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Influence of flupirtine on a G-protein coupled inwardly rectifying potassium current in hippocampal neurones

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1 Previous studies have shown that flupirtine, a centrally acting, non-opioid analgesic agent, also exhibits neuroprotective activity in focal cerebral ischaemia in mice and reduces apoptosis induced by NMDA, gp 120 of HIV, prior protein fragment or lead acetate as well as necrosis induced by glutamate or NMDA in cell culture. To study the potential mechanism of the neuroprotective action of flupirtine, we investigated whether flupirtine is able to modulate potassium or NMDA-induced currents in rat cultured hippocampal neurones by use of the whole-cell configuration of the patch-clamp technique.

2 We demonstrated that 1 μM flupirtine activated an inwardly rectifying potassium current (K_{ir}) in hippocampal neurones ($\Delta I = -39 \pm 18$ pA at -130 mV; $n = 10$). This effect was dose-dependent ($EC_{50} = 0.6$ μM). The reversal potential for K_{ir} was in agreement with the potassium equilibrium potential predicted from the Nernst equation showing that K_{ir} was predominantly carried by K^+. Furthermore, the induced current was blocked completely by Ba^{2+} (1 mM), an effect typical for K_{ir}.

3 The activation of K_{ir} by flupirtine was largely prevented by pretreatment of the cells with pertussis toxin (PTX) indicating the involvement of a PTX-sensitive G-protein in the transduction mechanism ($\Delta I = -3 \pm 6$ pA at -130 mV; $n = 8$). Inclusion of cyclic AMP in the intracellular solution completely abolished the activation of K_{ir} ($n = 7$).

4 The selective α_2-adrenoceptor antagonist SKF-86466 (10 μM), the selective 5-HT_{1A} antagonist NAN 190 as well as the selective $GABA_B$ antagonist 2-hydroxysaclofen (10 μM) failed to block the flupirtine effect on the inward rectifier.

5 Flupirtine (1 μM) could not change the current induced by 50 μM NMDA.

6 These results show that in cultured hippocampal neurones flupirtine activates an inwardly rectifying potassium current and that a PTX-sensitive G-protein is involved in the transduction mechanism.

Keywords: Flupirtine; inwardly rectifying potassium current; G-protein; NMDA; patch-clamp; hippocampus; neuroprotection

Introduction

Flupirtine is a centrally acting, non-opioid analgesic agent with muscle relaxant properties (Jakovlev *et al.*,1985; Nickel *et al.*, 1985; 1988; Vaupel *et al.*, 1989). Furthermore, previous studies have shown that flupirtine also exhibits neuroprotective activity in focal cerebral ischaemia in mice (Rupalla *et al.*, 1995) and reduces apoptosis induced by N-methyl-D-aspartate (NMDA), gp 120 of HIV, prion protein fragment or lead acetate (Perovic *et al.*, 1994; 1995) as well as necrosis induced by glutamate or NMDA (Rupalla *et al.*, 1995; Müller, personal communication). Neuronal cultures treated with flupirtine showed a higher viability compared with untreated control cultures (Perovic *et al.*, 1994).

However, the mechanism of the neuroprotective action of flupirtine is not yet understood. There is evidence that noradrenergic mechanisms are involved in the antinociceptive action of flupirtine, because selective destruction of noradrenergic pathways, depletion of neurotransmitters by reserpine and inhibition of the synthesis of noradrenaline (NA) diminished or even totally abolished the flupirtine effect. Inhibition of NA uptake by imipramine significantly increased and antagonists at α_2-adrenoceptors significantly decreased the flupirtine-induced antinociception (Szelenyi *et al.*, 1989; Nickel *et al.*, 1988). It is known that α_2-adrenoceptor agonists hyperpolarize the neuronal membrane by activating a G-protein-coupled inwardly rectifying K^+ current (Aghajanian & VanderMaelen, 1982; Suprenant & North, 1988).

Recently, it has been suggested that flupirtine shows NMDA receptor antagonistic properties (Osborne *et al.*, 1994; Schwarz *et al.*, 1994a,b). Also, it has been demonstrated, in our laboratory, that this drug inhibits the increase in the intracellular Ca^{2+} concentration induced by stimulation of cultured neurones with glutamate (Rupalla *et al.*, 1995). The aims of the present study were to investigate the possible modulation of inwardly rectifying K^+ currents by flupirtine, as well as the corresponding second messenger system(s) by use of the whole-cell configuration of the patch-clamp technique. Furthermore, we wanted to determine if α_2-adrenoceptors are involved in the flupirtine action under our experimental conditions and if flupirtine was able to influence NMDA-induced currents in hippocampal neurones.

Methods

Cell culture

Primary cultures of rat hippocampal neurones were prepared as described previously (Prehn *et al.*, 1993). Briefly, primary cultures were prepared from hippocampi of neonatal rats (Fischer 344). Hippocampi were dissected free of meninges, cut into small pieces, then treated with papain (0.1% on Leibovitz L-15 medium, 37°C) for about 15 min and after that triturated by repeated slow pipetting through a fire-polished pipette. A tyrosine inhibitor solution (1% in Minimal Essential Medium (MEM) containing 1% bovine serum albumin) was carefully layered at the bottom of the cell suspension. The tube was centrifuged at 600 r.p.m. and 20°C for 10 min. The cells were then resuspended in MEM containing 10% NU° serum, seeded into poly-L-lysine-coated Petri dishes at a final density of 3×10^5 cells/dish and grown in MEM supplemented with 10% NU° serum, 100 iu ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The cultures were kept in a humidified atmosphere of 5% CO_2 and 95% air at

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37°C. To inhibit the glial cell proliferation 20 μM 5-D-arabinofuranoside was added for 24 h. Cells were used for measurements 4–8 days after plating for the measurement of K^+ current and 6–10 days after plating for the NMDA-induced currents.

Recording conditions

Experiments were performed with the whole cell configuration of the patch-clamp technique (Hamill *et al.* 1981) at room temperature (20–22°C). Drugs were applied to the extracellular solution with a fast-application system (Bormann, 1992). Micropipettes were pulled from borosilicate glass with filament (Science Trading, Frankfurt, Germany) and heat polished at the tip. They had resistances between 8–10 MΩ after filling with the pipette solution. Current signals were obtained by means of an EPC-7 amplifier (List, Darmstadt, Germany), filtered at 10 KHz (unless otherwise indicated) and digitized by an ITC-16 interface (Instrutech, Elmont, U.S.A.). Generation of voltage steps, data storage and analysis were performed with AQUIRE and REVIEW software (Instrutech, Elmont, U.S.A.).

Recording solutions

Different pipettes and bathing solutions were used. For experiments on K^+ channels the pipettes were filled with a solution containing (in mM): KCl 140, $CaCl_2$ 1, $MgCl_2$ 2, TTX 0.01, EGTA 11 and HEPES 10 (pH 7.2; 295–300 mosmol). The bathing solution contained (in mM) NaCl 140, $MgCl_2$ 2, KCl 5, $CaCl_2$ 2 and HEPES 5 (pH 7.3; 305–315 mosmol).

For NMDA-induced currents the patch pipette contained (in mM) $CsCl_2$ 120, $MgCl_2$ 2, $CaCl_2$ 1, HEPES 10 and EGTA 11, magnesium-ATP 5 and glucose 10 (pH = 7.2; 295–300 mosmol). The bath solution contained NaCl 140 mM, KCl 5.4 mM, $CaCl_2$ 2 mM, HEPES 10 mM, glycine 1 μM, strychnine 0.1 μM (to block the glycine-activated Cl^- currents) and TTX 1 μM (pH 7.3; 305–315 mosmol). NMDA (50 μM) was applied for 20 s. The current values are expressed as mean amplitudes ± s.d. For these experiments only pyramidal neurones of 6–10 day old cultures were used.

Materials

Flupirtine [ethyl-2-amino-6(fluorobenzyl)-amino-3-pyridine carbamate] was a gift from Asta Medica (Frankfurt, Germany) and SKF-86466 (6-chloro-2,3,4,5-tetrahydro-3-methyl-1H-3-benzazepine) from Smith Kline Beecham Pharmaceuticals (King of Prussia, U.S.A.). Idazoxan, NAN 190 (= 1-[2-methoxyphenyl]-4-[4-(2-phthalimide)butyl]piperazine hydrobromide), 2-hydroxysaclofen and pertussis toxin were obtained from RBI (Cologne, Germany), adenosine 3′:5′-cyclic monophosphate (cyclic AMP) from Sigma (Gelsenkirchen, Germany).

Statistical analyses

Data are presented as means ± s.d. For statistical comparison unpaired *t* test was used.

Results

Activation of an inwardly rectifying potassium current by flupirtine

Flupirtine activated an inwardly rectifying K^+ current (K_{ir}) in about 85% of the neurones tested (Figure 1a and b and Table 1). The remaining 15% showed no response to flupirtine under these conditions. The observed effect was concentration-dependent (Figure 1c; $EC_{50} = 0.6$ μM). Figure 1a shows that 1 μM flupirtine enhanced the inward K^+ current activated by hyperpolarizing voltage steps from a holding potential of −70 mV in 10 mV steps in 10 mM $[K^+]_o$. This effect was nearly reversible after 5 min washing with flupirtine-free

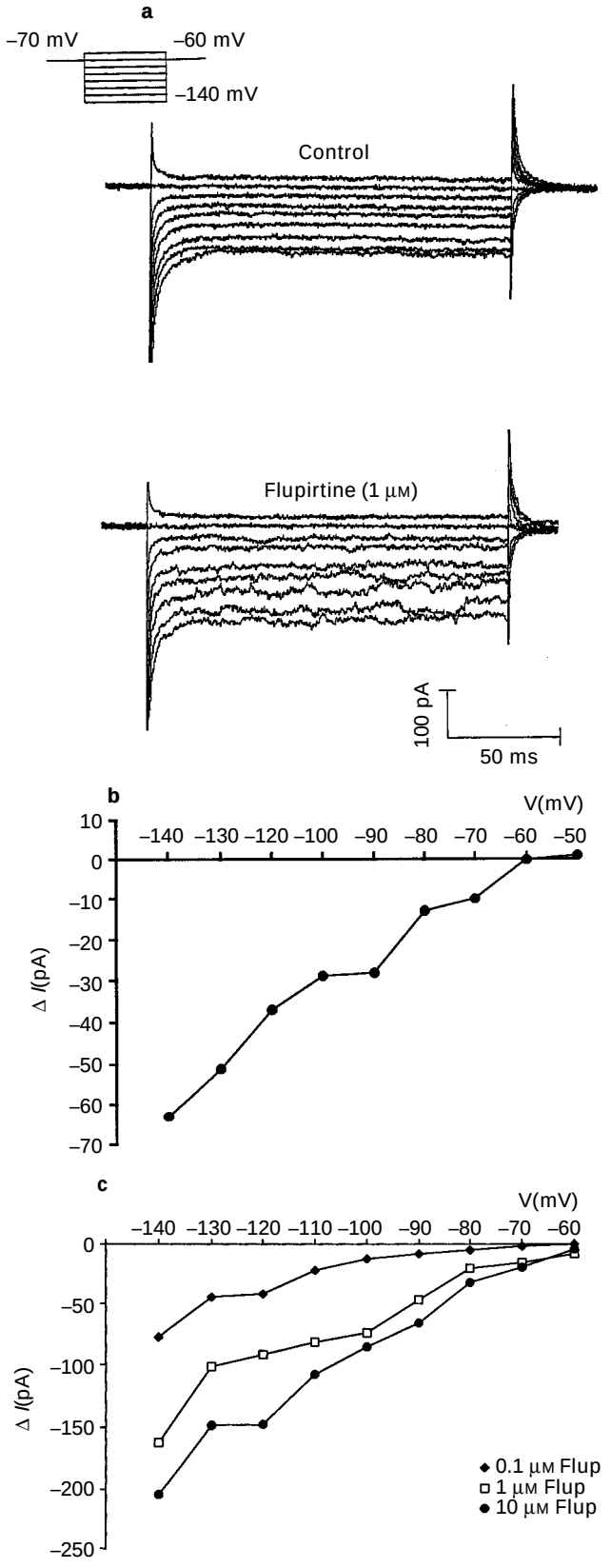

Figure 1 Activation of K_{ir} by 1 μM flupirtine. Currents were evoked in the absence (control) and presence of 1 μM flupirtine by 200 ms hyperpolarizing voltage steps over the range of −60 to −140 mV increments from a holding potential of −70 mV. (a) Original traces: currents induced by flupirtine were larger in response to hyperpolarizing voltage pulses than depolarizing ones indicating inwardly rectifying properties. (b) Current-voltage relation of the same cell as in (a). The effect is shown as difference, Δ*I*, of the current in absence and presence of flupirtine. (c) Concentration-dependence; for the flupirtine concentrations tested (0.1, 1 and 10 μM) the same cell was used. $[K^+]_o$ was 20 mM.

Table 1 Activation of K_{ir} by 1 μM flupirtine

$[K^+]_o$ (mM)	$\Delta I_{130\,mV}$ (pA)	V_o (mV)	E_K (mV)	n
10	-39 ± 18	-66 ± 3	-66	10
20	-54 ± 21	-51 ± 6	-49	7

From a holding potential of -70 mV voltage steps were made to -130 mV. The effects are shown as difference ΔI in the presence and absence of flupirtine. V_o = reversal potential of the current; $E_K = K^+$ equilibrium potential; $[K^+]_o$ = extracellular K^+ concentration; intracellular K^+ concentration = 140 mM

bathing solution. The current-voltage (*I*-V) relationship of the flupirtine-induced current (I_{flup}) exhibited marked inward rectification at potentials negative to the K^+ equilibrium potential (E_K; Figure 1b).

With 10 mM K^+ extracellular and 140 mM K^+ intracellular the mean value of the reversal potential of the induced current was close to the E_K predicted from the Nernst equation, indicating that I_{flup} is mainly carried by K^+. Increasing the external K^+ concentration shifted the reversal potential in the positive direction (see Table 1).

Effect of Ba^{2+}

As illustrated in Figure 2a, 1 mM $BaCl_2$ was able to block K_{ir} completely ($n=4$). For these experiments only neurones that had shown a response to flupirtine were used. Addition of Ba^{2+} to the bath produced a linear *I*-V relationship and shifted V_o in the positive direction (Figure 2b).

Pre-treatment with pertussis toxin

To determine whether a pertussis toxin (PTX)-sensitive G-protein is involved in the transduction mechanism, the cells were incubated with 200 ng ml^{-1} PTX for 14–36 h at 37°C before measurement. Under these conditions no obvious morphological differences could be observed between control cells and PTX pretreated cells. Figure 3 shows that PTX pretreatment largely prevented the activation of I_{flup}. In the untreated neurones 1 μM flupirtine elicited an inward current of $\Delta I_{-130\,mV} = -39\pm18$ pA ($n=10$), whereas in the pretreated neurones the induced current was $\Delta I_{-130\,mV} = -3\pm6$ pA ($n=8$).

Effect of intracellular cyclic AMP on I_{flup}

When 100 μM cyclic AMP was added to the pipette solution, the activation of I_{flup} was completely abolished ($\Delta I_{-130\,mV} = -1\pm3$ pA; $n=5$; Figure 3b). In 2 out of 7 neurones even the control current was reduced.

Determination of the receptor responsible for the activation of I_{flup}

It has been shown that idazoxan, an α_2-adrenoceptor antagonist, reduced the antinociception induced by flupirtine (Szelenyi *et al.*, 1989). When idazoxan and flupirtine (1 μM) were applied together the activation of I_{flup} was only slightly diminished ($\Delta I_{-130\,mV} = -19\pm18$ pA; $n=5$), whereas the pretreatment with idazoxan nearly prevented the response to flupirtine ($\Delta I_{130\,mV} = -2\pm4$ pA; $n=5$ for 100 μM idazoxan and 5 ± 5 pA for 10 μM idazoxan). However, we could observe a biphasic effect of idazoxan on the inward rectifier: immediately after application of idazoxan the inward current was reduced in 3 out of 5 neurones ($\Delta I_{-130\,mV} = +19\pm5$ pA; $n=3$), but with a delay of about 4 min an enhancement of the current was found in all neurones ($\Delta I_{-130\,mV} = -24\pm11$ pA; $n=3$). Therefore, we tested the selective α_2-adrenoceptor antagonist SKF-86466 (Reis *et al.*, 1994). SKF-86466 (10 μM) was not capable of preventing the activation of I_{flup} ($\Delta I_{-130\,mV} = -40\pm17$; $n=5$). Both 10 μM NAN 190, a selective 5-HT_{1A} antagonist, and 10 μM 2-hydroxysaclofen, a selective $GABA_B$ antagonist, could not block I_{flup}. The induced currents averaged -37 ± 17 pA and 34 ± 21 pA at -130 mV, respectively.

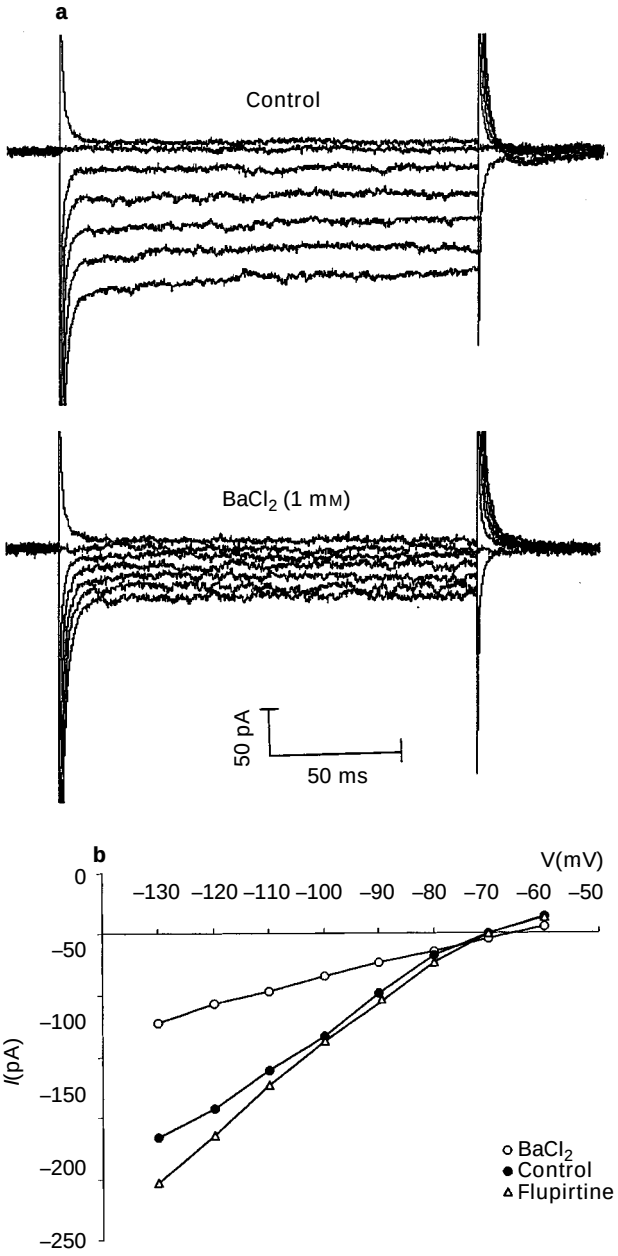

Figure 2 Effect of Ba^{2+} on K_{ir}. Values in (a) and (b) were from the same neurone. Voltage protocol and data analysis were identical to those used in Figure 1. (a) Series of whole cell currents measured in the absence (control) and presence of 1 mM $BaCl_2$. (b) Steady state *I*-V relation of the same neurone as in (a). In this experiment $[K^+]_o = 20$ mM.

Effect of flupirtine on the NMDA-induced current

Six to 10 day old pyramidal neurones were clamped at -70 mV holding potential and the NMDA inward current was elicited by 20 s application of 50 μM NMDA in the presence of 1 μM glycine. Addition of 5 mM magnesium-ATP to the intracellular solution nearly prevented the run-down of the NMDA-induced currents (98 ± 5; $n=3$). Flupirtine 1 mM did not change the current induced by NMDA (98 ± 4; $n=4$).

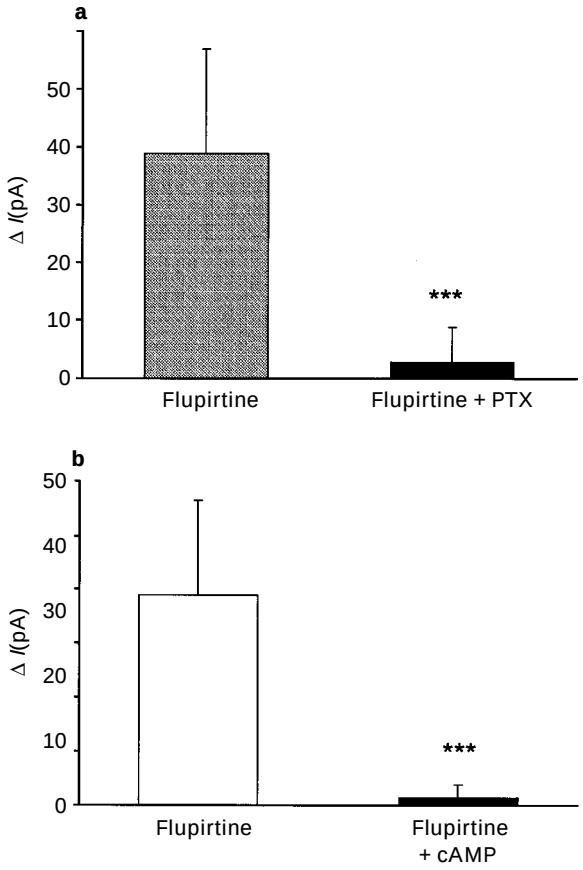

Figure 3 Blockade of flupirtine effect on K_{ir} by pertussis toxin (PTX) pretreatment and addition of cyclic AMP to the intracellular solution. Neurones were maintained at a holding potential of -70 mV and stepped to -130 mV for 200 ms. Flupirtine was used at a concentration of 1 μM. (a) The columns represent the current induced by flupirtine ($n=10$) as well as flupirtine plus PTX ($n=8$). The values are given as means $\pm$ s.d. Different from flupirtine-treated cells: ***$P<0.001$ (Student's t test). (b) The induced current (ΔI) was determined with ($n=5$) and without ($n=10$) addition of 100 μM cyclic AMP to the pipette solution. Values are given as means $\pm$ s.d. ***$P<0.001$ (Student's t test).

Discussion

Inwardly rectifying K^+ channels, which contain two transmembrane regions and a pore region, represent a new family of K^+ channels (Ho *et al*., 1993; Kubo *et al*., 1993a,b), with significant differences from classical voltage-gated K^+ channels (Pongs, 1992). They play a significant role in the maintenance of the resting membrane potential and controlling the excitability of the cell and are characterized by a conductance that increases under hyperpolarization and decreases under depolarization. A slight outward conductance at membrane potentials just above the E_K helps to keep the resting potential close to the E_K (Hille, 1992). It is known that the activation of G-protein activated inward rectifier K^+ (GIRK) channels, a member of the family of inward rectifier K^+ channels, leads to a hyperpolarization of the neuronal membrane. GIRK channels are gated by the PTX-sensitive G-protein directly (Huang *et al*., 1995), which couples to a variety of neurotransmitter receptors such as 5-HT_{1A}, $GABA_B$, μ-, κ- and δ-opioid, α_2-adrenoceptors and D_2 receptors (Suprenant & North, 1988; Loose & Kelly, 1990; Kim *et al*., 1995; for review see North, 1989). *In situ* hybridization experiments showed that GIRK1 is widely distributed in the rat brain including the hippocampus, dorsal raphe, substantia nigra and locus coerulus (Karschin *et al*., 1994), whereas GIRK2 was found mainly in the cerebellum, substantia nigra, olfactory bulb, cerebral cortex, hippocampus and pons (Kobayashi *et al*., 1995).

Moreover, it has been found that 5-HT_{1A} agonists, $GABA_B$ agonists, κ-agonists and D_2 agonists exhibit neuroprotective activity (Prehn *et al*., 1991; 1993; Tortella & Decoster, 1994; Farber *et al*., 1995; Lal *et al*., 1995; Liu *et al*., 1995). Recently Patil *et al*. (1995) showed that weaver mice have a mutation in the GIRK2 gene. Weaver mice are characterized by an almost complete loss of neurones in two brain regions: the granule cells of the cerebellum fail to differentiate and undergo apoptosis within the first two weeks after birth and the dopaminergic neurones of the substantia nigra probably die due to a necrosis.

Our results demonstrate that flupirtine activates an inwardly rectifying K^+ current. To characterize the induced current as K_{ir} we showed that V_o of the current was in agreement with the Nernst equation for K^+ transport across the membrane and that the current was completely blocked by Ba^{2+}. It has been found that K_{ir} is blocked by Ba^{2+}. In contrast, I_h, defined as an inwardly rectifying current, with both Na^+ and K^+ ions as charge carriers, slowly activated by hyperpolarization (Scroggs *et al*., 1994) is not influenced by Ba^{2+}. Furthermore, we showed that the activation of K_{ir} by flupirtine involves a PTX-sensitive G-protein in the transduction mechanism.

Interestingly, it has been found that opioids also activate GIRK (for example: Williams *et al*., 1988; Loose & Kelly, 1990; Chieng & Christie, 1994), whereas substance P raises excitability by reducing inward rectification (Stanfield *et al*., 1985). It is therefore reasonable to suggest that opioids and flupirtine use the same second messenger pathways but bind to different receptors.

Another important mechanism of K^+ channel activation in neurones is the reduction in the levels of cyclic AMP by neurotransmitters. For example, Andrade & Aghajanian (1985) observed that opioid- and α_2-adrenoceptor-induced hyperpolarization was reversed by cyclic AMP analogues in locus coerulus neurones. In our experiments the activation of K_{ir} by flupirtine was completely abolished by including cyclic AMP in the intracellular solution.

It is well established that central noradrenergic, 5-hydroxytryptaminergic and GABAergic pathways are involved in the mediation of pain transmission (Zemlan *et al*., 1980; Archer *et al*., 1986) and α_2 adrenoceptor agonists have been shown to produce antinociception (Luttinger *et al*., 1985). In the muscle relaxant action of flupirtine α_2-adrenoceptors are proposed to be involved (Schwarz *et al*., 1994b) and Szelenyi *et al*. (1989) found that idazoxan reduced the antinociception induced by flupirtine. Recently, it has been recognized that idazoxan, which was originally developed as a selective antagonist at α_2-adrenoceptors (Doxey *et al*., 1983), also binds to non-adrenoceptor, imidazoline binding sites (Brown *et al*., 1990) and we observed a biphasic effect of idazoxan on the inward rectifier. In our study we chose SKF-86466 as an α_2-adrenoceptor antagonist, because it does not bind to imidazoline binding sites (Reis *et al*., 1994). We showed that SKF-86466, the 5-HT_{1A} antagonist NAN 190 and the $GABA_B$ antagonist 2-hydroxysaclofen failed to block the activation of K_{ir} by flupirtine. In line with this, in direct receptor binding studies flupirtine showed no relevant affinity for α_1- and α_2-adrenoceptors, 5-HT_1 and 5-HT_2 receptors (Szelenyi *et al*., 1989).

Recently, it has been suggested that flupirtine acts like an NMDA-antagonist (Perovic *et al*., 1994). But no relevant affinity of flupirtine to the known binding sites of the NMDA receptor was observed (Osborne *et al*., 1996) and the NMDA-induced currents were not reduced by this drug in cultured hippocampal neurones or cortical neurones of the rat. Further investigations are necessary to determine the receptor responsible for this effect of flupirtine.

In conclusion, we demonstrated that flupirtine activates an inwardly rectifying K^+ current in cultured hippocampal neurones and that this effect is mediated via a PTX-sensitive G-protein. This could at least partly be responsible for the neuroprotective action of flupirtine.

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