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Flupirtine protects neurons against excitotoxic or ischemic damage and inhibits the increase in cytosolic Ca^{2+} concentration

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Abstract

We tested the effect of flupirtine against ischemic and excitotoxic neuronal damage as well as on the glutamate-induced rise in cytosolic calcium ion concentration ($=[Ca^{2+}]_i$). For in vivo experiments we used a model of focal cerebral ischemia in mice. The middle cerebral artery was permanently occluded and 48 h afterwards brain tissue was stained with neutral red, perfusion-fixed and the infarct surface was determined planimetrically. Pretreatment with flupirtine significantly reduced the infarct area (controls: 24.3 ± 4.8 mm^2, 1 mg/kg flupirtine: 20.1 ± 3.6 mm^2 and 10 mg/kg flupirtine: 19.5 ± 3.9 mm^2; $P < 0.05$), whereas postischemic application of flupirtine failed to reduce the infarct area. For in vitro studies, primary neuronal cultures were prepared from the hippocampi of newborn rats and excitotoxic damage was induced by exposing the cells to 500 μM L-glutamate for 30 min. We could demonstrate that flupirtine (1–10 μM) was capable of protecting neurons against glutamate-induced cytotoxicity. In order to elucidate the underlying mechanism of action, we tested the effect of flupirtine on the glutamate-induced rise in $[Ca^{2+}]_i$ using the Ca^{2+}-indicator fura-2. L-Glutamate added in a final concentration of 100 μM to the cultured cells for 16 s caused a rise in $[Ca^{2+}]_i$ from about 100 nM to 900 nM. Flupirtine (0.1–10 μM) reduced the glutamate-induced rise in $[Ca^{2+}]_i$ concentration dependently.

Keywords: Flupirtine; Focal cerebral ischemia; Primary neuronal culture; Glutamate; Ca^{2+}, cytosolic; Fura-2

1. Introduction

Flupirtine (ethyl-2-amino-6-(fluorobenzyl)-amino-3-pyridine carbamate) is a non-opioid, centrally acting analgesic drug (Jakovlev et al., 1985). α_2-Adrenoceptors appear to be involved in the analgesic effect since the α_2-adrenoceptor antagonist idazoxan attenuates its antinociceptive activity (Nickel et al., 1988). In addition, it has been shown that flupirtine has muscle relaxant properties at doses comparable with those exerting antinociceptive effects (Nickel et al., 1990). There is evidence that the NMDA receptor is involved in the muscle relaxant action of flupirtine. Recent studies have suggested a NMDA receptor antagonistic effect of flupirtine (Schwarz et al., 1994; Osborne et al., 1994). From these pharmacological effects it may be assumed that flupirtine could protect neurons against damage caused by ischemia or excitotoxicity. We therefore examined the neuroprotective activity of this drug using a mouse model of focal cerebral ischemia and primary cultures of hippocampal neurons from newborn rats. In order to elucidate the underlying mechanism of action, we tested the effect of flupirtine on the glutamate-induced rise in cytosolic Ca^{2+} concentration ($=[Ca^{2+}]_i$) in cultured hippocampal neurons.

2. Materials and methods

2.1. Drugs and drug administration

Flupirtine maleate was a gift from ASTA Medica (Frankfurt, Germany). Dizocilpine (MK-801) was obtained from RBI (Cologne, Germany). Stock solutions of flupirtine and dizocilpine were freshly prepared before each experiment by dissolving the drugs in physiological saline. Flupirtine was injected intraperitoneally 1 h before or 15 min after the induction of ischemia. Control animals received vehicle only.

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2.2. Focal cerebral ischemia in mice

Permanent middle cerebral artery occlusion was performed in male NMRI mice (Charles River, Sulzfeld, Germany) according to the method of Welsh et al. (1987). The animals were anesthetized with tribromoethanol (600 mg/kg i.p.), and the left middle cerebral artery was occluded by microbipolar electrocoagulation. During the surgical procedure, the body temperature was maintained at 37 ± 1°C by means of an infrared heating lamp. To prevent a decrease in body temperature, the animals were kept at an environmental temperature of 30°C up to 2 h after middle cerebral artery occlusion.

Forty-eight hours after middle cerebral artery occlusion, the mice were once again anesthetized and perfused intraperitoneally with the dye neutral red (1%, 0.5 ml). The brains were removed and stored for at least 24 h in phosphate-buffered saline (pH 7.4) containing 4% formalin. The unstained infarct area on the brain surface was determined planimetrically by means of an image analysis system (IBAS 2, Kontron, Eching, Germany) as described by Backhauß et al. (1992).

2.3. Cell culture

Mixed neuronal/glial primary cultures were derived from the hippocampi of newborn (P1) Fischer 344 rats. The dissected tissue chunks were treated with papain (0.1% in Leibovitz L-15 medium, Gibco) for 15 min at 37°C and mechanically triturated by repeated slow pipetting through a fire-polished glass pipette. A trypsin inhibitor solution (1% in minimal essential medium (MEM), Gibco, containing 1% bovine serum albumin) was layered under the cell suspension. The suspension was centrifuged at 600 rpm at 20°C for 10 min. Finally, the cells were resuspended in MEM containing 10% NU serum (Collaborative Research) and seeded into poly-L-lysine-coated 35 mm Petri dishes (Falcon) at a final density of 3×10^5 cells/dish. Cells for fluorescence measurements were seeded into Petri dishes containing a glass cover slip. The cells were grown in MEM supplemented with 10% NU serum, 100 IU/ml penicillin and 100 μg/ml streptomycin, and kept in a humidified atmosphere of 5% CO_2 and 95% air. After 2 days in vitro, glial cell proliferation was inhibited by adding 1 μM cytosine-β-D-arabinofuranoside for 24 h to the culture medium. Cells were used 8 days after plating.

2.4. Glutamate neurotoxicity

Exposure to glutamate was performed by transferring the cells from conditioned culture medium to serum- and glutamine-free MEM supplemented with 500 μM L-glutamate. After 30 min of incubation, the cells were returned to the L-glutamate-containing MEM conditioned culture medium removed previously. After 18 h, cell viability was determined by means of the trypan blue exclusion method. The cultures were incubated for 5 min with 0.4% trypan blue, which stains non-viable cells. Flupirtine was added to the cultures 30 min before and during glutamate incubation and remained in the cultures until the next day, when the neuronal damage was evaluated.

2.5. Calcium measurements

$[Ca^{2+}]_i$ was assessed by measurement of the fluorescence ratio of the Ca^{2+}-indicator dye fura-2 at 340 and 380 nm (Grynkiewicz et al., 1985). Cultures were loaded with a solution of 1.5 μM fura-2-acetoxymethyl ester (fura-2-AM, Sigma) in Hepes-buffered Hanks' balanced salt solution (HHBSS, pH 7.45, containing 137 mM NaCl, 5.4 mM KCl, 0.41 mM $MgSO_4$, 0.49 mM $MgCl_2$, 1.26 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.64 mM Na_2HPO_4, 3 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes) with 0.02% pluronic F127 detergent for 30 min at 37°C. Afterwards the cells were washed twice in HHBSS and incubated for another 30 min at 37°C. This time period was found to be sufficient for loading the cells with fura-2-AM and for hydrolysis of the permanent acetoxymethyl ester.

For fluorescence measurements, the glass cover slips were glued over holes in the bottom of 35 mm Petri dishes and placed on an inverted-stage Zeiss Axiovert 100 microscope. The cells were alternately illuminated with light of wavelengths 340 and 380 nm by computer-controlled switching of narrow-band interference filters in front of a 75 W xenon lamp. An additional 0.63 ND filter was used with the 340 nm filter. Light passed through a 425 nm dichroic mirror and a Zeiss LD Achroplan $40 \times /0.6$ NA fluorescence objective to reach the cells. Their fluorescence emissions (500–530 nm) were monitored by a CCD camera, model C 2400-87 (Hamamatsu, Herrsching, Germany). Images were then digitized as 256×256 pixels by 8-bit arrays with a computerized imaging system (Argus-50, Hamamatsu, Herrsching, Germany). The fluorescence ratio 340/380 nm was determined by dividing the image pairs.

2.6. Preparation of the Ca^{2+} calibration curve

A Ca^{2+} calibration curve was prepared according to the method of Grynkiewicz et al. (1985). Two stock solutions were prepared: one Ca^{2+}-free EGTA solution (containing 100 mM KCl, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 10 mM EGTA and 1 μM fura-2, pH = 7.2) and a second Ca^{2+}-EGTA solution (containing additionally 10 mM $CaCl_2$). Calibration buffers of different Ca^{2+} concentrations were

then prepared by mixing $0.3 \times n$ ml Ca^{2+}-EGTA solution and $0.3 \times (10 - n)$ ml EGTA solution ($n = 0$–10). Fluorescence images at 340 nm and 380 nm were obtained for each buffer and the fluorescence ratios of the images ($F340/380$ nm) were calculated and plotted as a calibration curve. Ratio $F340/380$ nm values of pixels were then automatically transformed to concentration values by the Argus-50 system. The system was calibrated after each experiment.

2.7. Determination of drug effects on $[Ca^{2+}]_i$

Neurons were stimulated first with 100 μM glutamate for 16 s to define the control increase in $[Ca^{2+}]_i$. Five minutes later the same neurons were stimulated again in the presence of defined concentrations of the drugs. To distinguish neurons from glia in our system, cells were stimulated with 50 mM potassium chloride (KCl) at the end of the experiment. Only neuronal cells respond to the application of KCl with a massive increase in $[Ca^{2+}]_i$. The drugs were dissolved in HHBSS and were applied to the cells by exchanging the buffer in the Petri dish.

3. Results

3.1. Focal cerebral ischemia

Preischemic administration of flupirtine reduced significantly the infarct area in the mouse brain after middle cerebral artery occlusion (17% reduction by 1 mg/kg and 20% reduction by 10 mg/kg flupirtine; Fig. 1). However, postischemic administration of flupirtine failed to reduce the infarct area (Fig. 2).

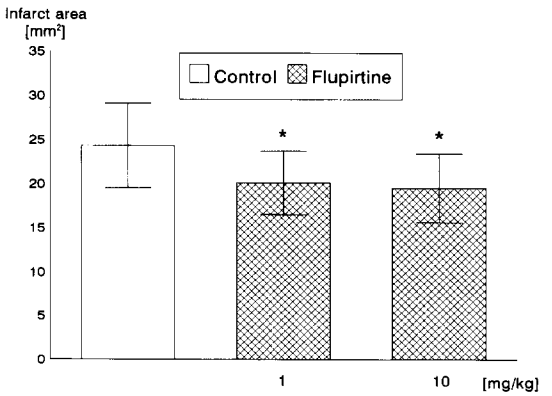

Fig. 1. Preischemic administration of flupirtine significantly reduced the infarct area after middle cerebral artery occlusion in mice. Flupirtine was administered intraperitoneally 1 h before middle cerebral artery occlusion. The animals were perfused with neutral red 48 h after middle cerebral artery occlusion. The unstained infarct area on the brain surface was calculated by means of an image analysis system. The values are given as means ± S.D. from $n = 11$–15 animals. Different from corresponding controls: $^{*}P < 0.05$ (ANOVA followed by Duncan test).

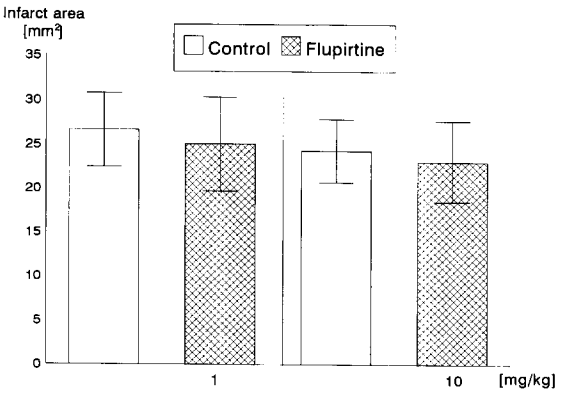

Fig. 2. Postischemic administration of flupirtine failed to reduce the infarct area after middle cerebral artery occlusion in mice. Flupirtine 1 mg/kg and 10 mg/kg was administered intraperitoneally immediately after middle cerebral artery occlusion. The animals were perfused with neutral red 48 h after middle cerebral artery occlusion. The unstained infarct area on the brain surface was calculated by means of an image analysis system. The values from two separate experiments are given as means ± S.D. ($n = 12$–17 animals).

3.2. Glutamate neurotoxicity

Exposure of the neuronal cultures to 500 μM glutamate for 30 min resulted in an increased number of trypan blue-stained neurons. While control cultures contained only 30 ± 9.7% damaged neurons, gluta-

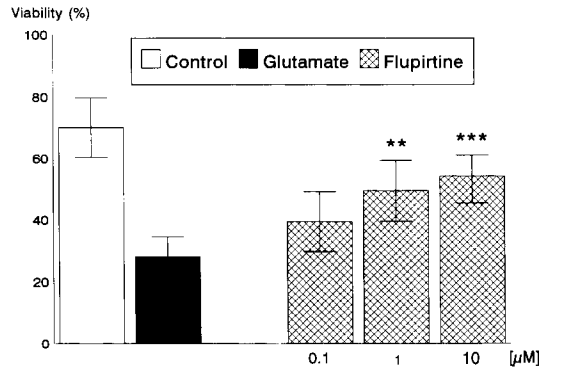

Fig. 3. Pretreatment with flupirtine significantly increased the percentage of viable cells after glutamate-induced excitotoxicity in primary cultures of neurons from rat hippocampi. Eight-day-old neuronal cultures from rat hippocampi were damaged by 500 μM L-glutamate for 30 min. Eighteen hours later cell viability was determined by trypan blue exclusion. Flupirtine was added to the cultures 30 min before and during glutamate incubation of the cultures until the next day, when cell viability was evaluated. The values are given as means ± S.D. from five experiments. Different from corresponding controls: $^{**}P < 0.01$; $^{***}P < 0.001$ (ANOVA followed by Duncan test).

Table 1
Flupirtine reduces the glutamate-induced increase in $[Ca^{2+}]_i$ in hippocampal neurons

1st Stimulus $[Ca^{2+}]_{i\,max}$ (nM)	Drug	2nd Stimulus $[Ca^{2+}]_{i\,max}$ (nM)
860 ± 250	–	840 ± 229
914 ± 110	0.1 μM flupirtine	808 ± 97 [a]
1004 ± 150	1 μM flupirtine	716 ± 178 [a]
1001 ± 137	10 μM flupirtine	630 ± 95 [a]
1134 ± 124	1 μM dizocilpine	757 ± 182 [a]

Neurons were stimulated first with 100 μM glutamate to define the control increase in $[Ca^{2+}]_i$ (1st Stimulus) and 5 min later the same neurons were stimulated again with 100 μM glutamate in the presence of defined concentrations of flupirtine or dizocilpine (2nd Stimulus). Data are given as means ± S.D. from 18–37 cells. Different from corresponding controls: [a] $P < 0.001$ (paired t-test). $[Ca^{2+}]_{i\,max}$ = maximal increase in $[Ca^{2+}]_i$ after stimulation.

mate-treated cultures showed a marked increase in injured neurons (72.2 ± 6.7%). Flupirtine (1–10 μM) added 30 min before and up to 18 h after glutamate incubation caused a concentration-dependent increase in cell viability (Fig. 3).

3.3. Calcium measurements

Double stimulation with 100 μM glutamate of hippocampal neurons was completely reversible. Flupirtine significantly attenuated the glutamate-induced increase in $[Ca^{2+}]_i$ at a concentration of 0.1 μM (Table 1). Both 1 μM flupirtine (Fig. 3) as well as 1 μM dizocilpine produced a similiar reduction of the glutamate-induced rise in $[Ca^{2+}]_i$ (32% reduction of the 1st stimulation with flupirtine and 37% with dizocilpine).

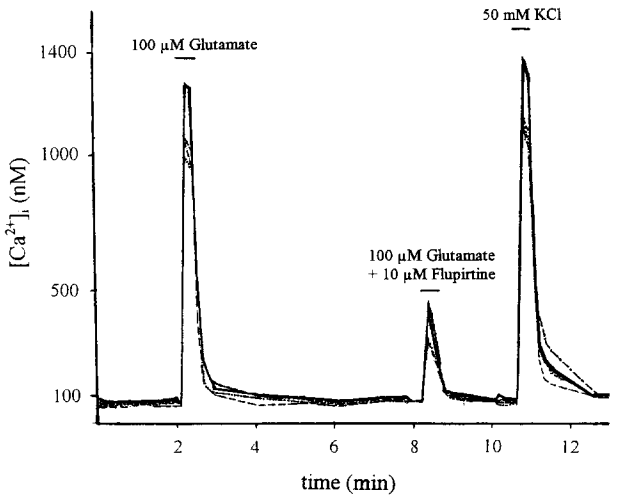

Fig. 4. Time course of $[Ca^{2+}]_i$ changes in seven cultured hippocampal neurons. Cells were stimulated by 100 μM glutamate to define the control increase in $[Ca^{2+}]_i$. Five minutes later the same cells were stimulated again with 100 μM glutamate in the presence of 10 μM flupirtine. To distinguish neurons from glia, cells were stimulated with 50 mM KCl at the end of each experiment. The bars indicate the period of drug application (about 16 s).

4. Discussion

In the present study we demonstrate that flupirtine protected brain tissue against ischemic damage. Augmented and prolonged activation of neuronal glutamate receptors is suggested to play a pivotal role in the development of ischemic neuronal damage (Choi, 1988). The overactivation of glutamate receptors causes an increased influx of Ca^{2+} and sodium ions (Na^+) via NMDA and non-NMDA receptor-operated channels. The neuronal membrane depolarizes and, in addition to the influx through the NMDA receptor-operated channels, Ca^{2+} enters the cell through the voltage-operated Ca^{2+} channels. The resulting intracellular Ca^{2+} accumulation is assumed to cause neuronal death (Siesjö, 1991). Since there is evidence that flupirtine may act on the NMDA receptor channel complex (Schwarz et al., 1994; Osborne et al., 1994), we tested its effect on glutamate-induced neuronal degeneration in cultured hippocampal neurons. Pretreatment with 1 or 10 μM flupirtine protected neurons against glutamate-induced excitotoxicity. Furthermore, we demonstrated that flupirtine reduced the glutamate-induced rise in $[Ca^{2+}]_i$ (Fig. 4). In line with the calcium hypothesis, it has been found that neuroprotective agents such as NMDA receptor antagonists can reduce the glutamate-induced increase in $[Ca^{2+}]_i$ (Hartley et al., 1993; Rothman and Olney, 1987). Our results demonstrate that flupirtine is able to inhibit the rise in $[Ca^{2+}]_i$ and based on this effect, flupirtine could exert a neuroprotective action in vivo and in vitro. The mechanism responsible for the reduction of the glutamate-induced rise in $[Ca^{2+}]_i$ is still not completely understood. Flupirtine could reduce the Ca^{2+} influx through NMDA receptor-operated Ca^{2+} channels. Furthermore, flupirtine could possibly interact with α_2-adrenoceptors (Nickel et al., 1988), which are located in the hippocampus (U'Prichard et al., 1979). Andrade and Aghajanian (1985) could demonstrate that activation of α_2-adrenoceptors induced hyperpolarization of the neuronal membrane in locus ceruleus neurons. Patch-clamp studies in our laboratory also showed that flupirtine induced hyperpolarization of the membrane in cultured hippocampal neurons (Jakob and Krieglstein, 1995). This hyperpolarization of the neuronal membrane may hamper the glutamate-induced depolarization and subsequently the Ca^{2+} influx through NMDA- and voltage-operated Ca^{2+} channels could be diminished. A reduction in high voltage activated Ca^{2+} influx by flupirtine has already been shown (Jakob and Krieglstein, 1995).

In summary, flupirtine reduced the infarct area after

middle cerebral artery occlusion in mice and protected neurons against excitotoxic damage. These effects could be due to the reduction of the increase in $[Ca^{2+}]_i$. Further investigations concerning the receptor systems involved in the reduction of the intracellular Ca^{2+} accumulation by flupirtine are necessary.

References

Andrade, R. and G.K. Aghajanian, 1985, Opiate- and α_2-adrenoceptor-induced hyperpolarizations of locus ceruleus neurons in brain slices: reversal by cyclic adenosine 3′:5′-monophosphate analogues, J. Neurosci. 5, 2359.

Backhauß, C., C. Karkoutly, M. Welsch and J. Krieglstein, 1992, A mouse model of focal cerebral ischemia for screening neuroprotective drug effects, J. Pharmacol. Methods 27, 27.

Choi, D.W., 1988, Glutamate neurotoxicity and diseases of the nervous system, Neuron 1, 623.

Grynkiewicz, G., M. Poenie and R.Y. Tsien, 1985, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, J. Biol. Chem. 260, 3440.

Hartley, D.M., M.C. Kurth, L. Bjerkness, J.H. Weiss and D.W. Choi, 1993, Glutamate receptor-induced $^{45}Ca^{2+}$ accumulation in cortical cell culture correlates with subsequent neuronal degeneration, J. Neurosci. 13(5), 1993.

Jakob, R. and J. Krieglstein, 1995, Flupirtine activates an inwardly rectifying potassium current in hippocampal neurons, Naunyn-Schmied. Arch. Pharmacol. 351, R162.

Jakovlev, V., R.D. Sofia, U. Achterrath-Tuckermann, V. Schlichtegroll and K. Thiemer, 1985, Untersuchungen zur pharmakologischen Wirkung von Flupirtin, einem strukturell neuartigen Analgetikum, Arzneim.-Forsch./Drug Res. 35, 30.

Nickel, B., J. Engel and I. Szelenyi, 1988, Possible involvement of noradrenergic descending pain-modulating pathways in the mode of antinociceptive action of flupirtine, a novel non-opioid analgesic, Agents Actions 23(1/2), 112.

Nickel, B., V. Jakovlev and I. Szelenyi, 1990, Einfluß von Flupirtin, verschiedener Analgetika und Muskelrelaxantien auf den Skelettmuskeltonus wacher Ratten, Drug Res. 40, 909.

Osborne, N.N., G. Pergande, F. Block and M. Schwarz, 1994, Immunohistochemical evidence for flupirtine acting as an antagonist on the *N*-methyl-D-aspartate (NMDA) and homocysteic acid-induced release of GABA in the rabbit retina, Brain Res. 667, 291.

Rothman, S.M. and J.W. Olney, 1987, Excitotoxicity and the NMDA receptor, Trends Neurosci. 10, 299.

Schwarz, M., F. Block and G. Pergande, 1994, *N*-Methyl-D-aspartate (NMDA)-mediated muscle relaxant action of flupirtine in rats, NeuroReport 5, 1981.

Siesjö, B., 1991, The role of calcium in cell death, in: Neurodegenerative Disorders: Mechanisms and Prospects for Therapy, eds. D. Price, A. Aguayo and H. Thoenen (John Wiley and Sons, Chichester) p. 35.

U'Prichard, D., W.D. Bechtel, B.M. Rouot and S.H. Snyder, 1979, Multiple apparent alpha-noradrenergic receptor binding sites in rat brain: effect of 6-hydroxydopamine, Mol. Pharmacol. 16, 47.

Welsh, F.A., T. Sakamoto, A.E. McKee and R.E. Sims, 1987, Effect of lactacidosis on pyridine nucleotide stability during ischemia in mouse brain, J. Neurochem. 49, 846.