10⁶ cells were plated in each 35-mm dish and grown to confluence. Cells were washed with 1 ml of medium and preincubated at 37°C for 10 min in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (2.5 mM) (Sigma-Aldrich, St. Louis, MO). Cells were incubated for an additional 3 min at 37°C with or without amoxapine-varying concentrations of 10, 50, and 100 μM. The medium was removed, and 0.5 ml of HCl (0.1 M) with 0.8% Triton X-100 (Sigma-Aldrich) was added to the plates. After 10-min incubation at room temperature, the lysate was removed from the plates and centrifuged for 2 min. The supernatant was collected and assayed for cAMP levels using a direct cAMP enzyme immunooassay kit (Sigma-Aldrich) according to the manufacturer's instructions.

**Immunocytochemistry.** To detect changes in cAMP immunoreactivity of cultured cortex neurons after amoxapine treatment, cultured cortex neurons were incubated for 10 min at 37°C in 50 μM amoxapine. Immunocytochemical assay was performed for the cultures using the procedure described as follows. Isolated cultured cortex neurons placed on the coverslips were first washed three times with 0.01 M phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde for 30 min, rinsed with PBS three times, and preincubated for 1 h in 6% normal donkey serum (v/v) in PBS plus 0.1% Triton X-100 at room temperature. The cells were then incubated with rabbit anti-cAMP antibody (1:200 dilution; Chemicon, Temecula, CA) for 2 h in a humidified air chamber. After incubation, they were rinsed with PBS three times and then further incubated with the secondary antibody donkey anti-rabbit IgG tagged with Texas Red (1:100 dilution; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 30 min at room temperature. Then the coverslips were rinsed twice in PBS and were mounted onto glass slides.

**DNA Constructs and Cell Transfection.** Total RNA was isolated from primary cultured mouse cortical neurons according to the manufacturer’s instructions (Qiagen Mini RNeasy; Qiagen, Valencia, CA). First-strand synthesis was performed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Amplification was performed with the following primer sets: primer Kv2.1: forward,
cyproheptadine, dibutyryl cAMP (db-cAMP), 5-HT, forskolin, risperidone, TEA, tetrodotoxin, H-89, 4-aminopyridine (4-AP), dihydroceramide C₄₅, ceramide, cytosein-β-d-arabinofuranoside, poly-L-lysine, and Dulbecco's modified Eagle's medium were purchased from Gibco Life Technologies (Carlsbad, CA). Jingzhaoxin-III (JZTX-III; molecular mass, 3919.3 Da) is a peptide toxin containing 36 amino acid residues with three disulfide bridges cross-linked in the pattern of I–IV, II–V, and III–VI (Cys4–Cys19, Cys11–Cys24, and Cys18–Cys31) isolated from the venom of the Chinese spider *Chilobrachys jingzhao*, and was the gift from Dr. Liang (Huanan Normal University, Changsha, China). Amoxapine and cyproheptadine solutions were prepared extemporaneously. First they were dissolved in methanol and then diluted in the bath with a final methanol concentration <0.1%. C₄₅-ceramide and risperidone were first dissolved in dimethyl sulfoxide (DMSO) and then diluted in the bath with a final DMSO concentration <0.1%. The 0.1% of methanol and DMSO did not produce the significantly effect on K⁺ currents.

The drug was ejected by gravity for 40 to 60 s from MSC-200 Manual Solution Changer (Bio-Logic SAS, Claiex, France). We tested the exact dilution rate by perfusion of a high concentration potassium solution. The exact dilution rate and the time for the drug to reach maximal concentration around the cell have been evaluated by measuring the absorbance at 260 and 280 nm. HEK293 cells were transfected using the calcium phosphate method. The average transfect efficiency is >80%. Two days after transfection, HEK293 cells with green or red fluorescence were further analyzed.

**Results**

Central neurons contain two main voltage-dependent outward K⁺ currents. One is the Iₖ, and the other is transient potassium current (Iₖ,trans). We first determined whether amoxapine affects either Iₖ,trans or Iₖ. Outward K⁺ currents were evoked by two sequential 200-ms depolarizing pulses 1 s apart to +40 mV from holding potentials of −100 and −50 mV, respectively (Fig. 1A). When the membrane potential was held at −100 mV, the depolarizing pulses elicited a global outward current (Iₖ,trans plus Iₖ) that activated rapidly (5–10 ms) and then decayed with time (Fig. 1A, left). However, at a holding potential of −50 mV, depolarization evoked only a slight inactivating or noninactivating outward K⁺ current described previously as a delayed rectifier Iₖ (Fig. 1A, right). Application of amoxapine significantly accelerated the decay phase of Iₖ,trans and reduced the Iₖ amplitude. In the presence of 5 mM TEA, a specific Iₖ,trans channel blocker, Iₖ was obtained by depolarizing pulses to +40 mV from a holding potential of −100 mV, and amoxapine did not significantly inhibit the Iₖ,trans current (Fig. 1B, left).

These results indicated that amoxapine selectively modulates Iₖ. Therefore, we investigated Iₖ,trans amplitudes in the presence of 5 mM 4-AP, which was used to block Iₖ,trans currents. Iₖ,trans were evoked by 200-ms depolarizing pulses over the range from −50 mV to +40 mV at 10-s intervals. Application of amoxapine produced a clear reduction in Iₖ,trans amplitudes (Fig. 1B, right). The inhibitory effect of amoxapine on Iₖ,trans was reversible, reaching its maximum effect within 50 to 100 s and recovered close to control levels after 2 min. The amoxapine-induced inhibitor effect on Iₖ was concentration-dependent at concentrations of 10 to 500 μM. The inhibition of Iₖ by amoxapine at 50, 100, and 300 μM was 20.7 ± 0.4% (n = 5), 31.7 ± 1.6% (n = 5), and 52.2 ± 2.19% (n = 5), respectively (P < 0.05 by one-way ANOVA). When the concentration of amoxapine was increased to 500 μM, Iₖ was inhibited by 72.5 ± 2.2% (n = 5, P < 0.05 by one-way ANOVA). The fitting of concentration-response curve was performed by using the Hill equation $y = 1/(1 + \left((C_{\text{50}}/\text{amoxapine})\right)^{n})$, where y is the inhibition rate in the presence of amoxapine.

![Fig. 1](https://jpet.aspetjournals.org/content/11032-001/1/439/F1.large.png)
amoxapine, IC_{50} is the half-maximal inhibition concentration of amoxapine, [amoxapine] is the concentration of amoxapine, and h is the Hill coefficient. With the Hill equation, we got the IC_{50} and Hill coefficient h, which were 211.36 ± 27.93 μM and 0.9255, respectively. Statistical analysis of concentration-response data is presented in Fig. 1C. Because amoxapine was dissolved by 0.1% methanol, we also examined the effect of methanol on I_A and I_K. As shown in Fig. 1D, neither I_A nor I_K was affected by 1% methanol (n = 10, P > 0.05 by paired t test).

We also studied the effects of amoxapine on the activation and inactivation of I_K using different experimental protocols. In the voltage activation protocol, membrane potential was held at −80 mV, and I_K was evoked by a 200-ms depolarizing pulse from a first pulse potential of −60 mV to +60 mV in 10-mV steps at 10-s intervals (Fig. 2A). When the depolarizing pulse was more positive than 10 mV, amoxapine significantly reduced the current amplitude throughout the activation voltage range (Fig. 2B). We obtained the I_K activation curve by plotting normalized conductance as a function of the command potential; data were analyzed using the equation $G_K = G_K(V_m - V_{rev})$, where $G_K$ is the membrane K⁺ conductance, $V_m$ is the membrane potential, and $V_{rev}$ is the reversal potential for K⁺. As shown in Fig. 2C, the activation curve shifted to the left in the presence of amoxapine. The current was half-activated at 4.59 ± 0.65 mV and −5.28 ± 0.36 mV in the absence and presence of 100 μM amoxapine, respectively (n = 10, P < 0.05 by paired t test), suggesting that amoxapine treatment significantly modified the voltage dependence of I_K channel steady-state activation.

To study the effects of amoxapine on steady-state inactivation of I_K, currents were elicited using 1-s conditioning prepulses from −80 mV to different membrane potentials before a 200-ms test pulse of +40 mV (Fig. 3, A and B). After normalizing each current peak to the maximal current amplitude obtained from the −80-mV prepulses as a function of the conditioning prepulse potential, we obtained an inactivation curve of I_K and calculated the Vh_{50} (the voltage at which the current amplitude was half-inactivated; Fig. 3C). This revealed that the inactivation curve was also significantly shifted to the left by the application of amoxapine. In five cells studied, the half-maximal inactivation voltage was 11.4 ± 2.6 mV and −3.9 ± 2.0 mV in the absence and presence of 100 μM amoxapine, respectively (n = 5, P < 0.05 by paired t test).

We then used a train protocol in conjunction with the protein phosphatase activator C6-ceramide (Gallego et al., 2005) to investigate whether amoxapine acts directly as an open-channel blocker. In this protocol amoxapine was applied to the bath solution for 1 min before channel opening (i.e., before I_K was elicited by the depolarizing pulse). The effects of amoxapine on I_K amplitudes elicited by the first and subsequent depolarizing pulses were recorded. As shown in Fig. 4A, the amplitude of the peak current evoked by the first pulse was reduced in the presence of 50 μM amoxapine. Thereafter, the current elicited by subsequent pulses decreased to a steady-state level approaching 79 ± 3.3% of control. This experiment indicated that current blocking can occur without previous channel opening. Furthermore, I_K amplitude was not affected by 20 μM C6-ceramide alone, whereas C6-ceramide abolished amoxapine inhibition of I_K. As shown in Fig. 4B, 20 μM C6-ceramide alone did not modify the I_K amplitude. In the presence of 20 μM C6-ceramide, the amoxapine-induced inhibitory effect on I_K amplitude was eliminated (n = 4, P < 0.05 by paired t test). Statistical analysis of the above data is shown in Fig. 4, C and D.
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To address whether the cAMP/PKA pathway contributed to amoxapine-mediated inhibition of $I_K$, we studied the effects of amoxapine in the presence of forskolin, which was an adenylate cyclase agonist (Fig. 5A). Perfusion of cortical neurons with 20 μM forskolin provoked a gradual decrease in $I_K$ amplitude similar to that produced by amoxapine. In the presence of forskolin, amoxapine inhibited $I_K$ by only 5.4 ± 1.2%. Perfusion with 20 μM db-CAMP (a membrane-permeable cAMP analog) (Sigma-Aldrich) produced a significant reduction (21.8 ± 1.0%) of $I_K$ and abolished amoxapine inhibition of $I_K$. In the presence of db-CAMP, amoxapine only inhibited $I_K$ by 4.7 ± 1.9% (Fig. 5B). The inhibition was not statistically significant (Fig. 5D; n = 5, P > 0.05 by paired t test).

We then used a selective PKA antagonist, H-89, to explore the role of PKA in the inhibition of $I_K$ by amoxapine. H-89 was added into the patch pipette solution and itself increased $I_K$ significantly to 139.7 ± 3.7% of control. Furthermore, 50 μM amoxapine only inhibited $I_K$ by 1.8 ± 0.87% in the presence of H-89, indicating that H-89 also abolishes the inhibitory effects of amoxapine (Fig. 5, C and D; n = 6).

The effects of amoxapine on intracellular cAMP levels were then studied using an anti-cAMP antibody. As shown in Fig. 6A, cAMP immunoreactivity was detected before the addition of amoxapine (Fig. 6A, left), but the levels were significantly increased when neurons were incubated with 50 μM amoxapine for 10 min (Fig. 6A, right). This indicated that amoxapine increases intracellular cAMP levels. We then used a direct cAMP assay to confirm amoxapine-induced changes in cAMP levels. As shown in Fig. 6B, cAMP levels were 16.5 ± 2.5 pM (n = 5) before the addition of amoxapine. When the cells were incubated with 10, 50, and 100 μM amoxapine for 3 min, cAMP levels were increased significantly to 21.8 ± 3.1 pM, 26.1 ± 2.7 pM, and 38.9 ± 4.6 pM, respectively (P < 0.05 by one-way ANOVA).

It was recently reported that amoxapine may be comparable in efficacy with the atypical antipsychotic risperidone, which has been shown by positron-emission tomography to have high serotonin (5-HT)2 occupancy (Kapur et al., 1999). Therefore, we investigated whether the 5-HT receptor might mediate the amoxapine-induced inhibition of $I_K$. As shown in Fig. 7A, 50 μM 5-HT inhibited $I_K$ by 21.5 ± 1.5% (n = 6). In the presence of 5-HT, however, amoxapine (50 μM) only inhibited $I_K$ by 2 ± 2.7% (n = 6). We further used the 5-HT2 receptor antagonist cyproheptadine to confirm the hypothesis. The result indicated that cyproheptadine (20 μM) alone significantly reduced $I_K$ amplitude by 24.4 ± 1.58% (n = 6, P < 0.05 by paired t test). With cyproheptadine in the bath solution, the 100 μM amoxapine-induced inhibitory effect on $I_K$ was decreased to 3.25 ± 0.8% (Fig. 6B; P > 0.05 by paired t test). Likewise, another 5-HT2 receptor antagonist, risperidone, has the same effect. The risperidone (0.02 μM) itself induced a significantly inhibition of $I_K$ by 20.1 ± 1.06% (n = 5, P < 0.05 by paired t test). In the presence of risperidone, 100 μM amoxapine-induced inhibitory effect on $I_K$ was only 2.28 ± 0.75% (Fig. 6C; P > 0.05 by paired t test). Moreover, high or low concentrations of cyproheptadine or risperidone had the similar effect (Fig. 7E). Because 5-HT receptors are G protein-coupled receptors that modulate multiple signal transduction pathways (Wada et al., 2006), we then used GTPγS to explore whether G protein-coupled receptors were involved in amoxapine inhibition of $I_K$. Internal infusion of GTPγS (10 μM) resulted in the gradual augmentation of $I_K$ amplitudes after membrane rupture (Fig. 7D). The amplitude was increased significantly to 1501.2 ± 284.5 pA in controls from 1231.7 ± 325.9 pA, an increase of 19.6 ± 1.2% (n = 8, P < 0.05 by paired t test). Moreover, after augmentation of $I_K$ amplitudes by internal application of GTPγS, 50 μM amoxapine only reduced $I_K$ by 3.9 ± 2.1% (n = 8, P > 0.05 by paired t test), significantly different from the reduction.

![Fig. 4. Effect of the protein phosphatase activator C6-ceramide on amoxapine inhibition of $I_K$. A, a train protocol was used to address whether amoxapine inhibition was selective for open channels. In this protocol current was elicited by a depolarizing pulse (control). Amoxapine was then applied to the bath solution 1 min before channel opening again by depolarizing pulses. The effects of amoxapine on the $I_K$ amplitudes elicited by the first and subsequent pulses were measured. Pulses were evoked by 200-ms steps from a −50 mV holding potential to +40 mV. B, protein phosphatase activator C6-ceramide (20 μM) abolished amoxapine inhibition of $I_K$. C and D, statistical analysis. Data come from four to seven independent cells; *, P < 0.05 compared versus controls.](image-url)
induced by 50 μM amoxapine alone (Fig. 7E). The abolition of amoxapine inhibition of \(I_K\) by either 5-HT receptor antagonist or GTP\(_S\) is consistent with the interpretation that amoxapine exerts its effects through the activation of 5-HT receptor.

It was previously reported that \(I_K\) channels are predominantly composed of Kv2 subunits of the Shaker family (Misonou et al., 2008). In particular, subunit Kv2.1, which is expressed at high levels in most mammalian central neurons, is a major contributor to \(I_K\) channels and plays a crucial role in regulating neuronal excitability (Murakoshi and Trimmer, 1999; Du et al., 2000). To address the potential role of Kv2.1 in amoxapine inhibition of \(I_K\) channel activity, we used JZTX-III, a toxin known to specifically eliminate the Kv2.1 subunit (Liao et al., 2007). To confirm the selectivity of JZTX-III, experiments were first carried out in HEK293 cells transfected with an expression vector encoding the Kv2.1 subunit. Kv2.1 currents were evoked by depolarizing pulse to 60 mV from a holding potential of 50 mV. As shown in Fig. 8A, the toxin significantly decreased the amplitude of the Kv2.1 currents, and application of 100 nM JZTX-III induced a 87.10 ± 1.72% decrease in Kv2.1 currents (\(n = 11, P < 0.05\) by paired t test). Inhibition was also observed in cortical neurons, where addition of 100 nM JZTX-III to the bath solution reduced \(I_K\) amplitudes by 28.5 ± 4.4% (\(n = 7\); Fig. 8B). We then investigated the effects of forskolin, db-cAMP, and H-89 on \(I_K\) amplitudes and amoxapine-induced \(I_K\) inhibition. Data are mean ± S.E.M. from five to nine cells; *, \(P < 0.05\) compared versus control.

Fig. 5. cAMP/PKA pathways mediate amoxapine inhibition of \(I_K\) in mouse cortical neurons. A, time course of changes of \(I_K\) amplitudes induced by 50 μM amoxapine in the presence of extracellular forskolin. Forskolin (20 μM) provoked a gradual decrease in \(I_K\) amplitude and abolished the effect of 50 μM amoxapine on \(I_K\) amplitude. The insets in the graphs show superimposed \(I_K\) traces from the initial control levels (after establishment of the whole-cell configuration) and after external infusion of forskolin. The time points (1, 2, 3) noted on the curves correspond to the superimposed \(I_K\) traces illustrated by the insets. B, time course of the changes in \(I_K\) amplitudes induced by amoxapine in the presence of db-cAMP (20 μM). db-cAMP decreased \(I_K\) amplitudes and eliminated the inhibitory effect of 50 μM amoxapine. C, time course of amoxapine inhibition in the presence of the PKA inhibitor, H-89. Intracellular H-89 (10 μM) provoked a gradual increase in \(I_K\) amplitudes and abolished the effect of 50 μM amoxapine on \(I_K\). D, statistical analysis of the effects of forskolin, db-cAMP, and H-89 on \(I_K\) amplitudes and amoxapine-induced \(I_K\) inhibition. Data are mean ± S.E.M. from five to nine cells; *, \(P < 0.05\) compared versus control.

Fig. 6. Amoxapine increases intracellular cAMP levels in mouse cortical neuron. A, cAMP immunoreactivity was detected by anti-cAMP antibody in the absence or presence of 50 μM amoxapine. The scale bar represents 20 μm. B, intracellular levels of cAMP measured by direct cAMP analysis in the presence of 10, 50, and 100 μM amoxapine for 3 min; *, \(P < 0.05\) compared versus multiple concentration of amoxapine by one-way ANOVA. Data come from five independent experiments.

induced by 50 μM amoxapine alone (Fig. 7E). The abolition of amoxapine inhibition of \(I_K\) by either 5-HT receptor antagonist or GTP\(_S\) is consistent with the interpretation that amoxapine exerts its effects through the activation of 5-HT receptor.
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Fig. 7. Effects of 5-HT, 5-HT$_2$ receptor antagonists, and GTP$\gamma$S on amoxapine inhibition of $I_K$. A, time course of changes in $I_K$ amplitudes induced by amoxapine in the presence of 20 $\mu$M 5-HT. 5-HT provoked a gradual decrease in $I_K$ amplitudes and abolished the inhibitory effects of 50 $\mu$M amoxapine. B and C, time course of changes in $I_K$ amplitudes induced by amoxapine in the presence of 20 $\mu$M cyproheptadine (CPD) or 0.02 $\mu$M risperidone (RFD). Cyproheptadine and risperidone both provoked a gradual decrease in $I_K$ amplitudes and abolished the inhibitory effects of 100 $\mu$M amoxapine on $I_K$. D, time course of changes in $I_K$ amplitudes induced by the application of 10 $\mu$M GTP$\gamma$S. Internal infusion of GTP$\gamma$S resulted in gradual augment of $I_K$ amplitude after membrane rupture and abolished $I_K$ inhibition by 50 $\mu$M amoxapine. E, statistical analysis of the effects of 5-HT, higher and lower concentration of 5-HT$_2$ receptor antagonist, and GTP$\gamma$S on $I_K$ amplitudes and amoxapine-induced $I_K$ inhibition. Data are mean ± S.E.M. from five to nine cells; *, $P < 0.05$ compared versus untreated cells. #, $P < 0.05$ compared versus controls without amoxapine.

Discussion

Previous studies have revealed that, in addition to specific inhibition of serotonin and/or norepinephrine transporters, TCAs also modulate several ion channels. Although the mechanism by which TCAs can affect ion channel activity has not been elucidated, previous reports have suggested a direct interaction between the drugs and the ion channels (Kim et al., 2007). It was reported that the drugs can block ion channel conductance by binding within the pore itself or by allosteric inhibition produced by binding outside the pore. For example, imipramine and diphenhydramine were reported to act as an open-channel blocker on Na$^+$ currents in rat hippocampal neurons (Yang and Kuo, 2002). Furthermore, it was reported that imipramine and structurally related compounds can directly inhibit transient K$_v$ currents in rat hippocampal neurons. It was hypothesized that there is a specific imipramine binding site consisting of an aliphatic and aromatic site on the external side of the channel pore (Kuo, 1998).

In contrast, the results reported here indicate that external amoxapine blocks $I_K$ with slow onset and reversible recovery. Moreover, amoxapine inhibited $I_K$ in the absence of depolarizing pulses, implying that amoxapine can block channel activity irrespective of whether the channel is already open. This would appear to exclude direct open-channel blockade and instead suggest that amoxapine is most likely to inhibit $I_K$ through intracellular biochemical processes, which in turn reduce $I_K$ channel activity.

Protein phosphorylation and dephosphorylation are central to the regulation of ion channel function and membrane excitability. Some voltage-dependent K$^+$ channels, such as I$_{A}$ and human ether-a-go-go-related gene channel, are known to be regulated by protein phosphorylation (Thomas et al., 1999; Anderson et al., 2000), even though it is not known whether such regulation occurs directly or via intermediary proteins. We reported previously that activation of cAMP/PKA pathway in cerebellar granule cells could induce fast modulation of $I_K$ amplitudes (Jiao et al., 2007). In the present study we have shown that activation of cAMP/PKA pathways produces a similar inhibitory effect on $I_K$ amplitudes in cortical neurons. Activation of cAMP/PKA pathways by either forskolin or db-cAMP abolished amoxapine inhibition of $I_K$. Furthermore, inhibition of the PKA pathway by intracellular H-89 produced a gradual increase in $I_K$ amplitudes after establishment of the whole-cell configuration, and H-89 completely prevented amoxapine inhibition of $I_K$. We report that amoxapine treatment led to a significant increase in intracellular levels of cAMP. We suggest that amoxapine inhibits $I_K$ by activating cellular cAMP/PKA pathways. Nevertheless, we have no evidence to indicate that $I_K$ channels are phosphorylated after amoxapine induction of the PKA pathway. Further experiments will be required to test this possibility.
Amoxapine, a dibenzoxazepine antidepressant, is highly lipid-soluble and membrane-permeable. It was previously reported that externally applied TCAs inhibit ion channel activity by binding to an extracellular site or by diffusing through the cell membrane to gain access to a binding site on the internal surface of the membrane (Kuo, 1998; Kim et al., 2007). However, our results argue against direct \( I_K \) blocking activity. It would be interesting to explore whether amoxapine activates the cAMP/PKA pathway by targetting an extracellular receptor or whether entry of the molecule into the cell is required.

A recent positron-emission tomography study in normal volunteers showed that, at a dose of 150 mg/day, amoxapine saturated 5-HT\(_2\) occupancy (98%) but displayed only modest D\(_2\) occupancy (63%) (Kapur et al., 1999). Polymerase chain reaction analysis of rat cortical neurons has revealed that the 5-HT receptor is well expressed in mouse cortical neuron (data not shown). Therefore, we examined whether amoxapine induction of the inhibition of \( I_K \) might be mediated by the 5-HT receptor using 5-HT, 5-HT\(_2\) receptor antagonists, and GTP\(\gamma\)S. The results showed that the 5-HT\(_2\) receptor and G proteins were associated with amoxapine inhibition of \( I_K \) because the amoxapine-induced effect was fully eliminated by 5-HT, 5-HT\(_2\) receptor antagonists, and GTP\(\gamma\)S. Moreover, we noted that, except 5-HT, 5-HT\(_2\) receptor antagonists have the inhibitory effect on \( I_K \) amplitude. The effect of cytoheptadine and risperidone on \( I_K \) was similar and not concentration-dependent (data are not completely shown). The above results were consistent with a previous report in which risperidone and ketanserin (5-HT\(_2\) receptor antagonist) inhibited \( I_K \) currents through a direct block mechanism in human and rat myocardium (Zhang et al., 1994). Even though cytoheptadine and risperidone could play a role as a \( K^+ \) channel blocker, it did not hinder their usefulness as the 5-HT\(_2\) receptor antagonists. Because amoxapine is highly lipid-soluble and membrane-permeable, whether this substance inhibits \( I_K \) by interacting at the intracellular faces of 5-HT receptors or \( I_K \) channels needs further exploration.

Electrophysiological studies have revealed that most mammalian neurons express multiple types of voltage-gated \( K^+ \) channels with distinct time- and voltage-dependent properties (Storm, 1990). The Kv2 (Shab) genes in many species encode components of sustained potassium current channels (Coetzee et al., 1999). Previous studies have indicated that neuronal \( I_K \) channels are composed of \( \alpha \)-subunits of the Kv2 subfamily. In particular, Kv2.1 subunit, which is highly expressed in most mammalian central neurons, is a major contributor to \( I_K \) channels and plays a crucial role in regulating neuronal excitability (Sarmiere et al., 2008). Recent studies confirmed by polymerase chain reaction (data not shown) have indicated that Kv2.1 is a major component of cortical neuron \( I_K \) channels (Guan et al., 2007). In the present study, we report that inhibition of Kv2.1 \( \alpha \)-subunits using a specific toxin (JZTX-III) reported to selectively block Kv2.1 \( \alpha \)-subunits (Liao et al., 2007) eliminated the inhibitory effect of amoxapine on \( I_K \), implying that Kv2.1 \( \alpha \)-subunits are central to amoxapine inhibition. We also report that JZTX-III significantly reduced \( I_K \) amplitudes in cortical neurons, arguing that Kv2.1 \( \alpha \)-subunits are an integral part of cortical \( I_K \) channels.

Amoxapine, a second-generation TCA, was introduced as an alternative to the traditional TCAs because of its shorter onset of action and reduced cardiotoxicity (Kudo et al., 2007). Because amoxapine is a relatively weak reuptake inhibitor, it was suggested that the molecule may have the properties of an atypical antipsychotic agent (Apiquian et al., 2005). The upper range of therapeutic plasma concentrations of amoxapine has been reported from 0.2 to 0.4 \( \mu \)g/ml (0.637–1.912 \( \mu \)M) in human body (Winic et al., 2001). However, acute overdose has reportedly caused severe adverse reactions, including seizure, coma, metabolic acidosis, and tachycardia, and some fatalities have been reported (Kudo et al., 2007). In the present study, amoxapine inhibited \( I_K \) with an IC\(_{50}\) value of 211.36 \( \mu \)M, a concentration significantly above the clinical dosage. Although it is possible that the effective cell surface concentration in our perfusion system might be lower than the concentration applied, it still appears that the concentrations used in our study are above the levels obtained after oral administration of the drug (Kudo et al., 2007). This would suggest that therapeutic levels of amoxapine do not produce a major blockade of neuronal \( I_K \).

A recent study compared the antipsychotic effects and side effect profiles of amoxapine versus haloperidol in a 6-week double-blind study of patients with schizophrenia. It was reported that amoxapine may have the similar effect with haloperidol as an antipsychotic (Chaudhry et al., 2007), as predicted by its affinity for 5-HT\(_2\) receptors. We report here that amoxapine shifts Kv2.1 activation toward the resting membrane potential, making it more easily activated, and thereby dampening neuronal excitability (Surmeier and Foe hring, 2004). This may represent a novel mechanism by which amoxapine exerts atypical antipsychotic properties.

In summary, this is the first report addressing the effects of amoxapine on \( I_K \) in cultured mouse cortical neurons. We report that amoxapine inhibits \( I_K \), an effect different from the previously described effects of TCAs on ion channel activity. Amoxapine inhibition of \( I_K \) was mediated by activation of cAMP/PKA pathways. Our experiments indicate that the Kv2.1 subunit is a major target for inhibition. Although the clinical relevance of amoxapine inhibition of \( I_K \) remains unclear, inhibition of \( K^+ \) channels by amoxapine may contribute to the antidepressant and antipsychotic activity of the molecule.

Acknowledgments

We thank Dr. Liang (The Key Laboratory of Protein Chemistry and Developmental Biology of Ministry of Education, College of Life Sciences, Hunan Normal University, Changsha, People’s Republic of China) for the JZTX-III.

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