Neurochemical Research, Vol. 30, No. 12, December 2005 (© 2005), pp. 1557–1565
DOI: 10.1007/s11064-005-8834-8

Effects of Retigabine on the Neurodegeneration and Extracellular Glutamate Changes Induced by 4-Aminopyridine in Rat Hippocampus *In Vivo**

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(Accepted August 23, 2005)

We have previously shown that microdialysis perfusion of the K^+ channel blocker 4-aminopyridine (4-AP) in rat hippocampus induces convulsions and neurodegeneration, due to the stimulation of glutamate release from synaptic terminals. Retigabine is an opener of the KCNQ2/Q3-type K^+ channel that possesses antiepileptic action and may be neuroprotective, and we have therefore studied its effect on the hyperexcitation, the neuronal damage and the changes in extracellular glutamate induced by 4-AP. Retigabine and 4-AP were co-administered by microdialysis in the hippocampus of anesthetized rats, with simultaneous recording of the EEG, and the extracellular concentration of glutamate was measured in the microdialysis fractions. In 70–80% of the rats tested retigabine reduced the 4-AP-induced stimulation of glutamate release and prevented the neuronal damage observed at 24 h in the CA1 hippocampal region. However, retigabine did not block the EEG epileptic discharges and their duration was reduced in only 20–25% of the tested animals. We conclude that the neuroprotective action of retigabine is probably due to the blockade of the 4-AP-induced stimulation of glutamate release. This inhibition, however, was not sufficient to block the epileptic activity.

KEY WORDS: 4-Aminopyridine; extracellular glutamate; hippocampus; microdialysis; neurodegeneration; retigabine.

INTRODUCTION

It has been established that excitotoxicity due to excessive glutamatergic transmission leads to epilepsy and neurodegeneration and seems to be an important factor in many neurological disorders. One experimental model *in vivo* to produce excitotoxicity due to overactivation of glutamate receptors by endogenous glutamate is the hippocampal administration of 4-aminopyridine (4-AP) (1,2). 4-AP is a K^+ channel blocker that stimulates the release of neurotransmitters in different CNS preparations *in vitro* (3–6), and in hippocampal slices produces epileptiform activity (7–10) and turns paired pulse facilitation into depression (11). The systemic administration of 4-AP induces convulsions in a variety of mammalian species, including man (12–15), and its microdialysis perfusion in the hippocampus produces glutamate-dependent seizures, EEG epileptiform discharges and neurodegeneration, effects that are prevented by *N*-methy-D-aspartate (NMDA) receptor antagonists (16,17).

K^+ channels play a major role in the control of several aspects of neuronal excitability, including

* Special issue dedicated to Dr. Simo S. Oja.

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resting membrane potential, responsiveness to synaptic inputs and neurotransmitter release (18–20). Because these functional properties of the K^+ channels reduce neuronal excitability, the possibility of facilitating the opening of these channels as a potential antiepileptic strategy has been recently stressed (18–21). In fact, mutations of K^+ channels, particularly of the KCNQ2/Q3 type, have been proposed as the main possible cause of certain kinds of epilepsy, such as the benign familial neonatal convulsions (BFNC) (22–26). The KCNQ-channels are responsible for the formation of native M-currents I_M, which play an important inhibitory regulatory role of neuronal excitability in the CNS. Suppression of the M-current results in membrane depolarization and increase in neuronal input resistance, making the cell more likely to fire action potentials and to release excitatory neurotransmitters (21,22,25). These data suggest that K^+ channel openers could possess anticonvulsant action and therefore they represent new tools for the treatment of diseases related to hyperexcitability.

Retigabine [*N*-(2-amino-4-(4-fluorobenzylamino)-phenyl) carbamic acid ethyl ester], is an opener of the KCNQ2/Q3 K^+ channels in neuronal and PC12 cells (27–32), and possesses anticonvulsant properties in several models of epilepsy *in vivo* (33), including amygdala kindling (34), maximal electroshock, pentylenetetrazole and picrotoxin (but not bicuculline) (35), as well as two genetic models of epilepsy, the audiogenic seizure DBA/2J mice and the genetically epilepsy-prone rats (36,37). *In vitro*, retigabine protects against epileptiform activity in the low Ca^{++} and low Mg^{++} model (38) and against seizure-like electrical events induced by 4-AP in hippocampal and entorhinal-hippocampal slices (8,39). In the latter preparation, retigabine also reduces the repetitive firing produced by depolarizing current injections and induces neuronal hyperpolarization (40).

The above data prompted us to study whether retigabine could protect against the epilepsy and the neurodegeneration produced by the glutamate-mediated excitotoxic action of 4-AP when perfused in rat hippocampus by reverse microdialysis.

EXPERIMENTAL PROCEDURES

Microdialysis and EEG Recording. Adult Male Wistar rats (230–250 g) were used in all experiments and handled according to the Rules for Research in Health Matters (Mexico), with approval of the local Animal Care Committee. The combined microdialysis and EEG recording procedure was as described previously (2,16,41). Briefly, the rats were anesthetized with 0.5–1.5% halothane in 95% O_2/5% CO_2 and secured in a stereotaxic frame. Previously water-flushed microdialysis cannulas (CMA/12, CMA Solna, Sweden) were positioned in the left dorsal hippocampus (coordinates: AP −3.6, L 2.4, V −3.9 from bregma, ref. 42) and continuously perfused, using a microinjection pump (CMA 100, Carnegie Medicin), at a rate of 2 μl/min with Krebs medium of the following composition (in mM): 118 NaCl, 1.2 KH_2PO_4, 4.7 KCl, 1.18 $MgSO_4$, 25 $NaHCO_3$, 10 glucose, 2.5 $CaCl_2$ (pH 7.4). After 1 h stabilization, fractions of 25 μl (12.5 min) were consecutively collected. The first three fractions collected were used to determine the basal extracellular levels of amino acids, 4-AP (Sigma, St. Louis, MO, USA) and retigabine (kindly provided by ASTA Medica, Dresden, Germany) were then perfused, and four additional fractions with normal medium were collected. Based on our previous results (2,16), in all the experiments 4-AP was perfused during one fraction (12.5 min) at a 17.5 or 35 mM concentration. Retigabine was either coperfused with 4-AP (12.5 min) or perfused during four fractions (50 min), two before 4-AP, one together with 4-AP and one after 4-AP, at a 1 mM concentration. This concentration was chosen on the basis of the effective dose against 4-AP in hippocampal slices (8,40), and considering the efficiency of the microdialysis membrane (7–11%, see below). Retigabine was dissolved in 0.075 M HCl (43) and then diluted in the perfusion medium, so that the final pH was 7.4.

In some experiments, retigabine, dissolved as described, was injected i.p. 12.5, 30 or 60 min before 4-AP perfusion, at a dose of 1 mg/kg. This dose was chosen after some preliminary experiments revealed that in the halothane-anesthetized animals higher doses (2.5, 5 and 8 mg/kg) produced depression of the EEG activity and respiratory difficulties. Control rats were injected i.p. with 0.075 M HCl.

The amino acid content of the 25 μl microdialysis perfusate fractions was measured by HPLC after *o*-phthaldialdehyde derivatization, as previously described (44,45). The values obtained were not corrected for the efficiency of the dialysis membrane, which as previously reported was 7–11% for the amino acids (46) and close to 11% for 4-AP (47).

EEG recording in the hippocampus was carried out simultaneously and continuously during the microdialysis procedure. The microdialysis probes were used as electrodes, as previously described (16,17,41). A Grass polygraph, with a low frequency filter at 3 Hz and high frequency filter at 100 Hz, was used for the EEG recording.

Histological Evaluation. Twenty-four hours after the experiment the rats were anesthetized with sodium pentobarbital and transcardially perfused with 250 ml of 0.9% NaCl followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. The brains were removed and prepared for histology as described (2,41). Brain coronal sections (40 μm thick) were obtained in a cryostat and stained with cresyl violet. For quantifying the cell damage in the cresyl violet-stained sections, healthy pyramidal neurons in the CA1 hippocampal region (identified as cells of >15 μm diameter with clear cytoplasm and morphology similar to that of control rats and of the contralateral side, which was not damaged) were counted in a 20× microscope field (30,000 μm^2) with the help of an image analyzer system (NIH Image 1.6). At least three brain sections obtained from three different animals for each experimental protocol were counted.

ANOVA followed by *t*-test were used for the statistical analysis of the changes in amino acid concentration and cell

counting. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The EEG seizures produced by 4-AP have been amply described in our previous communications (2,16,17). The coperfusion of retigabine with 35 mM 4-AP during one microdialysis fraction (12.5 min) did not significantly reduce the intensity nor the duration of the EEG epileptiform discharges in 10 out of 17 rats (Fig. 1), whereas in the remainder 7 animals the duration of the discharges was reduced by about 40% but their frequency doubled. The latency to the first discharge was similar in the animals treated only with 4-AP (21 ± 14 min, $n = 10$) and in those receiving retigabine (18 ± 2.1, $n = 17$). Very similar results were observed when retigabine was perfused during four microdialysis fractions as described in Experimental Procedures ($n = 12$, not shown).

The effect of 4-AP and of the combined treatment with 4-AP and retigabine on the level of extracellular glutamate is shown in Figs. 2 and 3. The basal concentration of extracellular glutamate was 11–15 pmoles/10 µl and, as previously described (2), perfusion with 17.5 and 35 mM 4-AP induced a transitory increase, reaching a concentration-dependent 50–75% peak increment over the basal value. Perfusion with retigabine alone during one (Fig. 2, middle panel) or four microdialysis fractions (not shown) did not modify the level of extracellular glutamate. When retigabine was coperfused with 17.5 mM or 35 mM 4-AP during one microdialysis fraction, a reduction of the stimulatory effect of 4-AP was observed (Fig. 2, bottom panel and Fig. 3, middle panel), and this apparent inhibitory effect became more notable when retigabine was perfused during four microdialysis fractions; in this case the increase in extracellular glutamate was completely blocked and even a small decrease was observed (Fig. 3, bottom panel). The changes produced by 4-AP in the other amino acids measured (aspartate, glutamine, taurine, glycine, alanine and GABA) were similar to those previously reported (2) and were not significantly modified by retigabine.

As previously described (16,17), perfusion of 17 mM or 35 mM 4-AP (Figs. 4 and 5) produced a notable neurodegeneration of CA1 and CA3 subfields of the perfused hippocampus and no damage in the contralateral one. Perfusion of retigabine alone was innocuous for hippocampal tissue, but in most of the rats studied it protected against the damage produced by the two doses of 4-AP tested (Figs. 4 and 5). As shown quantitatively for the CA1 region in Fig. 6, this protection was complete when retigabine was coperfused with 17.5 mM 4-AP during one microdialysis fraction in 12 of the 14 animals tested (Fig. 6, upper panel). With 35 mM 4-AP the protection was

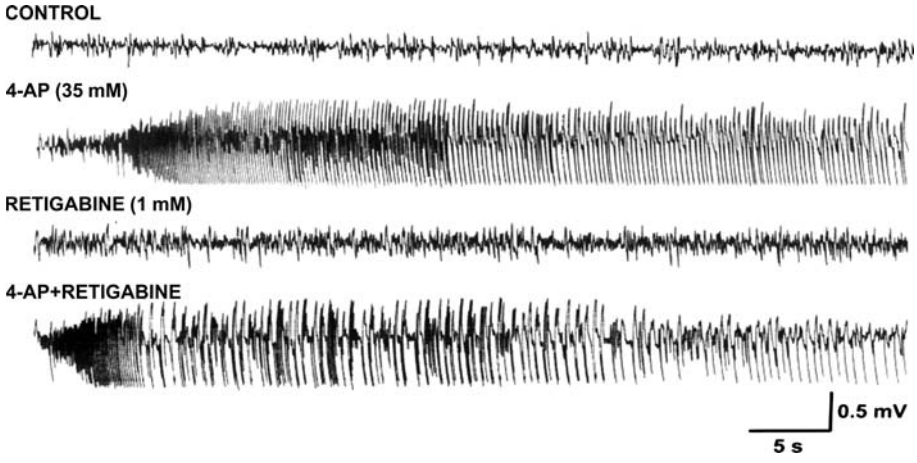

Fig. 1. Representative traces of the hippocampal EEG in halothane anesthetized rats. The control activity shows the high voltage, slow-wave activity characteristic of this type of anesthesia (41), that was slightly increased by the perfusion of retigabine alone. The main characteristics of the long (>1 min) epileptiform discharges induced by 4-AP, an initial hypersynchronic activity followed by trains of high amplitude spikes, were not significantly modified when retigabine was coperfused with 4-AP, although in 7 out of 17 animals their duration was reduced by 40% (see text in Results). Recordings correspond to 30 min after perfusion with Krebs medium (control) or with the drugs indicated, and are representative of 3 (control), 10 (4-AP), 3 (retigabine), and 17 (4-AP + retigabine) rats.

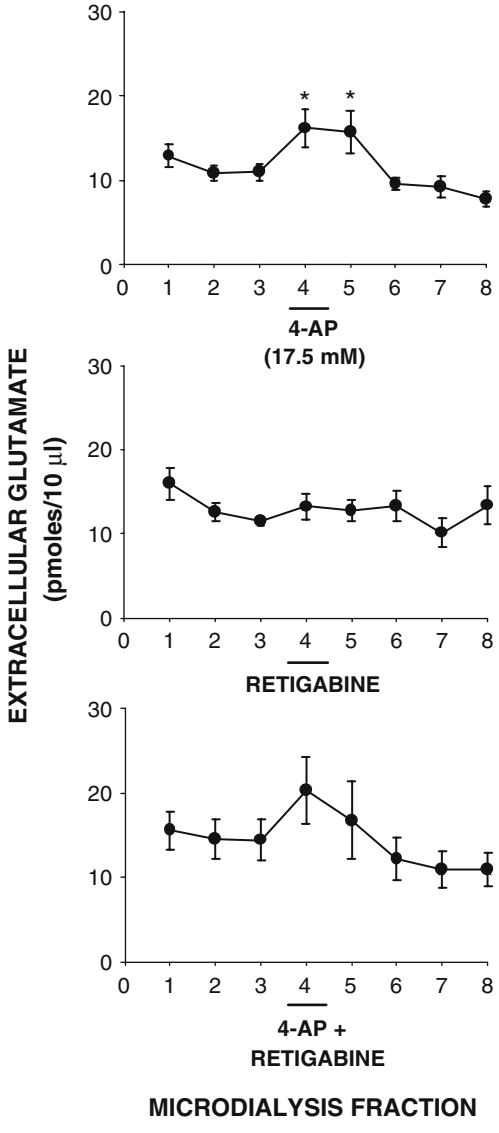

Fig. 2. Time course of the changes in extracellular glutamate concentration, measured in the collected microdialysis fractions (12.5 min each) induced by 17.5 mM 4-AP, 1 mM retigabine, and 4-AP + retigabine. Drugs were perfused during 12.5 min (horizontal bar). Mean values ± SEM for 8 rats in each group. $*P < 0.02$ as compared to the average of the basal three fractions. The peak value observed in the 4-AP + retigabine group was in the limits of significance ($P = 0.052$).

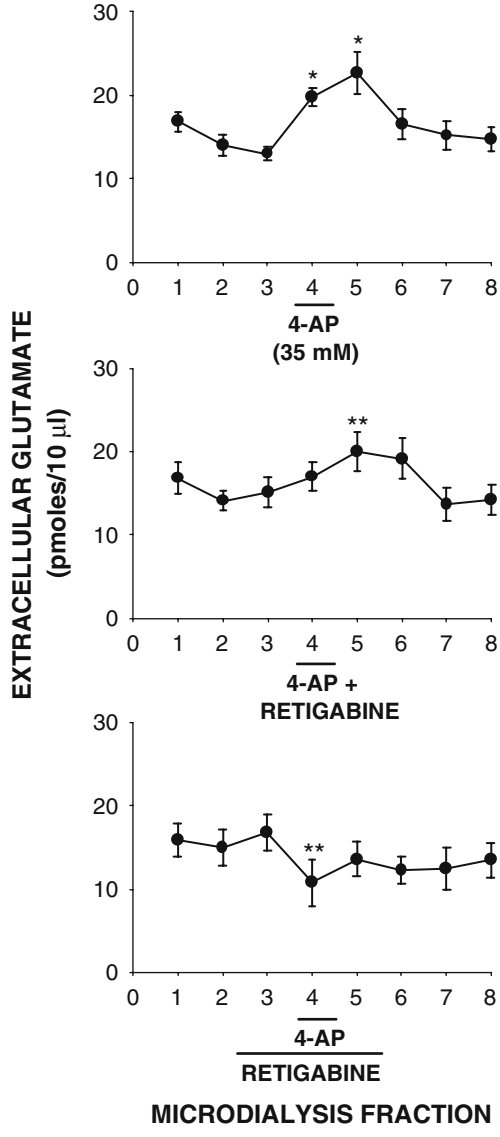

Fig. 3. Time course of the changes in extracellular glutamate concentration, measured in the collected microdialysis fractions (12.5 min each), induced by 35 mM 4-AP, 1 mM retigabine, and 4-AP + retigabine. 4-AP was perfused during 12.5 min, and retigabine during 12.5 min or 50 min (four microdialysis fractions), as indicated in the horizontal bars in each graph. Mean values ± SEM for 8 rats in each group. $*P < 0.005$, and $**P < 0.05$, as compared to the average of the corresponding basal three fractions.

equally effective but only in 11 out of 16 rats studied; in the 5 unprotected animals the damage was similar to that with 4-AP alone (Fig. 6, bottom panel, second and third pairs of columns). A better protection from 35 mM 4-AP-induced damage was obtained when retigabine was perfused during four microdialysis fractions. In this case 9 out of 12 rats were totally protected and in the remainder 3 the protection was about 50% (Fig. 6, bottom panel, fourth and fifth pairs of columns).

The histological analysis of the rats treated with retigabine i.p. before the perfusion of 35 mM 4-AP showed partial protection in only about 20% of the animals (3 of 18, 4 of 19 and 3 of 12 rats for the groups receiving retigabine 12, 30 or 60 min before 4-AP, respectively) and therefore the results are not

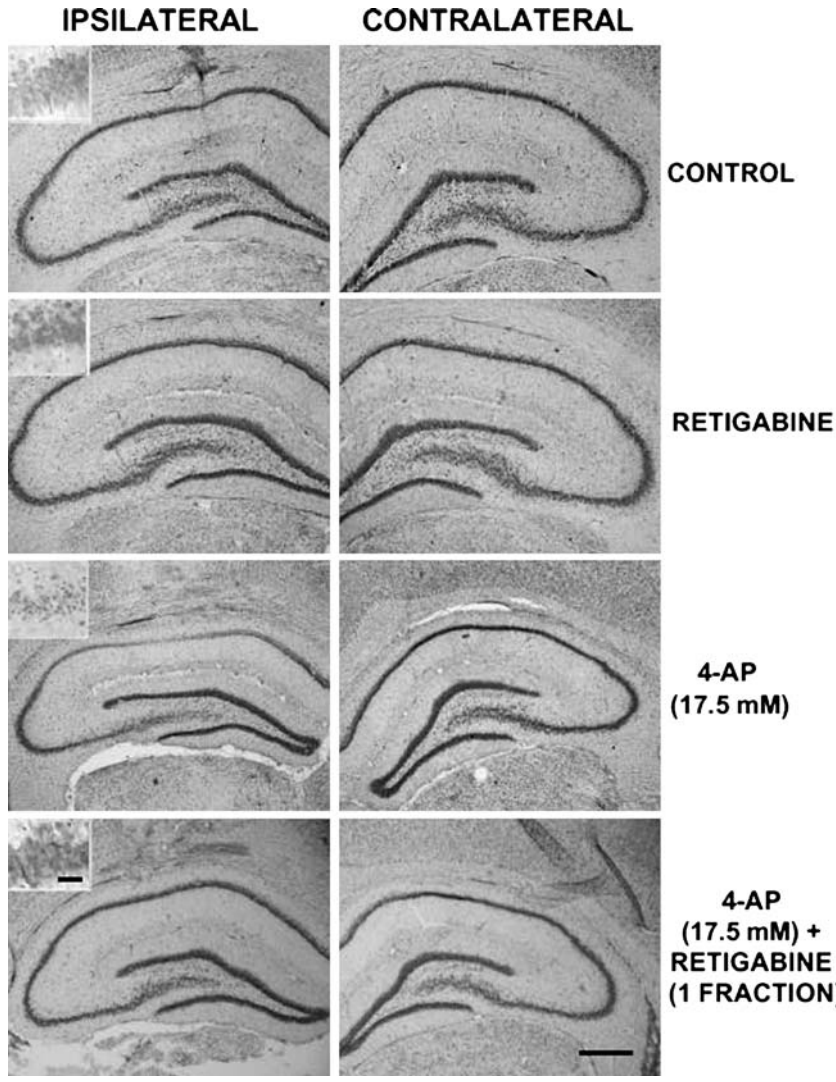

Fig. 4. Hippocampal neurodegeneration induced by 17.5 mM 4-AP, 24 h after the experiment, and protection by 1 mM retigabine perfusion as indicated in Fig. 2. Retigabine alone caused no damage (compare with the control rats, perfused only with Krebs medium, and with the contralateral hippocampus), and protected against the damage produced by 4-AP. See Fig. 6 for quantitative data. Each pair of micrographs (ipsilateral and contralateral) belongs to the same rat, and is representative of 3 (control), 3 (retigabine) 10 (4-AP), and 12 (4-AP + retigabine) animals. The insets are magnifications of the CA1 subfield close to the cannula tract, and show the shrinkage of the somas and the pyknotic nuclei of the damaged neurons, whereas in the protected rats the neurons look bigger and with a clear cytoplasm, as in the control tissue. Bars = 600 μm and 60 μm in the insets.

quantitatively shown. The EEG epileptiform activity in all these animals was similar to that observed after 4-AP alone.

DISCUSSION

The most important new finding of the present work is that perfusion of retigabine prevents the neuronal damage induced by 4-AP in the hippocampus, and that this protection occurs concomitantly with an inhibition of the stimulatory effect of 4-AP on glutamate release, as determined by microdialysis. Although the damage produced by the two doses of 4-AP used, 17.5 and 35 mM, was similar, retigabine was more efficient against the lower dose, since its perfusion during only 12.5 min (one microdialysis fraction) was sufficient to completely protect 86% of the animals, whereas perfusion during 50 min was necessary to obtain a similar protection against

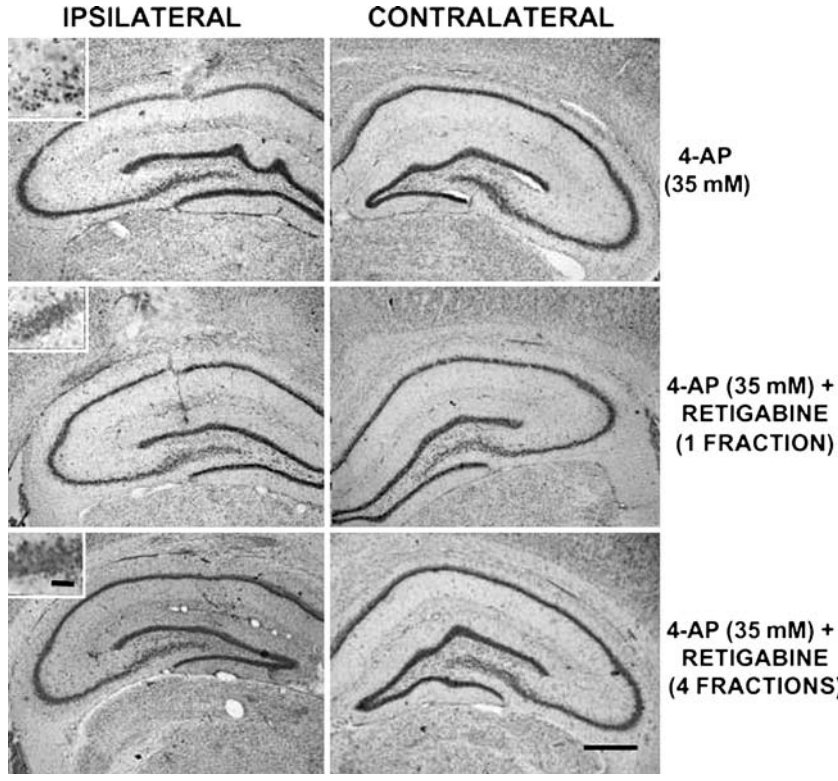

Fig. 5. Hippocampal neurodegeneration induced by 35 mM 4-AP, 24 h after the experiment, and protection by 1 mM retigabine perfusion during one (12.5 min) or four (50 min) microdialysis fractions, as indicated in Fig. 3. Note that under both experimental conditions retigabine protected against the notable neurodegeneration produced by 4-AP. However, when retigabine was perfused during 12.5 min only 11 out of 16 rats were protected, and when perfused during 50 min 9 of 12 animals were protected, as shown quantitatively in Fig. 6. See Fig. 5 for other details.

35 mM 4-AP. This protective effect may be due to the inhibition of glutamate release induced by 4-AP, since under all experimental conditions tested the perfusion of retigabine produced both effects. In support of this interpretation, we have previously shown (16) that when the release of glutamate stimulated by 4-AP is diminished by blockade of action potentials with tetrodotoxin, the damage produced by the drug is greatly reduced. However, in contrast to tetrodotoxin, which also notably prevented the epileptiform EEG discharges, retigabine was unable to protect from EEG seizures. This differential effect was also observed when ω-conotoxin, a well established blocker of the N-type presynaptic Ca^{2+} channels, was coapplied with 4-AP (2,16), and may be explained because the convulsant action of 4-AP involved both an increased excitatory glutamate-mediated transmission and an increment in neuronal firing frequency (2). In agreement with this interpretation, it has been shown that when retigabine was administered systemically after status epilepticus induced by i.p. kainic acid, it notably protected from the neurodegeneration occurring in the piriform cortex, although it was ineffective in the hippocampus (48). The latter observation is also in accordance with our present observation of the lack of protective effect of the systemic administration of retigabine against 4-AP, at the relatively low dose that we could use because of its toxic effect in the halothane anesthetized rats. In addition, we have recently shown that the neurodegeneration produced by 4-AP is a delayed phenomenon that can be blocked by NMDA receptor antagonists, whereas the seizures occur immediately and once established cannot be stopped (17; 49).

The inhibitory effect of retigabine on the 4-AP-induced glutamate release observed in the present work *in vivo* can be correlated with recent findings in hippocampal synaptosomes (50). In this preparation, retigabine notably inhibited the K^+-depolarization-evoked release of [^{3}H]labeled norepinephrine, D-aspartate (used as a substitute of glutamate because

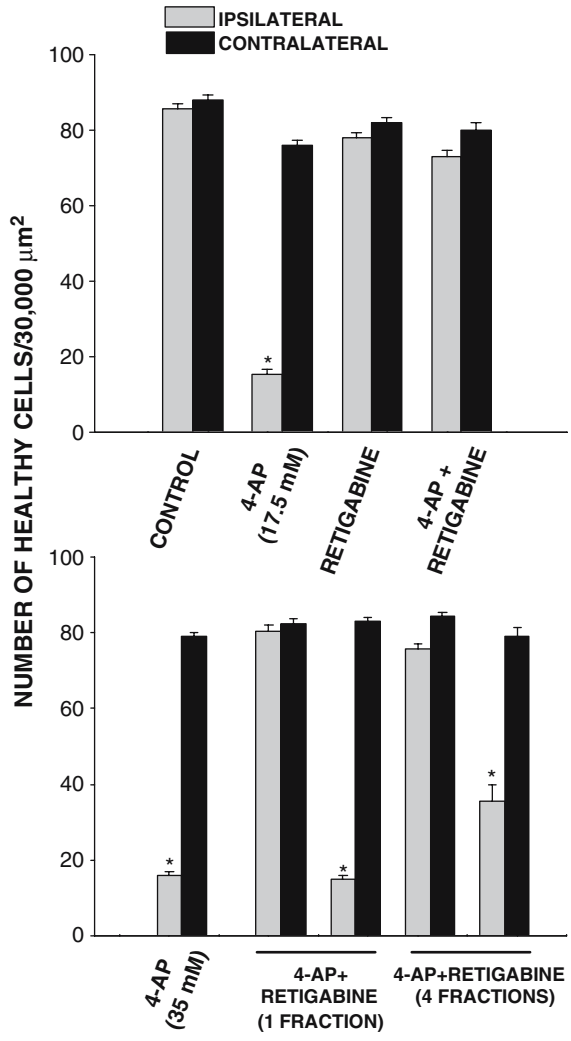

Fig. 6. Number of undamaged pyramidal neurons in the CA1 subfield of the hippocampus, 24 h after the perfusion of 4-AP (17.5 and 35 mM) and retigabine (1 mM), as indicated in Figs. 2 and 3. The neuronal loss caused by 17.5 or 35 mM 4-AP was similar. Retigabine alone was innocuous, but totally prevented the damage caused by 17.5 mM 4-AP in 12 of 14 rats (upper graph, last pair of columns). With 35 mM 4-AP, retigabine perfusion during one fraction completely protected 11 rats and failed to protect 5 rats (bottom graph, second and third pairs of columns, respectively). A better protection was observed with retigabine perfusion during four fractions, since in nine rats the protection was total and in three of them the protection was partial (bottom graph, fourth and fifth pairs of columns, respectively). Means ± SEM for at least three brain sections per rat and three rats in each group. $^*P < 0.001$ as compared to the control or the contralateral value.

of its resistance to metabolism) and GABA. In addition, retigabine blocked [^{3}H]norepinephrine release induced by 100 μm 4-AP, although D-aspartate was not studied under this condition. In this work it is also shown that antibodies against the KCNQ2 subunit of the channel responsible for the K^+ M current prevented the retigabine-induced inhibition of [^{3}H]norepinephrine release evoked by K^+-depolarization, suggesting that these channels play a regulatory role in neurotransmitter release in hippocampal nerve endings. This may be the mechanism of the inhibition by retigabine of the 4-AP-induced glutamate release in the present experiments. Other related data in mouse brain indicate that 1 mg/kg systemic retigabine may reduce the concentration of glutamate and glutamine and, at higher doses, decrease GABA transaminase activity (43).

In contrast to the amply reported anticonvulsant action of retigabine, both *in vitro* and *in vivo* (see the Introduction), few studies have addressed its potential neuroprotective effects. Besides the already mentioned protection against kainate-induced damage in piriform cortex after status epilepticus, to our knowledge the only study in this respect has been carried out in PC12 cells. Retigabine protected these cells against glutamate-induced necrosis, although it is noteworthy that, in contrast to the results reported here, this effect of glutamate is not related to excitotoxicity, because the NMDA receptor antagonist MK-801 failed to protect the cells and NMDA itself was innocuous (51).

Other potential mechanisms to account for the anticonvulsant properties of retigabine, that might also be involved in its neuroprotective effect against 4-AP shown here, are its effects on neurotransmitter amino acids. In hippocampal slices, retigabine prevented the increase in the *de novo* synthesis of glutamate and GABA induced by 4-AP, and by itself stimulated GABA synthesis (52). Furthermore, in cultured cortical neurons retigabine potentiated GABA-induced currents. We cannot discard that these mechanisms may be involved in the neuroprotection by retigabine described here, but, interestingly, we did not observe any change in the extracellular GABA concentration, as detected by our microdialysis procedure. In addition, we have previously found that GABAergic compounds and GABA uptake blockers, such as muscimol, isoguvacine, aminooxyacetic acid, nipecotic acid and NNC-711, were totally ineffective to protect from both the epilepsy and the hippocampal neurodegeneration induced by 4-AP (16). Moreover, a comparison of the retigabine concentration–response curves for its K^+ channel opening action and its effect on GABA-induced currents indicate that the channel opening is approximately 100 times more sensitive to retigabine than the GABA-current (53). Thus, we conclude that

the GABA potentiating effect of retigabine may be of minor importance for its neuroprotective effect in comparison to its K^+ channel opening activity. This conclusion is supported also by our previous work (16) showing that, similarly to retigabine, the ATP-sensitive K^+ channel opener diazoxide inhibited about 55% the extracellular glutamate increase and partially protected from neuronal death, but not from seizures, induced by intrahippocampal 4-AP.

In conclusion, our results demonstrate that retigabine protects against the neuronal death, but not against the epileptiform activity, produced as a consequence of an excess of endogenous glutamate release from hippocampal synaptic endings induced by 4-AP *in vivo*. Because this neuroprotective effect correlates with an inhibitory action on the stimulation of glutamate release by 4-AP, and in view that retigabine is an opener of the voltage-sensitive KCNQ2/3 K^+ channels that hyperpolarize the nerve endings and thus regulate neurotransmitter release, we suggest that this is probably the main mechanism of its protective action.

ACKNOWLEDGMENTS

This work was supported by DGAPA, UNAM (project IN213703) and CONACYT (project SEP42668). The authors are grateful to Asta Medica (Dresden, Germany) for their kind gift of retigabine and to Federico Jandete for the histological procedures.

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