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Flupirtine and retigabine prevent L-glutamate toxicity in rat pheochromocytoma PC 12 cells

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Abstract

Flupirtine is an analgesic drug thought to have NMDA receptor antagonistic and antiapoptotic effects. We investigated the effects of Ethyl-2-amino-6-(4-(4-fluorbenzyl)amino)-pyridine-3-carbamamic acid, maleate (flupirtine) and the related compound *N*-(2-amino-4-(4-fluorobenzylamino)-phenyl)-carbamic acid, ethyl ester) (retigabine) (Desaza-flupirtine) on the toxicity of L-glutamate and L-3,4-dihydroxyphenylalanine (L-DOPA) in rat pheochromocytoma PC 12 cells in vitro. Both drugs (10 μM) markedly decreased nonreceptor-mediated necrotic cell death in PC 12 cultures treated with L-glutamate (10 mM) for 72 h. In contrast, apoptosis induced by L-DOPA (250 μM) after 48 h was not affected by either substance. While L-DOPA elicited massive generation of reactive oxygen intermediates, L-glutamate-induced cell death was accompanied by only slightly increased levels of reactive oxygen intermediates. Flupirtine and retigabine exerted anti-oxidative effects in PC 12 cultures independent of their ability to prevent cell death. Further examination of the protective action of flupirtine and retigabine against L-glutamate toxicity showed that it had no influence on monoamine oxidase (monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4., MAO) activity. Thus, flupirtine and retigabine provided protection against cystine deprivation and L-glutamate toxicity but did not protect against L-glutamate under cystine-free conditions indicating that both compounds are sufficiently effective to compensate the oxidative stress elicited by cystine deprivation but not excessive activity of monoamine oxidase after L-glutamate treatment.

Keywords: Flupirtine; Retigabine; Glutamate toxicity; PC12 cells; Reactive oxygen intermediates; Glutathione

1. Introduction

Flupirtine (Katadolon®) is a non-opiate analgesic drug with muscle relaxant properties which has been used clinically for more than 10 years (Friedel and Fitton, 1993; Vaupel et al., 1989). Several lines of evidence suggest that Ethyl-2-amino-6-(4-(4-fluorbenzyl)amino)-pyridine-3-carbamamic acid, maleate (flupirtine) acts as an *N*-methyl-D-aspartate (NMDA) receptor antagonist. For example the intracellular rise of Ca^{2+} levels in rat cortical neurons following treatment with L-glutamate is reduced by flupirtine as well as neuronal damage resulting from focal cerebral ischemia in mice and rat (Block et al., 1997; Rupalla et al., 1995). Other NMDA receptor-mediated processes, which are affected by flupirtine, include: release of γ-aminobutyric acid (GABA) in the rabbit retina (Osborne et al., 1994), muscle relaxant action in rats (Schwarz et al., 1994) and motor behaviour and response to L-DOPA in a rat model of Parkinson's disease (Schmidt et al., 1997; Schwarz et al., 1996). However, no data supporting the notion that flupirtine has an affinity for any of the characterized binding sites associated with the NMDA receptor could be obtained from binding studies (Osborne et al., 1998). Comparison of therapeutically relevant serum concentrations of flupirtine with data derived from whole cell patch clamp experiments suggest that the NMDA antagonizing effect is mediated via interaction of flupirtine with an inwardly rectifying potassium channel. In cell culture studies, flupirtine protected the neuronal and

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retinal cells from cytotoxicity induced by various stimuli such as β-amyloid peptide (Muller et al., 1997), experimental ischemia (Osborne et al., 1997), human immunodeficiency virus type 1 (HIV-1) coat protein gp 120 (Perovic et al., 1994) and reactive oxygen species (Lorenz et al., 1998). Additionally, direct antioxidant properties of flupirtine have been reported (Gassen et al., 1998). The related compound retigabine has been reported to show anti-convulsive and anti-epileptic properties presumably by interaction with neuronal potassium channels (Armand et al., 1999; Rostock et al., 1996; Rundfeldt, 1997, 1999).

L-Glutamate is thought to be involved in the pathogenesis of a number of different neurodegenerative diseases (Choi, 1988; Greenamyre et al., 1985; Lees, 1993). In general, two distinct mechanisms of glutamate toxicity have been characterized. Excitotoxicity occurs through the activation of different classes of glutamate receptors (Choi, 1992; Michaels and Rothman, 1990; Olney, 1969). The second mechanism is termed oxidative glutamate toxicity and affects cell types expressing no or only little amounts of glutamate receptors via disturbances of cellular redox homeostasis. Both pathways are thought to contribute to necrotic as well as apoptotic neuronal cell death under conditions of both acute and chronic neurologic diseases (Ankarcrona et al., 1995; Beal, 1992; Bonfoco et al., 1995). Two effects by which L-glutamate exerts its toxicity in neuronal cells devoid of glutamate receptors have been reported. First, competitive inhibition of cystine uptake from the culture medium leads to intracellular shortage of glutathione (GSH) (Murphy et al., 1989). This results in an insufficient antioxidant defense against reactive oxygen intermediates and, ultimately, cell death. Second, it has been reported that inhibitors of monoamine oxidase (monoamine: oxygen oxidoreductase (deaminating), EC 1.4.3.4., MAO) as well as inhibitors of monoamine uptake prevent generation of reactive oxygen intermediates induced by treatment with L-glutamate and reduce its toxicity in neuronal cell cultures (Maher and Davis, 1996).

In the present study, we investigated the effect of flupirtine and the related compound retigabine on nonreceptor-mediated L-glutamate toxicity in rat pheochromocytoma cells PC 12 in vitro.

2. Materials and Methods

2.1. Cell culture

Undifferentiated PC12 cells (Greene and Tischler, 1976) were maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 5% fetal calf serum, 10% horse serum and 50 μg/ml penicillin/streptomycin. Cells were subcultivated every 5–7 days as described earlier (Soldner et al., 1999). For measurement of viability and generation of reactive oxygen intermediates, cells were seeded in 24- or 96-well plates coated with poly-L-lysine at 100,000 cells/ml. Drugs were dissolved in phosphate buffered saline (PBS), pH 7.4, or ethanol and filtered sterile. Concentration of the solvent in the incubation media was in no case above 1%, vehicle treated cultures were used as controls.

2.2. Viability assays

To assess directly the number of live and dead cells after treatment, cells were seeded in 24-well plates and treated 24 h after seeding. At the end of each experiment cells were trypsinized and pelleted together with cells of the culture supernatant. After staining for 10 min with 0.2% Trypan blue solution live (unstained) and dead (Trypan blue positive) cells were counted in a hemocytometer chamber. In addition, cellular viability was evaluated by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. After 2-h incubation with MTT (0.5 mg/ml) at 37°C, cells were lysed in dimethyl sulfoxide (DMSO). Extinction at 570 nm was determined on a plate photometer (Dynatech MRX). In some experiments cellular viability was assessed by the reduction of the water-soluble dye Alamar Blue (BioSOURCE, Fleurus, Belgium) after incubation at 37°C for 2 h according to the manufacture's guidelines. Cells were read on a Cytofluor II plate reader (Molecular Dynamics) at 530-nm excitation and 590-nm emission wavelength. Untreated cells were used for background readings. For staining of surviving adherent cells, plates were incubated for 10 min with 0.5% crystalviolet dissolved in 20% methanol. Plates were rinsed with water and stained cells were lysed in 50% ethanol, 0.1 M sodiumcitrate before determining extinction at 550 nm.

2.3. Electron microscopy

For ultrastructural examination, cultures were fixed with 0.2 M sodiumcacodylatebuffer (pH 7.2) containing 2.5% glutardialdehyde and 0.8% paraformaldehyde, osmificated and embedded into epon by standard procedures. Ultrathin sections were contrasted with uranyl acetate and lead citrate and studied with an EM 900 (Zeiss).

2.4. Measurement of reactive oxygen intermediates

To determine the level of reactive oxygen intermediates, cultures were incubated 1 h before measurement with 6′-carboxy-2′7′-dichlorodihydrofluoresceindiacetate di(acetoxymethylester) (C-DCH$_2$F; 10 μM), which is rapidly oxidized to the 2′7′-dichlorofluorescein-derivative by reactive oxygen intermediates in the cell (LeBel et al., 1992). To measure dichloroflourescein fluorescence, cells were rinsed with PBS and read on a Cytofluor II plate reader (Molecular Dynamics) at 485-nm excitation and 530-nm emission wavelength. Untreated cells were used for background readings.

2.5. Glutathione assay

Glutathione was assayed as total glutathione (GSX), i.e. the sum of the reduced and oxidized forms (GSH + 2 * GSSG) by a modified enzymatic microtiter plate assay (Baker et al., 1990; Tietze, 1969). Briefly, cells were washed in PBS, lysed in 1% sulfosalicylic acid, centrifuged at 11,000 × *g* for 10 min and GSX content was determined after addition of 5,5′-dithiobis-2-nitrobenzoacid to aliquots of the supernatant at a final concentration of 10 mM by absorbance changes at 405 nm for 10 min. Protein content was determined using the Lowry method (Lowry et al., 1951).

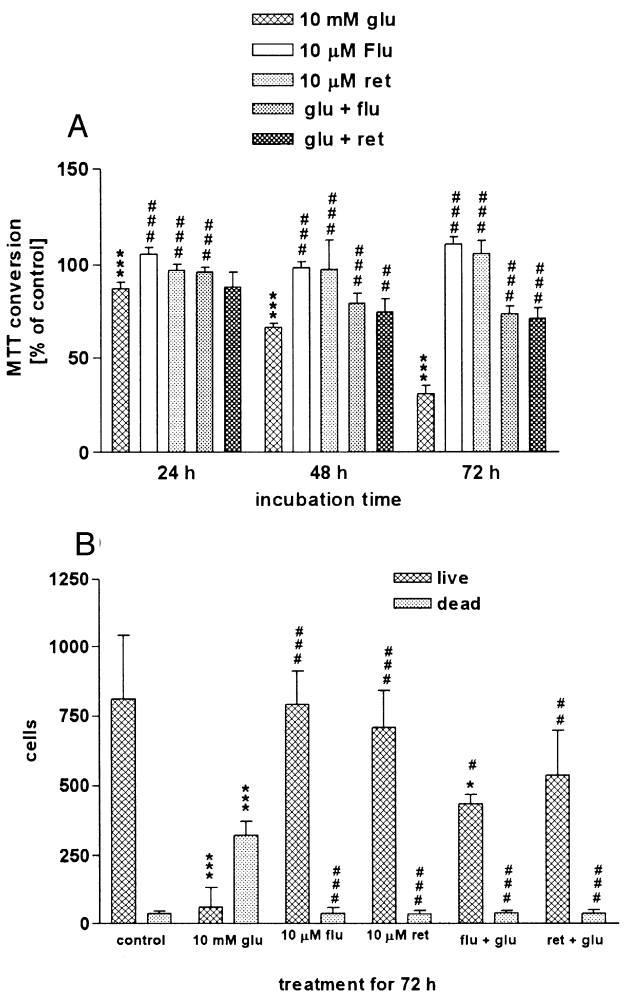

Fig. 1. Effects of flupirtine and retigabine on L-glutamate-induced PC 12 cell death. PC 12 cells were seeded at 100,000/ml in (A) 96- or (B) 24-well plates. Twenty-four hours after seeding cells were treated as indicated (glu = L-glutamate, flu = flupirtine, ret = retigabine). At the end of each treatment cultures were stained with MTT (A) or Trypan blue (B) as described in Section 2. Data are presented as mean ± S.D. with (A) $n = 10$ and (B) 4, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control treatment at the corresponding time point, respectively. $\#P < 0.05$; $\#\#P < 0.01$; $\#\#\#P < 0.001$ vs. 10 mM L-glutamate treatment (B).

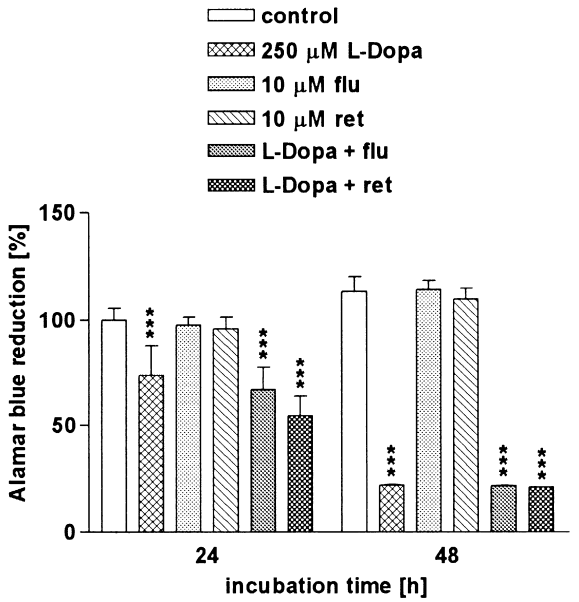

Fig. 2. Effects of flupirtine and retigabine on L-DOPA-induced PC 12 cell death. PC 12 cells were seeded at 100,000/ml in 96-well plates and 24 h after seeding cells were treated as indicated (flu = flupirtine, ret = retigabine). At the end of each treatment, cultures were stained with Alamar blue as described in Section 2. Data are presented as mean ± S.D. with $n = 10$. $^{***}P < 0.001$ vs. control treatment at the corresponding time point.

2.6. Measurement of monoamine oxidase activities

Two different assays were tested for estimation of monoamine oxidase activities. The method described by Szutowicz utilises a colorimetric reaction of horse radish peroxidase and 2,2′-azinodi(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to measure hydrogen peroxide formed during oxidation of amines by monoamine oxidase (Szutowicz et al., 1984).

For direct measurement of H_2O_2 generated by monoamine oxidase, we performed the H_2O_2-measuring technique described by Jiang et al. (1990), which relies upon the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} (catalyzed by sorbitol) under acidic conditions followed by reaction of the latter cation with the dye xylenol orange. PC 12 cells were grown to confluency in 800-ml flasks and harvested by trypsination. Since monoamine oxidase is located in the outer mitochondrial membrane we employed mitochondrial fractions isolated from PC 12 cells as described before (Seyfried et al., 1999) for measurement of enzyme activities. Fifty microliters of the mitochondrial extract, containing 50–100 µg protein, were incubated with 10 mM tyramine, 3 mM sodiumazide in 10 mM sodiumphosphate buffer, pH 7.4 at 37°C for 30 min. This reaction mixture was then diluted 1:10 in assay mixture (final concentrations 200 µM xylenolorange, 500 µM $FeSO_4$, 200 mM sorbitol and 25 mM H_2SO_4) and

allowed to stand at room temperature for 45 min. Thereafter, optical density at 560 nm was determined.

2.7. Materials

DMEM, fetal calf serum, horse serum, Dulbecco's PBS, penicillin and streptomycin were obtained from Gibco Life technologies (Heidelberg, Germany). The fluorescent dye C-DCH$_2$F, was obtained from Molecular Probes (Groningen, Netherlands). All other laboratory chemicals were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany). The drugs Ethyl-2-amino-6-(4-(4-fluorbenzyl)amino)-pyridine-3-carbamamic acid, maleate (flupirtine) and retigabine (*N*-(2-amino-4-(4-fluorobenzylamino)-phenyl)-carbamic acid, ethyl ester; D-23129) were kindly supplied by Arzneimittelwerk Dresden (Dresden, Germany).

2.8. Statistics

All experiments were performed at least in triplicate and repeated at least once. Statistical evaluation of the raw data was performed using one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test. Data are presented as mean ± S.D.

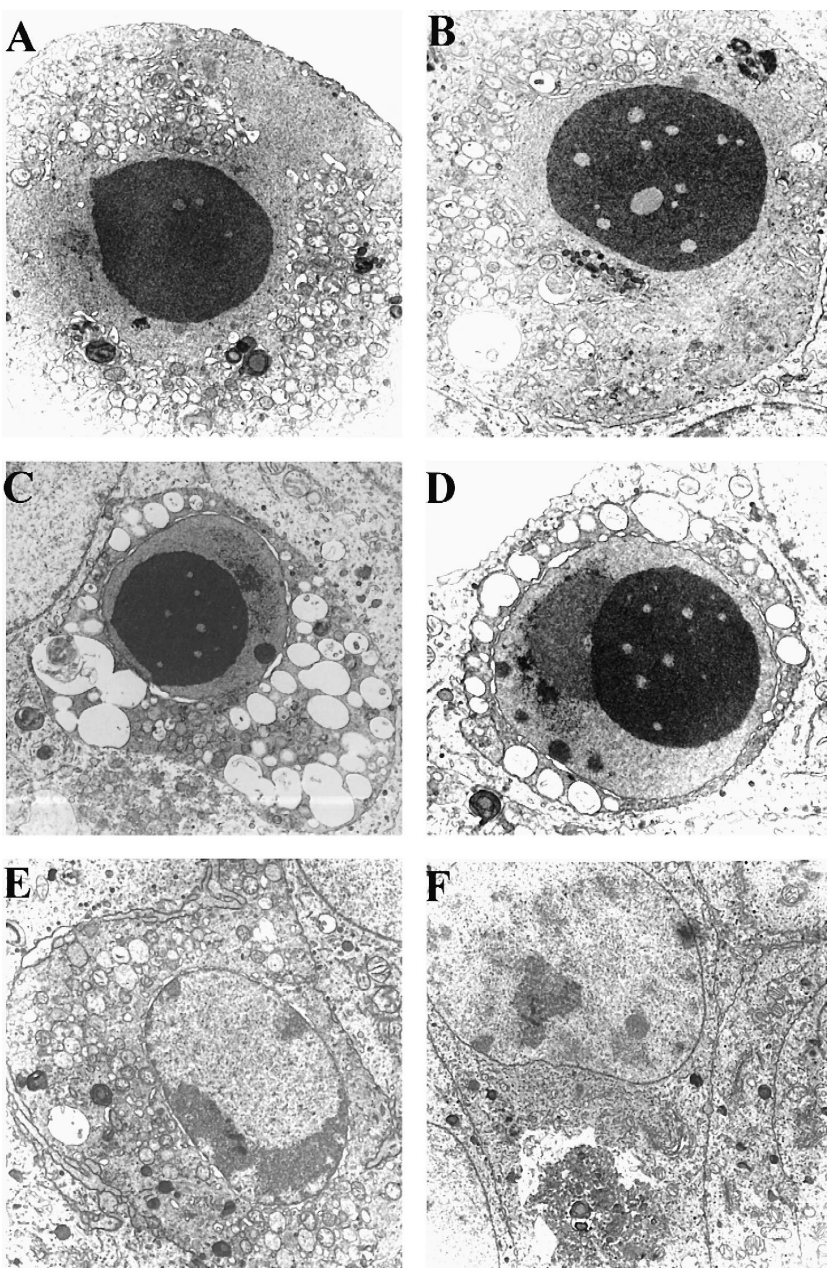

Fig. 3. Electron microscopy of PC 12 cells after treatment with L-glutamate and L-DOPA. PC 12 cells were seeded at 100,000/ml in 250-ml flasks and grown until 50% confluency. Cultures were treated with 250 μM L-DOPA for 48 h (A, B, C, D) and 10 mM L-glutamate for 72 h (E, F), fixed and processed further for ultrastrucutral examination as described in Section 2.

3. Results

3.1. L-Glutamate toxicity

Several reports confirm that PC12 cells express no or only negligible amounts of NMDA receptor protein when cultivated in undifferentiated state, i.e. without nerve growth factor (NGF), despite the presence of mRNA-transcripts corresponding to some of the NMDA receptor subunits (Casado et al., 1996; Schubert et al., 1992). To verify that L-glutamate causes cell death in our system by a nonreceptor-mediated mechanism, we performed cytotoxicity experiments over a broad range of L-glutamate concentrations using different viability assay methods. Whereas concentrations of L-glutamate up to 1 mM seemed to augment cell proliferation, treatment with 10 mM L-glutamate lead to a time dependent loss of cellular viability. This decrease of viability was not aggravated when cells were treated with 50 mM L-glutamate (data not shown). Similar results were obtained by crystalviolet staining of surviving adherent cells (data not shown). Control experiments demonstrated that NMDA was not toxic to the cells and dizocilpine, 5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801) — an NMDA channel blocker, did not attenuate L-glutamate toxicity in our system (data not shown) in accordance with published data (Schubert et al., 1992). Therefore, a 10-mM concentration L-glutamate was chosen for subsequent experiments investigating the effect of flupirtine and retigabine (Fig. 1). Both compounds (10 μM) significantly attenuated the loss of viability. After 72 h, conversion of MTT was elevated from 30% of control in L-glutamate treated cells to 73% and 71% of control during co-treatment with flupirtine or retigabine, respectively. To validate these protective effects and to directly determine live and dead cells we conducted Trypan blue exclusion assays after 72 h (Fig. 1). Treatment with L-glutamate for 72 h resulted in a 94% decrease of Trypan blue negative cells as compared to vehicle treated cultures. Co-incubation with flupirtine or retigabine (10 μM) preserved 87% and 66%, respectively of live cells as compared with controls. Correspondingly, the number of dead cells was increased in L-glutamate treated cultures more than eight-fold and was at control levels in cultures co-treated with flupirtine or retigabine. Staining of PC 12 cultures with the Alamar blue dye or crystalviolet yielded similar results and confirmed the protective action of flupirtine and retigabine against L-glutamate toxicity in PC 12 cells (data not shown).

3.2. L-DOPA toxicity

To test the effects of flupirtine and retigabine in a second paradigm of neuronal cell death we chose treatment of PC 12 cells with L-DOPA, which has been reported to cause apoptotic cell death in PC 12 cells (Walkinshaw and Waters, 1995). A concentration of 250 μM of L-DOPA resulted in 78% cell death after 48 h as estimated by Alamar blue reduction (Fig. 2). Co-incubation with flupirtine or retigabine (10 μM) failed to ameliorate cellular viability. Also, at 100 μM, both compounds were not protective (data not shown).

3.3. Morphological analysis of cell death

To re-evaluate the mode of cell death induced by L-glutamate and L-DOPA, we performed ultrastructural examination of PC 12 cultures fixed after treatment with

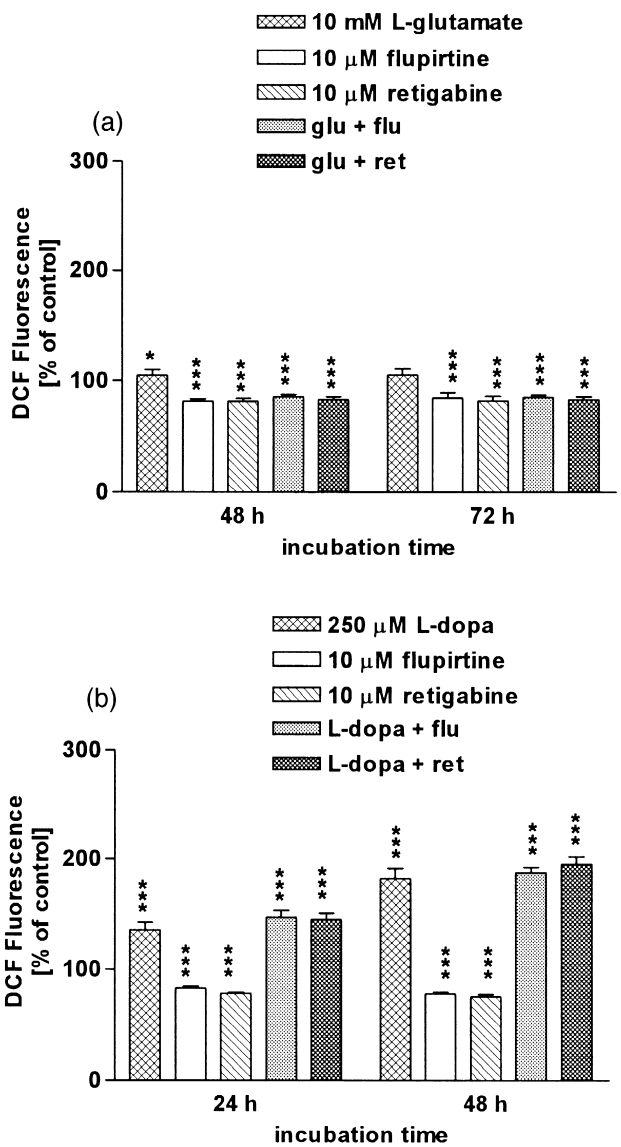

Fig. 4. Effects of flupirtine and retigabine on L-glutamate- and L-DOPA-induced generation of reactive oxygen intermediates. PC 12 cells were seeded at 100,000/ml in 96-well plates and 24 h after seeding cells were treated as indicated (glu = L-glutamate, flu = flupirtine, ret = retigabine). At the end of each treatment, cultures were stained with 6′-carboxy-2′7′-dichlorodihydrofluresceindiacetate di(acetoxymethylester) as described in Section 2. Data are presented as mean ± S.D. with $n = 10$. $^{***}P < 0.001$ vs. control treatment at the corresponding time point.

either toxin (Fig. 3). L-DOPA elicited morphological characteristics typical for apoptosis, i.e. strong condensation of heterochromatin, cell shrinkage and vacuolisation with intact plasma membranes. Cells treated with L-glutamate displayed normal nuclei but necrotic cytoplasm with swollen mitochondria and ruptured membranes. At earlier timepoints of L-glutamate treatment, less necrotic but no apoptotic cells were found (not shown). Visualizing of DNA-strand breaks by terminal deoxynucleatidyl-transferase mediated dUTP nick end labeling (TUNEL) showed negligible numbers of TUNEL-positive cells in control cultures. Cells treated with L-glutamate for 24 h displayed normal size and morphology accompanied by a weak and diffuse staining corresponding to beginning necrosis whereas cells treated with L-DOPA displayed an intensely stained and condensed, apoptotic morphology (not shown).

3.4. Generation of reactive oxygen intermediates

L-Glutamate increased cellular formation of reactive oxygen intermediates only after 48 h, this increase did not reach statistical significance after 72 h (Fig. 4). Flupirtine as well as retigabine lowered basal levels of reactive oxygen intermediates in PC 12 cultures to 85% and 83%, respectively, after 72 h. Such antioxidant effect was also present in cultures, which had received co-treatment with L-glutamate and flupirtine or retigabine, respectively.

In contrast, L-DOPA clearly increased cellular generation of reactive oxygen intermediates to 135% and 181% of controls after 24 and 48 h, respectively. Although flupirtine and retigabine lowered the basal reactive oxygen intermediates level, the L-DOPA-induced increase of reactive oxygen intermediates generation was not affected by either drug.

Table 1
Effect of cystine withdrawal on the viability of PC 12 cells

	24 h	48 h	72 h	96 h
CM	100 ± 7.3	166.9 ± 13.0	205.4 ± 16.9	301.4 ± 24.4
Cys-	46.8 ± 3.5[a]	44.0 ± 4.4[a]	35.7 ± 7.5[a]	27.9 ± 1.5[a]
Flu (cys-)	86.3 ± 6.4[b]	132.0 ± 21.0[b]	115.9 ± 22.9[b]	45.8 ± 16.0
Ret (cys-)	97.7 ± 5.9[b]	132.6 ± 11.0[b]	101.1 ± 8.4[b]	94.5 ± 15.3[b]
Glu (cys-)	41.7 ± 2.4	31.7 ± 6.6	22.4 ± 0.3	21.4 ± 0.1
glu and flu (cys-)	74.0 ± 4.4[b]	54.4 ± 5.6	33.1 ± 1.8	26.0 ± 1.7
glu and ret (cys-)	79.7 ± 2.6[b]	61.1 ± 7.3	37.5 ± 2.3	31.8 ± 14.2

PC 12 cells were seeded at 100,000/ml in 96-well plates and 24 h after seeding cells were switched to cystine-free medium containing drugs as indicated (CM: switch to complete medium, cys-: switch to cystine-free medium, flu: 10 μM flupirtine, ret: 10 μM retigabine, glu: 10 mM L-glutamate). At the end of each treatment cultures were stained with Alamar blue as described in Section 2. Data are presented as mean % ± S.D. ($n = 10$) of controls (switch to complete medium).

[a] $P < 0.001$ vs. control (CM) at the respective timepoint.

[b] $P < 0.001$ vs. switch to cystine-free medium (cys-) at the respective timepoints.

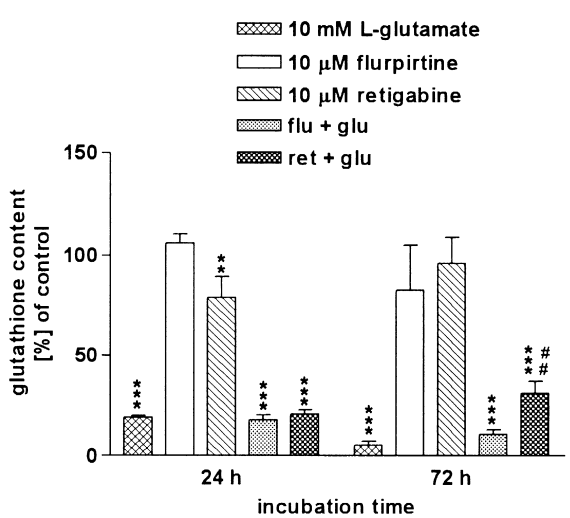

Fig. 5. Effects of flupirtine and retigabine on L-glutamate-induced depletion of cellular glutathione. PC 12 cells were seeded at 100,000/ml in 10-cm dishes and grown until 50% confluency. Cultures were treated as indicated (glu = L-glutamate, flu = flupirtine, ret = retigabine). At the end of each treatment, cells were lysed in 1% sulfosalicylic acid and processed for glutathione measurement as described in Section 2. Data are presented as mean % ± S.D. ($n = 3$) of controls. Glutathione content of control cultures was 6.278 ± 0.531 nmol/mg protein. *** $P < 0.001$ vs. control treatment at the corresponding time point. ## $P < 0.01$ vs. 10 mM L-glutamate treatment.

3.5. Glutathione levels

Flupirtine has been reported to rescue neuronal cells from L-glutamate-induced cell death through an increase of

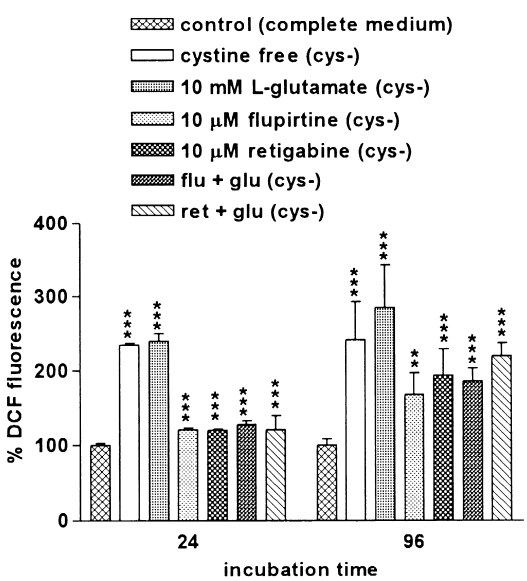

Fig. 6. Generation of reactive oxygen intermediates in PC 12 cells during cultivation in cystine-free medium. PC 12 cells were seeded at 100,000/ml in 96-well plates and 24 h after seeding cells were switched to cystine-free medium containing drugs as indicated. At the end of each treatment, cultures were stained with 6′-carboxy-2′7′-dichlorodihydrofluoresceindiacetate di(acetoxymethylester) as described in Section 2. Data are presented as mean ± S.D. with $n = 10$. ** $P < 0.01$; *** $P < 0.001$ vs. control treatment (switch to complete medium) at the corresponding time point, respectively.

Table 2
Effect of monoamine oxidase-inhibitors on the viability of L-glutamate-treated PC 12 cells

	24 h	48 h	72 h
Con	100 ± 6.7	126.3 ± 6.3	157.1 ± 6.5
10 mM glu	81.5 ± 12.9	79.8 ± 3.9 *	62.3 ± 6.4 *
10 μM clo	101.8 ± 13.7	129.1 ± 12.7	160.2 ± 5.2
100 μM clo	128.2 ± 10.9 *	140.2 ± 7.6	157.7 ± 1.4
10 μM par	98.6 ± 8	133.8 ± 3.5	162.9 ± 13.3
100 μM par	100.9 ± 4.9	127.7 ± 6.3	165.6 ± 15.1
10 mM glu and 10 μM clo	84.5 ± 2.6	98.5 ± 7.7 *	87.9 ± 13.2 *
10 mM glu and 100 μM clo	111.1 ± 16.7	123.3 ± 6.6	122.2 ± 8.6 *
10 mM glu and 10 μM par	86.9 ± 7.3	86.2 ± 11.6 *	67.4 ± 6.6 *
10 mM glu and 100 μM par	86.7 ± 4.1	77.4 ± 37.5 *	60 ± 2.7 *

PC 12 cells were seeded at 100,000/ml in 96-well plates and 24 h after seeding cells were treated as indicated (glu: L-glutamate, clo: clorgyline, par: pargyline). At the end of each treatment cultures were stained with Alamar blue as described in Section 2. Data are presented as mean % ± S.D. ($n = 10$) compared to the control value at 24 h.
* $P < 0.001$ vs. control at the respective timepoint.

cellular glutathione levels (Muller et al., 1997; Perovic et al., 1997). We determined glutathione levels in PC 12 cultures subjected to treatment with L-glutamate and flupirtine or retigabine (Fig. 5). Neither flupirtine nor retigabine increased glutathione levels. L-glutamate depleted cellular glutathione in a time dependent manner to 18.9% and 5.1% of controls after 24 and 72 h, respectively. Co-treatment with retigabine resulted in a significant preservation of glutathione 72 h treatment while flupirtine effects did not reach statistical significance ($p = 0.053$). Similarly, in experiments studying the effects of flupirtine or retigabine on glutathione depletion induced by 50 mM L-glutamate or 250 μM L-DOPA neither drug significantly preserved cellular glutathione (data not shown).

3.6. Involvement of cellular cystine uptake

3.6.1. Cellular viability

Preliminary experiments verified that addition of cystine (final concentration 1 mM) to complete medium attenuated L-glutamate toxicity in PC 12 cells (data not shown). To investigate whether the antioxidant action of flupirtine and retigabine is related to the inhibition of cellular cystine uptake imposed by L-glutamate, we carried out cytotoxicity experiments in PC 12 cultures that were switched from complete medium containing 200 μM cystine to cystine-free medium at the start of each treatment (Table 1). As assessed by Alamar blue reduction, control cultures maintained in complete medium proliferated up to 301% of the 24 h value after 96 h. Switching cells to cystine-free medium elicited a rapid loss of viability to 27% of the 24 h value after 96 h. Addition of 10 mM L-glutamate did not enhance further the effect of cystine deprivation.

Flupirtine as well as retigabine prevented the loss of viability in cystine-free medium although compared to untreated controls no further proliferation was observed. Co-incubation of L-glutamate and either flupirtine or retigabine in cystine-free medium delayed viability loss during 24 and 48 h but failed to markedly improve viability after 96 h. Essentially, the same results were obtained by crystalviolet staining (data not shown).

3.6.2. Generation of reactive oxygen intermediates

Measurement of reactive oxygen intermediates revealed a rapid increase in PC 12 cells switched to cystine-free medium to 234% compared with control cultures (switched

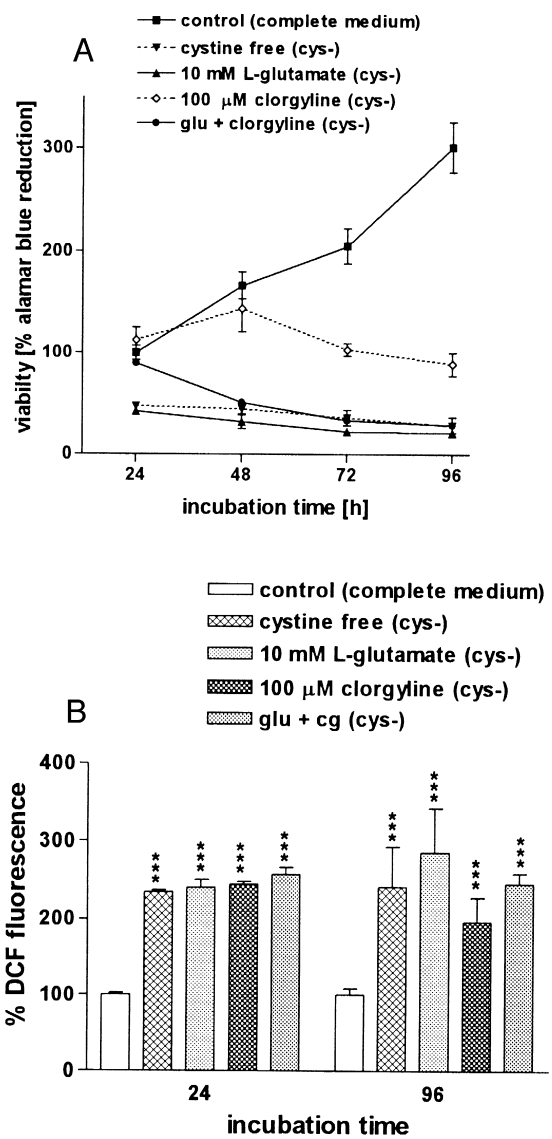

Fig. 7. Effect of monoamine oxidase — Inhibition in PC 12 cells maintained in cystine-free medium. PC 12 cells were seeded at 100,000/ml in 96-well plates and 24 h after seeding cells were switched to cystine-free medium containing drugs as indicated. At the end of each treatment, cultures were stained with (A) Alamar blue or (B) 6′-carboxy-2′7′-dichlorodihydrofluoresceindiacetate di(acetoxymethylester) as described in Section 2. Data are presented as mean % ± S.D. ($n = 10$) of controls. *** $P < 0.001$ vs. control treatment (switch to complete medium).

to complete medium) after 24 h and 241% after 96 h (Fig. 6). Treatment with L-glutamate in cystine-free medium did not potentiate this reactive oxygen intermediates signal. Both flupirtine and retigabine partially suppressed formation of reactive oxygen intermediates after 24 h and still significantly attenuated it after 96 h even when applied in combination with L-glutamate.

3.7. Involvement of monoamine oxidase

3.7.1. Cellular viability

To examine a possible influence of monoamine oxidase in our paradigm of nonreceptor-mediated L-glutamate-induced cell death, we tested the effect of monoamine oxidase-inhibition in PC 12 cells treated with L-glutamate (Table 2). Clorgyline at micromolar concentrations selectively and irreversibly inhibits the monoamine oxidase-A isoform (Denney et al., 1982; White and Glassman, 1977), which is the predominant monoamine oxidase activity present in PC 12 cells (Naoi et al., 1987; Youdim et al., 1986). Pargyline inhibits both monoamine oxidase isoforms at milimolar concentrations (White and Glassman, 1977); however, it has been noted that at submilimolar concentrations pargyline does not inhibit monoamine oxidase activity in PC 12 cells to the same degree as clorgyline (Basma et al., 1990). This is consistent with our results: clorgyline at a 10- and 100-μM concentration significantly blocked the decrease of viability caused by L-glutamate (87% and 122%, respectively of control 24 h value after 72-h treatment) whereas pargyline did not halt L-glutamate toxicity. Again, crystalviolet staining of surviving adherent cells yielded similar results.

3.7.2. Effect of clorgyline in cystine-free cultures

Clorgyline prevented loss of viability when PC 12 cultures were switched to cystine-free media, but failed to enable further cell growth leading to 88% of the 24 h control value after 96 h (Fig. 7). Comparable to flupirtine and retigabine, clorgyline had no beneficial effect on PC 12 cells treated with 10 mM L-glutamate in cystine-free

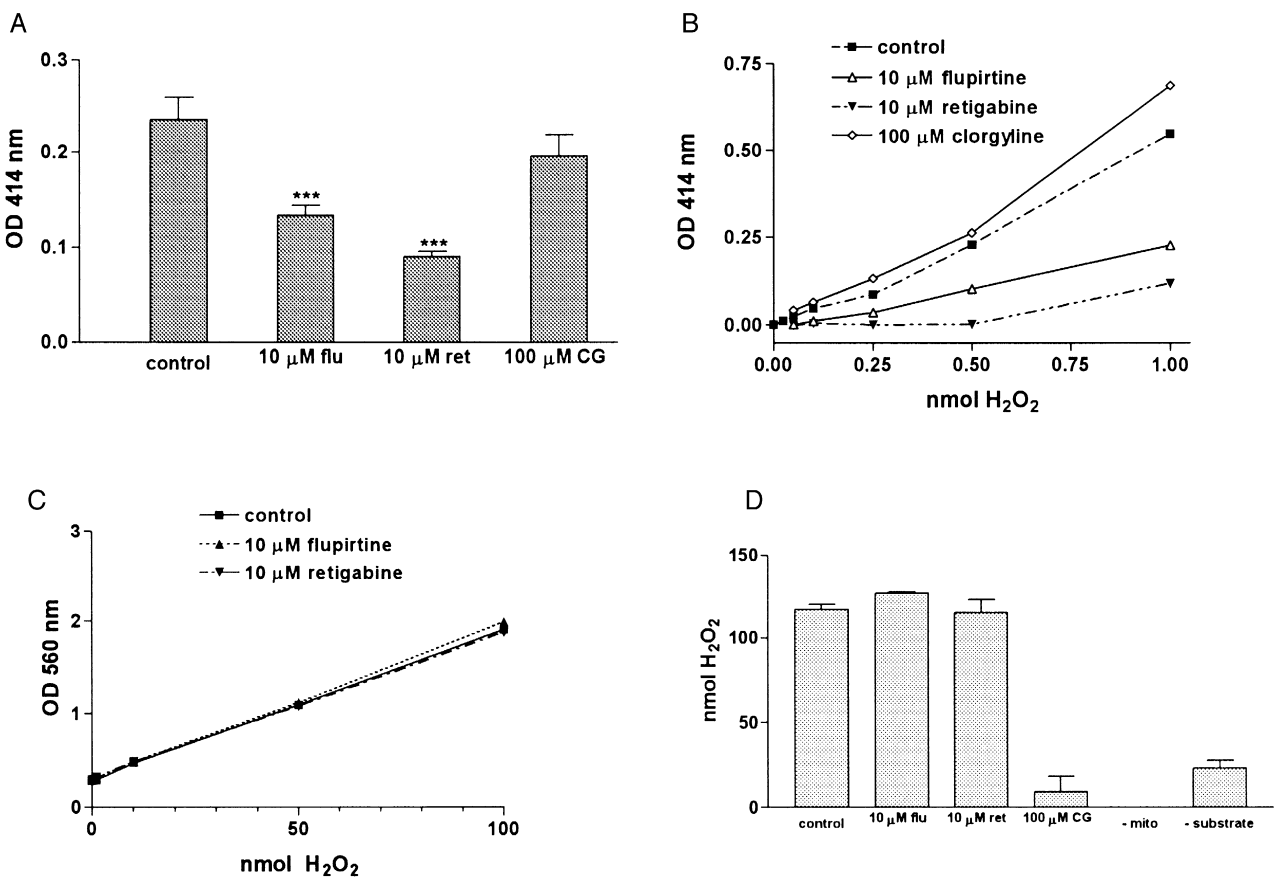

Fig. 8. Effects of flupirtine and retigabine on monoamine oxidase-activitiy in PC 12 cells. A first set of experiments was conducted to check a possible influence of flupirtine and retigabine on the ABTS/peroxidase detection system, as described in Section 2. After 15 min, the optical density at 414 nm as a measure of basal ABTS oxidation was determined (A) in samples containing the reaction mixture and the indicated compounds (flu: flupirtine, ret: retigabine, CG: clorgyline). (B) Second, the assay was performed with exogenously added H_2O_2 under the same conditions. (C) Measurements using the method of Jiang et al. as described in Section 2 ascertained that flupirtine and retigabine do not interfere with the formation of the Fe^{3+}/xylenolorange dye following the addition of exogenous hydrogen peroxide. (D) The effects of the presence of flupirtine, retigabine and clorgyline on the generation of hydrogen peroxide by mitochondria were calculated using a standard curve obtained with exogenous hydrogen peroxide.

media. Measurements of reactive oxygen intermediates in PC 12 cultures in cystine-free media showed a minor, not significant protective action of clorgyline against the formation of reactive oxygen intermediates induced by cystine shortage.

3.7.3. Assessment of monoamine oxidase activities

To investigate whether flupirtine and retigabine might exert a direct influence on monoamine oxidase activity, we prepared mitochondrial fractions for measurement of enzymatic activities from PC 12 cells as described (Seyfried et al., 1999). The assay method reported by Szutowicz et al. (1984) relies on the oxidation of ABTS by hydrogen peroxide generated by monoamine oxidase activity. However, control experiments demonstrated that flupirtine and retigabine react with the chromophore itself, so that any signal generated by hydrogen peroxide is quenched (Fig. 8). Indeed, it has been shown that the reduction of the ABTS radical cation can be used as an indicator of antioxidant properties (Re et al., 1999). Therefore, we tested the effect of flupirtine and retigabine on monoamine oxidase activity employing a second assay method. Initial measurements of exogenous hydrogen peroxide showed that neither flupirtine nor retigabine interfere with the peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} (catalyzed by sorbitol) and the subsequent reaction of the latter cation with the dye xylenol orange (Jiang et al., 1990) (Fig. 8). The generation of hydrogen peroxide by mitochondria isolated from PC 12 cells during incubation with a substrate of monoamine oxidase was not altered by both compounds; whereas, clorgyline completely inhibited monoamine oxidase activity.

4. Discussion

It is widely accepted that excessive extracellular L-glutamate contributes to neurodegenerative events in various pathological scenarios (Beal, 1992; Choi, 1992). Whether excitotoxins may induce either classical apoptotic and necrotic types of death (Ferrer et al., 1995), or atypical forms (Sohn et al., 1998) seems to depend on neuronal maturity (Chihab et al., 1998; Portera-Cailliau et al., 1997a), expression and subtype composition of glutamate receptors (Portera-Cailliau et al., 1997b) and severity of the insult (Yoon et al., 1998). The morphology of L-glutamate-treated PC 12 cells clearly provides evidence for a necrotic cell death. Whether this represents at least partly secondary necrosis following a limited extent of apoptosis cannot be quantitatively excluded by morphological analysis. We did not detect increased numbers apoptotic cells in cultures treated with L-glutamate for 24 h, 48 and 72 h neither by electron microscopy nor by TUNEL-staining. Comparison of the treatment conditions, i.e. concentration and duration, with published data, reveals that cell death following excessive activation of glutamate receptors generally occurs within a shorter time span and excitotoxin concentrations markedly lower than applied in this study (Bonfoco et al., 1995; Cheung et al., 1998). This is in agreement with previous studies investigating glutamate toxicity in PC 12 cells (Froissard and Duval, 1994; Pereira et al., 1998; Schubert et al., 1992). Thus, we conclude that the cytotoxicity observed by us is mainly necrosis and not mediated by glutamate receptors.

Both drugs did not confer protection against L-DOPA-induced toxicity in PC 12 cells. The morphological analysis of L-DOPA treated PC 12 cells showed a pattern typical for apoptosis confirming an earlier study (Walkinshaw and Waters, 1995). This lack of protection is in contrast to numerous studies reporting the ability of flupirtine to prevent apoptosis elicited by several different insults in neurons (Lorenz et al., 1998; Muller et al., 1997; Osborne et al., 1997). Therefore, the anti-apoptotic action of flupirtine appears to depend on the specific trigger of the apoptotic insult. To our knowledge, no reports exist describing an antiapoptotic action of flupirtine in a paradigm of L-DOPA-induced apoptosis. Taken together, it is our interpretation that flupirtine and retigabine do not specifically interact with a signalling component of one or more apoptotic pathways but rather exerts an unspecific antioxidant action which is only protective against apoptosis if reactive oxygen intermediates are a crucial early step of the apoptotic cascade.

That flupirtine and retigabine have antioxidative capacity is demonstrated by the measurements of reactive oxygen intermediates. Both compounds significantly suppressed basal formation of reactive oxygen intermediates in PC 12 cultures even when cultivated without cystine. Furthermore, both compounds acted to reduce the ABTS radical cation. In contrast, flupirtine as well as retigabine were ineffective against L-DOPA-induced generation of reactive oxygen intermediates. On the other hand, L-glutamate toxicity was accompanied by only marginally elevated reactive oxygen intermediates levels but was clearly attenuated by both drugs. From the latter experiments, one might suggest that the protective action against L-glutamate is not related to an anti-oxidative property. Considering the loss of viability caused by L-glutamate that occurs over a time span of 72 h, another explanation could be that L-glutamate gradually depletes cellular antioxidant defence without directly generating reactive oxygen intermediates. This is supported by experiments using cystine-free maintained cultures. The time course of viability loss caused by cystine shortage displays a severity intermediate between the rapid effects of L-DOPA and the gradual progress of L-glutamate toxicity. Furthermore, even the combined challenge of L-glutamate plus absence of cystine did not result in an accelerated viability loss as compared to treatment with L-DOPA. It appears that the increase in cellular levels of reactive oxygen intermediates

elicited by treatment with L-DOPA is the result of an active generation of reactive oxygen intermediates, presumably via autooxidation of L-DOPA itself (Basma et al., 1990). In contrast, under conditions of L-glutamate treatment or cystine depletion formation of reactive oxygen intermediates occurs because of decreased intracellular defense. While flupirtine and retigabine are capable of counteracting the effects of a limited extent of oxidative stress under conditions of cystine shortage they fail to scavenge excessive reactive oxygen intermediates generated by L-DOPA treatment. This is supported by their inability to deactivate exogenous hydrogen peroxide as judged by the Fe^{2+}/xylenolorange assay.

Since several authors reported that flupirtine rescued neuronal cells by elevation of intracellular glutathione (GSH) levels (Muller et al., 1997), we determined the cellular levels of this important cellular antioxidant. Consistent with published data L-glutamate leads to a rapid depletion of GSH preceding the actual onset of cell death (Froissard et al., 1997; Pereira and Oliveira, 1997). Retigabine provided a minor significant preservation of cellular glutathione after 72 h while flupirtine failed to preserve the cellular GSH levels significantly. This finding makes any interaction with enzymes of glutathione synthesis unlikely and provides evidence for an antioxidant effect of both drugs thereby sparing cellular antioxidants under conditions of oxidative stress. Furthermore, it seems that protection by either compound is distinct from their mode of action in paradigms of apoptotic or receptor mediated cell death. In another study induction of the antioxidant and antiapoptotic gene *bcl-2* by flupirtine was suspected as possible cause for increased cellular GSH levels (Perovic et al., 1997). To verify the level of bcl-2 expression in PC 12 cells during treatment with flupirtine and retigabine, we performed western blot analysis but did not detect a rise in bcl-2 protein levels following treatment with both drugs (data not shown).

Two components of nonreceptor-mediated L-glutamate neurotoxicity have been elucidated: First, inhibition of cystine uptake by extracellular glutamate gives way to an intracellular shortage of thiol-containing antioxidants, i.e. glutathione thereby leading to increased formation of reactive oxygen intermediates, damage to macromolecules and finally cell death (Murphy et al., 1989; Pereira and Oliveira, 1997). Second, Maher and Davis (1996) demonstrated that increased activity of monoamine oxidase as well as other oxidase reactions can contribute to L-glutamate toxicity via excessive generation of hydrogen peroxide again resulting in oxidative stress. To gain further insight into the mechanism by which flupirtine and retigabine protect PC 12 against L-glutamate, we undertook experiments aimed to distinguish the two known pathways of nonreceptor-mediated L-glutamate toxicity. That flupirtine and retigabine protect the viability of PC 12 cells maintained in cystine-free medium can be explained via an antioxidative effect, which does not involve cellular uptake of cystine or GSH supply. The addition of L-glutamate in this scenario nearly abrogates the beneficial effects of both compounds. In such a case, an effect of L-glutamate other than blockade of cystine uptake must contribute to a combined challenge that cannot be sufficiently counteracted anymore. Under the assumption that the inhibition of monoamine oxidase is the sole effect of clorgyline, the experiments with this compound provide evidence that monoamine oxidase activity is a component of L-glutamate toxicity in PC 12 cells. From the fact that clorgyline ameliorates viability in cystine-deprived cultures, we conclude that monoamine oxidase activity at least contributes to the cell-damaging events following exposure to cystine-free medium. However, flupirtine and retigabine neither modulate the activity of monoamine oxidase nor directly inactivate hydrogen peroxide. In turn, clorgyline lacks any anti-oxidative effect as estimated by the ABTS radical cation assay. The protective effects of clorgyline and flupirtine or retigabine are very similar under each the conditions tested. Therefore, we propose that either the inhibition of monoamine oxidase or the attenuation of the effects of reactive oxygen intermediates generated in part by monoamine oxidase (Pizzinat et al., 1999) is equally efficient in counteracting the oxidative insult elicited by L-glutamate.

In summary, we demonstrated for the first time the protective effect of flupirtine and retigabine in nonreceptor-mediated L-glutamate-induced necrosis. In case of retigabine so far anti-epileptic (Armand et al., 1999) and anticonvulsant (Rundfeldt, 1997; Rostock et al., 1996) but no anti-oxidative properties have been described. Recently a molecular target for retigabine has been identified to be a neuronal delayed-rectifier voltage-gated potassium channel (KCNQ2/KCNQ3) in vitro (Rundfeldt and Netzer, 2000). Since retigabine activates a K^+ current in differentiated but not in undifferentiated PC 12 cells (Rundfeldt, 1999), it is likely that this compounds exerts an even more pronounced beneficial effect in receptor mediated L-glutamate toxicity. Correspondingly, the numerous NMDA receptor antagonistic effects of flupirtine seem to be related to interaction with a potassium channel (Kornhuber et al., 1999). The antioxidative properties effective in nonreceptor-mediated L-glutamate toxicity presented in this work an are not in contradiction with these findings. Taken together, both compounds promise to be interesting drugs in the treatment of various neurodegenerative pathologies involving oxidative stress as well as excessive stimulation of glutamate receptors, independent of the mode of cell death.

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