Amoxapine Inhibits Delayed Outward Rectifier K^+ Currents in Cerebellar Granule Cells via Dopamine Receptor and Protein Kinase A Activation

Guang Yang, Meng-Hua Zhou, Zhen Ren, Jia-Jie Xu and Yan-Ai Mei

Institutes of Brain Science, School of Life Sciences and State Key Laboratory of Medical Neurobiology, Fudan University, Shanghai

Key Words

Amoxapine • I_K • PKA • Cerebellar granule neuron • D_1 -like receptor

Abstract

Background: Although tricyclic antidepressants amoxapine is proposed to target 5-HT and D2 receptors, very few studies have addressed the effect of amoxapine on molecular and cellular mechanisms via receptor pathways. In this study, we test the effect of amoxapine on rat cerebellar granule neurons (CGNs) to address this possibility. **Methods:** CGNs cell culture, whole-cell current recording using a patch-clamp technique, western blot and non-radioactive detection analysis of phosphorylated protein kinase A (PKA) were used. **Results:** Amoxapine inhibits delayed rectifier potassium (I_K) current in a dose-dependent manner and modulates inactivation properties in CGNs. Those effects were not eliminated by preincubation with 5-HT or 5-HT receptor antagonists, but abolished by dopamine and $D1/D5$ receptor antagonists. Application of $GTP\gamma S$ and inhibitor of the G_s signalling cascade abolished the amoxapine-induced effect on I_K . The application of forskolin or dibutyryl-cAMP mimicked the inhibitory effect of amoxapine on I_K . Western blotting for

phosphorylated PKA revealed that amoxapine significantly increased the intracellular levels of phosphorylated PKA, a marker of PKA activation. **Conclusion:** Amoxapine inhibits I_K currents in rat CGNs via cAMP/PKA-dependent pathways, as in mouse cortical neurons we reported earlier, but that involves $D1$ -like receptors instead of 5-HT receptors.

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Introduction

Psychological depression is major health problem for which pharmacological treatment is not yet entirely satisfactory. Tricyclic antidepressants (TCAs) were the first successful antidepressants and have been widely used for the treatment of depression and other psychiatric disorders [1, 2]. The therapeutic effects of TCAs are thought to be mediated by inhibition of 5-HT reuptake in the brain [3]. However, in addition to blocking the reuptake of 5-HT, most TCAs also bind to other neurotransmitter receptors such as muscarinic, adrenergic and histamine receptors [3], and it has been suggested that these secondary properties could explain some of the differences in the clinical actions and adverse side-effects of TCAs [3].

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Dr. Mei Yan-Ai
School of Life Sciences and Institutes of Brain Science,
Fudan University, Shanghai 200433 (PR China)
Tel. +86-21-55664711,
E-Mail yamei@fudan.edu.cn, yanaimei@yahoo.com

Amoxapine is a second-generation TCA as an alternative to traditional TCAs because of its faster action and, in view of its reduced cardiotoxicity, it is now widely used for the treatment of patients who cannot tolerate the adverse cardiovascular side-effects. Amoxapine was found to exert antipsychotic effects in animal models [4] and was tested as a single-drug treatment for psychotic depression. In systematic double-blind trials, amoxapine was found to have an efficacy comparable to that of typical antidepressant and antipsychotic drugs [5-7]. Furthermore, because amoxapine is now off-patent and can be produced at generic prices, the drug could potentially rival other typical antipsychotics. An understanding of the molecular and cellular mechanisms underlying the therapeutic effects and/or side effects of amoxapine is therefore of importance. Although it has been proposed that amoxapine can also target 5-HT and D₂ receptors [8, 9], few studies, with the exception of our previous work on mouse cortical neurons [10], have addressed the effect of amoxapine on molecular and cellular mechanisms via 5-HT and dopamine receptor pathway.

In contrast to earlier theories, recent data now indicate that the function of the cerebellum is not restricted to balance and motor control, and the region contributes to several aspects of higher brain function including emotional regulation and cognition [11]. Cerebellar granule neurons (CGNs) are small glutamatergic interneurons; the brain contains between 10¹⁰ and 10¹¹ CGNs making them by far the most numerous neuron in the CNS [12]. CGNs receive inputs from mossy fibers or the axons of brush cells [13], and CGNs axonal projections form the parallel fibers of the cerebellum that terminate on the dendrites of Purkinje neurons. Golgi cells, stellate cells and basket cells also receive inputs from CGNs parallel fibers. Because of their abundance and extensive interactions with other cell types in the cerebellum, CGNs are likely to play a central role in cerebellar function.

Primary CGNs display several different outward K_v currents, including I_A , I_K , and the non-inactivating K⁺ current $I_{K(so)}$ [14, 15]. Potassium (K) channel activity is known to determine the frequency and shape of neuronal action potentials, and also controls the strength of synaptic contacts between neurons [16, 17]. Modification of the activity of CGNs potassium channels therefore has a profound influence on the CGNs firing properties, interactions with other cells, and cerebellar function [18]. Nevertheless, few studies have addressed the possibility that antidepressants might exert their pharmacologic

effects by modulating the activity of voltage-gated K⁺ channels in CGNs.

In a previous study we reported that amoxapine inhibited I_K currents in cortical neurons and that drug action was associated with 5-HT receptor targeting and the modulation of cAMP/PKA-dependent pathways. In this study, we investigated the effects of amoxapine on I_K currents in CGNs and report that amoxapine inhibits I_K currents in CGNs via a dopamine receptor.

Materials and Methods

Cell culture

Cells were derived from cerebellum of 7–8 day Sprague-Dawley rat pups as described previously [19]. Isolated cells were plated onto 35 mm diameter Petri dishes coated with poly-L-lysine (10 µg/ml) at a density of 10⁶ cells/ml. Cultured cells were incubated at 37°C under 5% CO₂ in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, glutamine (5 mM), insulin (5 µg/ml), KCl (25 mM), and 1% antibiotic-antimycotic solution. All experiments were carried using primary CGNs after 5–7 days in culture.

Patch-clamp recordings

Whole-cell currents of CGNs were recorded using a patch-clamp technique. Prior to I_K current recording the culture medium was replaced with a bath solution containing (in mM): NaCl 140, KCl 2.5, HEPES 10, MgCl₂ 1 (pH adjusted to 7.4 using NaOH). Soft glass recording pipettes were filled with an internal solution containing (in mM): K gluconate 135, KCl 10, HEPES 10, CaCl₂ 1, MgCl₂ 2, EGTA 10 (pH adjusted to 7.3 using KOH). The pipette resistance was 5–7 MΩ after filling with the internal solution. All recordings were performed at room temperature (23–25°C).

Data acquisition and analysis

All currents were recorded using an Axopatch 200B amplifier (Axon Instrument, Foster City, CA, USA) operated in voltage-clamp mode and a Pentium computer connected to the recording equipment via a Digidata 1332 analog-to-digital (A/D) interface. Current was digitally sampled at 100 µs (10 kHz). Current signals were filtered by a 5 kHz three-pole Bessel filter. Currents were corrected online for leak and residual capacitance transients by a P/4 protocol. Data acquisition and analysis were performed with pClamp 8.01 software (Axon Instruments, Foster City, CA, USA) and/or Origin8.0 (Microcal Analysis Software, Northampton, MA, USA). The current is analyzed by calculating the mean of the sample points from 130 ms to 200 ms of a 200 ms pulse because the I_K amplitude have been stable in this duration. Statistical analysis was performed using the Student *t*-test with non-paired comparison or paired comparisons where appropriate. Values are given as means ± SEM, with *n* as the number of cell tested. *p* < 0.05 was considered to indicate statistical significance. One-way ANOVA was employed for multiple comparisons.

Western blot analysis of phosphorylated PKA (pPKA)

Rat CGNs were lysed in NP40 lysis buffer (HEPES 20, NaCl 150, EDTA 2, Na₃VO₄ 0.1, NaF 50, in mM, NP-40 0.5%, glycerol 10%) with a protease inhibitor cocktail (Sigma-Aldrich, USA). Lysates were mixed with an equal volume of Laemmli sample buffer and boiled for 5 min. Samples were electrophoresed by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with Tris-buffered saline Tween (TBST; Tris-base 10mM, NaCl 0.15mM, Tween-20 0.05%) containing 10% nonfat milk, blots were probed with rabbit polyclonal antibodies against phosphorylated PKA (1:1000) (sc-32968, Santa Cruz biotechnology, CA, USA) or mouse monoclonal antibodies against GAPDH (1:10000) (KC-5G4, Kangchen Bio-tech, Shanghai China) respectively, at 4°C overnight. After washing, membranes were treated with anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase (HRP) at room temperature. Immunoreactivity was detected using a chemiluminescence substrate (Pierce Protein Research Products, Rockford, IL, USA) with X-ray film and analyzed by Quantity One software (Bio-Rad Laboratories, USA).

cAMP assay

cAMP levels were measured using a rat cAMP ELISA kit (Yili, Minhang, SH, PRC). It is an enzyme linked immunosorbent assay. Briefly, Rat CGNs were plated in 35-mm dish and lysed in 70 μ l NP40 lysis buffer with the presence of IBMX (Sigma-Aldrich, St. Louis, MO, USA). After 10 minutes incubation on ice, the lysate was removed from the plates and centrifuged for 5 minutes at 4°C. The supernatant was collected and assayed for cAMP levels according to the manufacturer's instructions. The optical densities of samples were obtained with Thermo Multiskan MK3 Microplate Photometer.

Protein kinase A activity assay

PKA activity was analyzed with a PepTag Non-Radioactive Detection of cAMP-Dependent Protein Kinase from Promega (Madison, WC, USA) according to the instructions. This assay is based on changes in the net charge of fluorescent peptide substrates by PKA phosphorylation. The difference of net charge allows the phosphorylated or nonphosphorylated substrate to be separated on an agarose gel at neutral pH. The fluorescence intensity of the phosphorylated substrates represented the activity of PKA.

Reagents

Amoxapine, guanosine 5'-[γ thio]-triphosphate (GTP γ S), forskolin, dibutyryl cyclic AMP (db-cAMP), cyproheptadine, 5-hydroxytryptamine (5-HT), Rp-adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt hydrate (Rp-cAMP), (S)-(-)-Sulpiride, R(+)-SCH-23390 hydrochloride, Tetraethylammonium chloride, dopamine hydrochloride, insulin, poly-L-lysine and DMEM were purchased from Sigma-Aldrich (St. Louis, MO, USA). SKF 83566 hydrobromide was purchased from Tocris Bioscience (Ellisville, MS, USA). Fetal calf serum and antibiotic-antimycotic solution were obtained from Gibco Life Technologies (Grand Island, NY, USA). Amoxapine

Fig. 1. Concentration-dependent and reversible inhibition of cerebellar granule neurons I_K amplitudes by amoxapine. A, I_K traces were recorded in the absence and presence of different concentration of amoxapine (AXP). Currents were evoked by sequential 200 ms depolarizing pulses from a holding potential of -50 mV to 40 mV at 10 s intervals. B, I_A traces recorded in the absence and presence of 50 μ M AXP. Currents were elicited by depolarizing pulse from -100 mV to +40 mV with TEA in the bath solution. C, Concentration-response curve was fitted using the Sigmoidal dose response function (variable slope). Data are means $\pm$ SEM from 5 to 7 cells.

solutions were prepared extemporaneously. First they were dissolved in methanol and then diluted in the bath with a final methanol concentration <0.1%. The 0.1% of methanol did not produce the significantly effect on K⁺ currents. The drug was ejected by gravity for 40-60 seconds from MSC-200 Manual Solution Changer (Bio-Logic-Science Instruments, France).

Results

To investigate the effects of amoxapine on I_K amplitudes in CGNs, a holding potential of -50 mV was

Fig. 2. Amoxapine does not modulate steady-state activation of I_K . A, I_K recording with an activation voltage protocol in the absence (top) or presence (bottom) of 10 μ M amoxapine. Cells were held at -50 mV and depolarized in 10 mV steps from -50 mV to $+90$ mV at 10 s intervals. B, I_K voltage-dependent activation curves in the absence or presence of amoxapine. C, Plot of the normalized conductance as a function of the command potential in the absence or presence of amoxapine; data points were fitted using the Boltzmann function. The voltage-dependent activation curve was not shifted in the presence of amoxapine. Data are means $\pm$ SEM from 6 cells.

Fig. 3. Amoxapine alters steady-state inactivation of I_K . A, I_K recorded in the absence (top) or presence (bottom) of 10 μ M amoxapine. A 1 s conditioning pre-pulse from -50 mV to $+40$ mV in 10 mV increments was applied before the test pulse to $+40$ mV. The voltage protocol is shown below the current recordings. B, Steady-state inactivation curves of I_K in the absence or presence of amoxapine obtained from the current traces shown in 3A. The abscissa shows the conditioning pre-pulse potential. C, Current amplitudes corresponding to each test pulse were normalized to the maximal currents and plotted against the pre-pulse potential. Normalized current values were fitted to a Boltzmann function. The steady-state inactivation curve was significantly shifted by amoxapine towards hyperpolarization. Data are means $\pm$ SEM from 6 cells.

applied to inactivate the I_A [20], I_K current was then evoked by 200 ms depolarizing pulses over the range from -50 mV to $+40$ mV at 10 s intervals. Application of amoxapine extracellularly produced a clear reduction in I_K current amplitudes (Fig. 1A). The inhibitory effect of amoxapine on I_K was partly reversible, reaching its maximum effect within 50–100 s and recovering close to control levels after 1–2 min. The amoxapine-induced effect on I_K was also concentration-dependent: inhibition of I_K by amoxapine at 1 μ M, 10 μ M and 50 μ M was $9.2 \pm 0.8\%$ ($n = 6$), $21.1 \pm 2.6\%$ ($n = 6$) and $68.1 \pm 2.4\%$ ($n = 10$), respectively ($P < 0.05$ by one-way ANOVA). However, when the concentration of amoxapine was increased to 100 μ M, no further inhibition is seen ($55.1 \pm 2.4\%$, $n = 5$). On

contrast, as shown in Fig. 1B, amoxapine did not significantly inhibit I_A currents obtained by depolarizing pulses to $+40$ mV from a holding potential of -100 mV in the presence of a specific I_K channel blocker, 20 mM TEA (Fig. 1B). Statistical analysis of concentration–response data is presented in Fig. 1C. The data was fitted with Sigmoidal dose response function, in which the formula is $y = A_1 + (A_2 - A_1) / (1 + 10^{(\text{LOG}x_0 - x)p})$ and drug doses, parameter x , were log transformed, where y is the inhibition rate in the presence of amoxapine, p is the variable Hill slope, A_1 means bottom asymptote, A_2 means top asymptote, and $\text{LOG}x_0$ represents center. With the equation we got the IC_{50} and parameter p , which was 13.27 μ M and 1.02, respectively. The concentration-effect

Fig. 4. Effects of train protocol and GTP γ S on amoxapine inhibition of I_K . A, A train protocol was used to address whether amoxapine (AXP) inhibition of I_K is selective for open channels. In this protocol, I_K was elicited by a depolarizing pulse (control); amoxapine was then applied to the bath solution 2 min before channel opening again by depolarizing pulses, and the effects of amoxapine on I_K amplitudes elicited by the first and subsequent pulses were measured. Pulses were evoked by 200 ms and stepping from a -50 mV holding potential to +40 mV. B, Time-course of changes in I_K amplitudes induced by the application of 10 μ M GTP γ S. Internal infusion of GTP γ S resulted in gradual reduction of I_K amplitudes after membrane rupture and abolished I_K inhibition by 10 μ M amoxapine. C, Time-course of changes in I_K amplitudes induced by the application of 10 μ M NF 449. External perfusion of NF 449 abolished I_K inhibition by 10 μ M amoxapine. D, Statistical analysis of the inhibitory effect of 10 μ M amoxapine alone (Control), 10 μ M amoxapine with train protocol (train protocol), GTP γ S and NF 449 on I_K amplitudes and amoxapine-induced I_K inhibition. Data are means $\pm$ SEM from 5-12 cells; *, $P < 0.05$ for amoxapine alone versus control; #, $P < 0.05$ for GTP γ S versus control.

relationship is in good agreement with the experimental formula, and also consistent with the concept of drugs primarily through cellular receptors. In order to eliminate the Ca^{2+} -activated potassium channel in this study, we used the bath solution without Ca^{2+} . In the fact, we did not found the different effect of amoxapine on I_K current with or without Ca^{2+} in the bathing solution. Amoxapine induces the inhibition of I_K by $19.68 \pm 1.72\%$ ($n = 5$) with 1 mM Ca^{2+} in extracellular solution, and there is no significant difference with the inhibition rate ($21.1 \pm 2.6\%$) without Ca^{2+} in extracellular solution.

We next studied the effects of amoxapine on the steady-state activation and inactivation of I_K using different experimental voltage protocols. In the voltage-activation protocol, membrane potential was held at -50 mV and I_K was evoked by a 200 ms depolarizing pulse from a first pulse potential of -50 mV to +90 mV in 10 mV steps at 10 s intervals (Fig. 2A). The I-V curve was obtained by plotting current amplitude as a function of the command potential (Fig. 2B); this revealed that when the depolarizing pulse was more positive than 10 mV, 10 μ M amoxapine

significantly reduced the current amplitude throughout the activation voltage range (Fig. 2B). By plotting normalized conductance as a function of the command potential we obtained the I_K activation curve. The data were then analyzed using the equation $G_K = I_K / (V_m - V_{\text{rev}})$, 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 Figure 2C, the activation curve was shifted towards hyperpolarization in the presence of amoxapine. The current was half-activated at 21.31 ± 1.40 mV and 16.28 ± 2.07 mV in the absence and presence of 10 μ M amoxapine, respectively ($n = 6$, $P > 0.05$ by Student's t -test), indicating that amoxapine treatment does not modify the steady-state activation properties of I_K channels.

To study the effects of amoxapine on steady-state inactivation of I_K , currents were elicited using 1 s conditioning pre-pulses from -50 mV to different membrane potentials before a 200 ms test pulse of +40 mV (Fig. 3A); Currents corresponding to each test pulse were then plotted as a function of conditioning pre-pulses (Fig. 3B). After normalizing each current to the maximal

Fig. 5. Effects of 5-HT, 5-HT₂ receptor antagonists on amoxapine inhibition of I_K . A, B, Time-course of changes in I_K amplitudes induced by amoxapine (AXP) in the presence of 20 μ M 5-HT or 20 μ M cyproheptadine (Cyp). The insets in the graphs show superimposed I_K traces from the initial control levels (after establishment of the whole-cell configuration), after external infusion of 5-HT or cyproheptadine, and after external infusion of amoxapine. The time points (1, 2, 3) noted on the curves correspond to the superimposed I_K traces illustrated by the insets. C, Time course of the changes in I_K amplitude obtained with normal intracellular solution as control. D, Statistical analysis of the effects of 5-HT and cyproheptadine on I_K amplitudes and amoxapine-induced I_K inhibition, including higher concentration of 5-HT and cyproheptadine. Data are means $\pm$ SEM from 4 to 5 cells. *, $P < 0.05$ versus controls without 5-HT and cyproheptadine; #, $P < 0.05$ versus controls without amoxapine.

current amplitude obtained from the -50 mV pre-pulses as a function of the conditioning pre-pulse potential, we obtained an inactivation curve for I_K and calculated V_{h50} , the voltage at which the current amplitude was half-inactivated (Fig. 3C). In 6 cells studied, the half-maximal inactivation voltage was -6.41 ± 2.12 mV and -17.69 ± 2.23 mV in the absence and presence of 10 μ M amoxapine respectively ($n = 6$, $P < 0.05$ by Student's t -test). This revealed that the inactivation curve was significantly shifted towards hyperpolarization by the application of amoxapine.

We then used a train protocol to investigate whether amoxapine acts directly as an open-channel blocker or exerts its action via intracellular signal transduction. 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 magnitudes elicited by the first and subsequent depolarizing pulses were recorded. As shown in Fig. 4A, the inhibition of the current amplitude evoked by the first pulse was $20.16 \pm 4.43\%$ in the presence of 10 μ M amoxapine, similar to the inhibitory effect induced by 10 μ M amoxapine alone. This experiment indicated that current blocking can occur without prior channel

opening, and the signal transduction could therefore be involved. We used the non-hydrolyzable GTP analog and G-protein activator GTP γ S to explore the potential involvement of G-protein-coupled receptors (GPCRs) in amoxapine inhibition of I_K . Internal infusion of GTP γ S (10 μ M) resulted in a gradual decrease of I_K amplitudes after membrane rupture (Fig. 4B). Moreover, following inhibition of I_K amplitudes by internal application of GTP γ S, 10 μ M amoxapine only reduced I_K by $4.28 \pm 0.56\%$ ($n = 12$), and the magnitude of the reduction was significantly different from that obtained with 10 μ M amoxapine alone ($P < 0.05$ by non-paired t -test). Similar to the result with GTP γ S, the inhibitor of Gs, 10 μ M NF 449 reduced the inhibitory effect of 10 μ M amoxapine on I_K to $2.85 \pm 1.08\%$ ($n = 7$, Fig. 4C). This result indicated that G-protein, especially Gs, activation is required for amoxapine inhibition of I_K . Statistical analysis of the above data is shown in Fig. 4D.

It was recently reported that positron emission tomography (PET) on normal volunteers revealed that amoxapine at a dose of 150 mg/day saturated 5-HT₂ occupancy (98%) but displayed only modest D₂ occupancy (63%) [21]. We therefore used 5-HT and the 5-HT₂ receptor antagonist cyproheptadine in the bath

Fig. 6. Effects of dopamine, D_2 and D_1/D_5 receptor antagonists on amoxapine-induced inhibition of I_K . A–D, Time-course of changes in I_K amplitudes induced by amoxapine (AXP) in the presence of dopamine, (S)-(-)-Sulpiride (Sulp), R(+)-SCH-23390 (SCH) and SKF 83566 (SKF). Dopamine provoked a modest decrease in I_K amplitudes and eliminated the inhibitory effects of 10 μ M amoxapine on I_K . E, Statistical analysis of the effects of dopamine, 20 μ M (S)-(-)-Sulpiride, 20 μ M R(+)-SCH-23390 and 5 μ M SKF 83566 on I_K amplitudes and amoxapine-induced I_K inhibition. Data are means $\pm$ SEM from 6 to 8 cells. *, $P < 0.05$ versus corresponding control cells (dopamine, Sulpiride and R(+)-SCH-23390); #, $P < 0.05$ versus controls without amoxapine.

solution to investigate whether amoxapine-induced inhibition of I_K currents is mediated via 5-HT receptors. As presented in Fig. 5A and 5B, both 5-HT and cyproheptadine significantly inhibited I_K by $17.6 \pm 3.2\%$ ($n = 5$) and $21.7 \pm 1.8\%$ ($n = 4$), respectively. However, in the presence of 5-HT or cyproheptadine, 10 μ M amoxapine still inhibited I_K by $19.1 \pm 2.0\%$ ($n = 5$) and $20.8 \pm 2.1\%$ ($n = 4$), respectively. Also with a higher concentrate of 5-HT (100 μ M) or cyproheptadine (200 μ M) in the bath, amoxapine remain an inhibition of I_K by $21.5 \pm 5.3\%$ ($n = 4$) or $23.0 \pm 2.4\%$ ($n = 4$), an extent of inhibition that was not significantly different from that obtained with 10 μ M amoxapine alone ($P > 0.05$ by non-paired t -test). Fig. 5C shows that the amplitude of I_K was stable during the recording time following establishment of the whole-cell configuration. Statistical analysis of these data is shown in Fig. 5D.

We then used dopamine and antagonists of the D_1/D_5 or D_2 receptors in the bath solution to address whether the dopamine receptors might mediate amoxapine-induced inhibition of I_K currents. Application of 20 μ M dopamine inhibited of I_K by $9.4 \pm 1.2\%$ ($n = 8$) and, in the presence of dopamine, 10 μ M amoxapine only inhibited I_K by $2.9 \pm 0.8\%$ (Fig. 6A, $n = 6$). Although the D_2 receptor antagonist sulpiride were able to reduce I_K amplitudes, sulpiride failed to eliminate amoxapine-induced inhibition of I_K and, in the presence of sulpiride, the extent of inhibition of I_K by 10 μ M amoxapine ($20.82 \pm 1.87\%$, $n = 7$) was not significantly different from that induced by 10 μ M amoxapine alone ($P > 0.05$ by non-paired t -test, Fig. 6B). By contrast, D_1/D_5 receptor antagonist, R(+)-SCH-23390 or SKF 83566 abolished amoxapine-induced I_K inhibition (Fig. 6C and 6D). In the presence of 20 μ M R(+)-SCH-23390 or 5 μ M SKF 83566 in the bath solution,

Fig. 7. cAMP/PKA pathways mediate amoxapine-induced inhibition of I_K . A–C, Time-course of changes of I_K amplitudes induced by 10 μ M amoxapine (AXP) in the presence of extracellular 20 μ M forskolin, 20 μ M db-cAMP and 20 μ M Rp-cAMP. Forskolin, db-cAMP and Rp-cAMP provoked a gradual decrease in I_K amplitudes and abolished the effect of 10 μ M amoxapine on I_K . D, Statistical analysis of the effects of forskolin, db-cAMP and Rp-cAMP on amoxapine-induced I_K inhibition. Data are means $\pm$ SEM from 4–5 cells. *, $P < 0.05$ versus control group with amoxapine alone. E, Intracellular levels of cAMP measured by cAMP analysis in the presence of 50 μ M amoxapine and 20 μ M forskolin for 2 min; *, $P < 0.05$ by one-way ANOVA. Data from 5 independent experiments. F, The steady-state inactivation curve was obtained in the present or absence of cAMP. Data are means $\pm$ SEM from 4 cells.

10 μ M amoxapine only inhibited I_K by $5.47 \pm 1.26\%$ ($n = 8$) and $2.79 \pm 0.79\%$ ($n = 8$), an extent that was significantly different from that induced by 10 μ M amoxapine alone ($P < 0.05$ by non-paired t -test). Notably, when a higher concentration (50 μ M) of R(+)-SCH-23390 was used in the bath solution, 10 μ M amoxapine provoked an modest augmentation of I_K amplitude ($7.55 \pm 3.92\%$). Statistical analysis of these data is shown in Fig. 6E. These results argue that amoxapine inhibition of I_K amplitudes in CGNs is mediated via the dopamine D_1 and/or D_5 receptors.

Agonist binding to D_1/D_5 receptors is known to activate adenylyl cyclase and stimulate intracellular cAMP synthesis. To address whether cAMP/PKA pathway activation contributes to amoxapine-mediated inhibition of I_K we studied the effects of PKA agonism or antagonism on the action of amoxapine. Perfusion of CGNs with 20 μ M forskolin provoked a gradual decrease in I_K amplitude similar to that produced by amoxapine. In

the presence of forskolin, amoxapine only inhibited I_K currents by $1.2 \pm 2.1\%$ (Fig. 7A, $n = 5$). The membrane-permeable analog of cAMP, db-cAMP, also mimicked amoxapine inhibition of I_K , and reduced the extent of I_K inhibition by 10 μ M amoxapine to $2.88 \pm 2.14\%$ (Fig. 7B, $n = 4$). Moreover, when the membrane-permeable selective PKA antagonist Rp-cAMP, which was not inhibit the activation of Epac [22] was added in the bath solution, 10 μ M amoxapine only inhibited I_K currents by $0.82 \pm 1.82\%$ (Fig. 7C, $n = 5$). Statistical analysis of the above data is shown in Fig. 7D. We also used a direct cAMP assay to detect the effect of amoxapine on cAMP levels. As shown in Fig. 7E, when the cells were incubated with 50 μ M amoxapine or 20 μ M forskolin for 2 minutes, cAMP levels were significantly increased by $70.95 \pm 30.45\%$ and $38.52 \pm 26.59\%$ compared with control group (without amoxapine and forkolin) ($P < 0.05$ by one-way ANOVA). In addition, the inactivation curve was also significantly shifted towards hyperpolarization by the application of

Fig. 8. Amoxapine increases the phosphorylation level of PKA (pPKA).

A. Western blot analysis of pPKA levels in the presence and absence of amoxapine with or without 20 μ M R(+)-SCH-23390. Quantitative analysis the levels of pPKA normalized to levels of the internal control (GAPDH, glyceraldehyde 3-phosphate dehydrogenase); values are means from 3-6 independent experiments. *, $P < 0.05$ versus control. B. PepTag assay to measure activity of cAMP-dependent PKA in response to amoxapine. The upper bands represents the nonphospho-substrates, and the lower bands represents the substrates phosphorylated by activated PKA. Positive, negative referred to 2 μ g/ml activated PKA catalytic subunit and double distilled water, respectively. The control was CGNs treatment without amoxapine. Quantitative analysis the levels of activated PKA normalized darkness of lower bands to that of positive control. *, $P < 0.05$ versus control. values are means from 3 independent experiments.

Fig. 9. Proposed model depicting the mechanisms likely involved in the decrease of I_K by amoxapine in CGNs. Amoxapine (AXP), mainly acting through the dopamine-specific receptor D_1/D_5 , decreases I_K via the adenylate cyclase (AC)/protein kinase A (PKA) signaling pathways. AXP might have lower affinity for D_2 -like subtypes. G_s , G_i , G protein coupled to AC; Fos, Forskolin; Sulp, (S)-(-)-Sulpiride; SCH, R(+)-SCH-23390; (+), activation; (-), inhibition; solid line, higher affinity; dashed line, lower affinity.

cAMP. In 4 cells studied, the half-maximal inactivation voltage was -4.38 ± 1.16 mV and -12.34 ± 1.35 mV in the absence and presence of 20 μ M db-cAMP, respectively ($P < 0.05$ by Student's t -test). These results suggest that cAMP/PKA pathway activation mediates amoxapine inhibition of I_K in cerebellar neurons.

To confirm the involvement of cAMP/PKA pathways in amoxapine inhibition of I_K we used western blotting in conjunction with a phospho-specific antibody to measure levels of phosphorylated PKA (pPKA) in response to amoxapine. As shown in Fig. 8A, there was a significant increase in pPKA levels after incubation of CGNs for 2 min with 10 μ M and 50 μ M amoxapine. Moreover, the D_1/D_5 receptor antagonist R(+)-SCH-23390 significantly eliminated the effects of 10 μ M amoxapine on pPKA accumulation, and this paralleled the abolition of amoxapine-mediated inhibition of I_K induced by R(+)-SCH-23390 (data from 3-6 independent

experiments). We also used PepTag assay to measure activity of cAMP-dependent protein kinase (PKA) in response to amoxapine (Fig. 8B). Similar with the result shown in Fig. 8A, there was a significant increase in activated PKA levels after incubation of CGNs for 2 min with 10 μ M and 50 μ M amoxapine (data from 3 independent experiments).

Discussion

Psychological depression is major health problem for which pharmacological treatment is not yet entirely

satisfactory. Most prescribed antidepressants enhance extracellular levels of monoamines by inhibiting 5-HT and/or noradrenaline uptake transporters [23]. Previous studies have revealed that, in addition to specific inhibition of serotonin and/or norepinephrine transporters, TCAs also modulate several ion channels, and a direct drug interaction with ion channels has been suggested [24]. For example, fluoxetine was reported to act as an open-channel blocker for Kv3.1 currents [24], and doxepin also had direct inhibitory effects on the human *Ether-a-go-go*-related gene (hERG) potassium channels [25]. We previously reported that amoxapine inhibits I_K currents in mouse cortical neurons by activating 5-HT receptors and downstream cAMP/PKA pathways [10]. We report here that, in contrast to results obtained with cortical neurons, in rat cerebellar neurons amoxapine modulates I_K currents independently of the 5-HT receptor. Instead, amoxapine inhibition of CGNs I_K currents was dependent on dopamine-receptor activation and was abolished by a specific D_1/D_5 receptor inhibitor.

The effects of amoxapine on I_K amplitudes in rat CGNs were comparable to those seen in cortical neurons. Amoxapine inhibited I_K currents 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 suggests instead that amoxapine is most likely to inhibit I_K in CGNs through intracellular biochemical processes that in turn reduce I_K channel activity, as in cortical neurons [10]. In the present study we report that activation of cAMP/PKA pathways by either forskolin or db-cAMP eliminated further amoxapine inhibition of I_K currents in CGNs. Furthermore, activation of G-proteins downstream of GPCRs with intracellular GTP γ S also abolished I_K inhibition by amoxapine. Notably, amoxapine treatment led to a significant increase in intracellular levels of cAMP and phosphorylated PKA. Together these results argue that amoxapine effects on I_K do not involve a direct interaction between the drug and the ion channel. Instead, amoxapine targets GPCRs that activate intracellular cAMP/PKA pathways, and these then modulate channel activity.

A PET study in normal volunteers demonstrated that, at a dose of 150 mg/day, amoxapine almost completely saturated 5-HT₂ occupancy (98%) but displayed only modest D₂ occupancy (63%) [21]. In a previous study on mouse cortical neurons we reported that amoxapine inhibits I_K in these cells by activating 5-HT receptors [10, 26]. However, we now report that 5-HT₂ receptor antagonists and 5-HT have no effect on amoxapine

inhibition of I_K in rat CGNs. Instead, the application of dopamine or dopamine receptor antagonists was able to eliminate amoxapine-induced inhibition of I_K , indicating that dopamine receptor was targeted. This is consistent with the results reported by Frederik et al., 5-HT₂ receptor may not express in rat CGNs [27]. It may explain why there are different receptors involved in amoxapine-induced I_K inhibition on mouse cortical neurons and rat CGNs.

At least five distinct subtypes of GPCR dopamine receptors are known. Two D₁-like receptor subtypes (D₁ and D₅) couple to the G-protein G_s, and that the D₂-like subfamily (D₂, D₃, and D₄) are prototypic GPCRs that inhibit adenylyl cyclase and activate K⁺ channels [28]. Although both D₁ and D₂ receptor subtypes have been detected in rat cerebellum [29], a selective D₁/D₅ receptor antagonist could effectively eliminate amoxapine-induced inhibition of I_K and PKA activation, indicating that a D₁-like receptor subtype is associated with the I_K inhibition by amoxapine in CGNs. Moreover, when a higher concentration of the D₁/D₅ receptor antagonist was employed, amoxapine induced a modest increase in I_K amplitudes instead. Overall the results are consistent with the possibility that amoxapine might activate both D₁ and D₂-like dopamine receptor in rat CGNs, but that it has significantly higher affinity for D₁-like subtypes. Because D₁-like and D₂-like receptors appear to have opposing effects on adenylyl cyclase, the modest inhibition of I_K by dopamine could reflect the fact that both D₁ and D₂ receptor types are expressed in CGNs [29]. This also partly explains why no further inhibition is seen when the high concentration of amoxapine was used. In addition, we also observed that 5-HT₂, D₁/D₅ and D₂ receptor antagonists all exert significant inhibitory effects on I_K . The effects of cyproheptadine, sulpiride, and SCH-23390 on I_K amplitudes were similar, consistent with our previous study on cortical neurons [10], and with a report that in human and rat myocardium the 5-HT₂ receptor antagonists risperidone and ketanserin inhibit K⁺ currents through a direct channel blocking mechanism [30]. However, despite direct inhibition of K⁺ channels, these antagonists failed to abolish the inhibitory effects of amoxapine.

The upper range of therapeutic plasma concentrations of amoxapine has been reported to vary between 0.2 to 0.4 μ g/ml in humans (0.637 to 1.912 μ M; [31]. In some cases, the concentration of amoxapine in brain was roughly 10-fold higher than its plasma concentration [32]. In cortical neurons, amoxapine inhibited I_K currents with an IC₅₀ value of 211.36 μ M, a concentration significantly above the clinical dosage.

However, the IC_{50} value of amoxapine for inhibition of I_K in rat CGNs was only 13.27 μ M, a significantly lower concentration than that required in rat cortical neurons, and markedly closer to the clinically effective concentrations. Although 5-HT receptors are expressed in the cerebellum [33], this finding suggests that amoxapine has significantly higher affinity for D_1/D_5 receptors than for 5-HT receptors, and its action on rat CGNs is predominantly via targeting of D_1/D_5 receptors.

Structural and functional neuroimaging studies have emphasized the potential role of the cerebellum in the pathophysiology of psychiatric disorders, and it has been suggested that integrated models of neuropsychiatric disorders must include the cerebellum and its neural connections [34, 35]. Because information transmission in the nervous system is relayed by changes in the frequency of neuronal firing, pathological changes in firing frequency can give rise to neurological and psychological disorders [36]. Voltage-gated K^+ channels play an important role in defining the action potential waveform, and selective modulators of these channels are anticipated to be of therapeutic utility in such disorders. Theoretically, under conditions in which action potential firing is decreased (e.g. in depression and cognitive dysfunction) K_v channel blockers might restore normal firing [36]. Although amoxapine inhibition of I_K could represent a novel mode of action for this second-generation TCA antidepressant, further studies will be required to address whether I_K inhibition via dopamine receptor targeting underlies the antipsychotic action of amoxapine.

In summary, this is the first report of amoxapine modulation of I_K currents via dopamine receptor targeting in cultured rat cerebellar neurons. We report that amoxapine inhibits I_K currents, an effect different from the previously described effects of tricyclic antidepressants

on ion-channel activity and on cortical neurons. Although the clinical relevance of inhibition of I_K currents remains unclear, it is possible that amoxapine inhibition of K^+ channels in CGNs could contribute to the antidepressant and antipsychotic activity of the drug. A proposed model illustrating the signaling cascade associated with amoxapine in the cerebellar neurons is shown in Fig. 9. Amoxapine, acting through the dopamine receptor, increases the I_K via Gs activity and cAMP/PKA pathway.

Abbreviations

cAMP (cyclic adenosine monophosphate); CGN (Cerebellar granule neuron); Cyp (Cyproheptadine); db-cAMP (dibutyl cyclic adenosine monophosphate); DMEM (Dulbecco's Modified Eagle's Medium); GTP γ S (guanosine 5'-[γ thio] triphosphate); hERG (Human *Ether-a-go-go* Related Gene); I_A (transient potassium current); I_K (delayed rectifier potassium current); Rp-cAMP (Rp-adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt hydrate); SCH (R(+)-SCH-23390 hydrochloride); SKF (SKF 83566); Sulp ((S)-(-)-Sulpiride); TEA (tetraethylammonium chloride); TCAs (Tricyclic antidepressants).

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