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Reversal by Flunarizine of the Decrease in Hippocampal Acetylcholine Release in Pentylenetetrazole-Kindled Rats

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ABSTRACT. The aim of our study was to evaluate the effect of the non-selective calcium antagonist flunarizine on hippocampal acetylcholine (ACh) release with the microdialysis technique in freely moving rats after long-term concomitant administration of pentylenetetrazole (PTZ) in comparison with rats treated long-term with PTZ (kindled animals). The basal extracellular concentration of ACh in the hippocampus of rats treated with PTZ alone was significantly reduced relative to that of vehicle-treated rats (2.04 ± 0.2 vs 3.94 ± 0.3 pmol per 20-min sample; $P < 0.01$). Administration of flunarizine (7.5 mg/kg i.p.) before each PTZ injection prevented this decrease in basal ACh output (3.75 ± 0.4 pmol per 20-min sample). On the contrary, the expression of PTZ-induced kindling was not prevented by administration of flunarizine. The specific antagonistic effect of flunarizine on the kindling-induced decrease in hippocampal ACh release is shared by the selective antagonist of the L-type calcium channel, nifedipine, but not by the dopamine D_2 antagonist, (−)-sulpiride, suggesting that the decrease in Ca^{2+} overload by a blockade of the L-type calcium channel may be responsible for the protective action on cholinergic neurons exerted by flunarizine. These data also suggest a potential therapeutic role for flunarizine in counteracting impairment of hippocampal cholinergic activity. BIOCHEM PHARMACOL **58**;1:145–149, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. kindling; acetylcholine release; pentylenetetrazol; flunarizine; nifedipine; (−)-sulpiride

Repeated systemic injection of an initially subconvulsive dose of PTZ,† a blocker of the γ-aminobutyric acid type A receptor-associated chloride channel [1], results in the appearance of convulsive activity as well as in the development of kindling in rats [2]. We have recently shown that PTZ-induced kindling is accompanied by a selective decrease in basal ACh release and a parallel increase in the density of postsynaptic muscarinic receptors in the hippocampus of freely moving rats [3, 4].

Kindling, which represents an experimental model of epilepsy [5], interferes with the performance of animals in various learning and memory paradigms [6, 7], and epileptic patients manifest memory deficits [8]. Becker and Grecksch [6] showed that flunarizine, a calcium antagonist with anticonvulsant activity [9], markedly reduced the impairment in learning capacity of kindled rats when administered concomitantly with PTZ. With the use of *in vivo* brain microdialysis, we have now measured the extracellular concentration of ACh in the hippocampus of freely moving rats after 8 weeks' treatment with PTZ in the absence or presence of flunarizine. The aim of our study was to evaluate if the long-term administration of flunarizine prevented the PTZ-induced decrease in hippocampal basal ACh release.

Extensive investigations demonstrate that flunarizine is a non-selective antagonist for voltage-dependent Ca^{2+} channels, although it shows relative specificity for the L-type [10]. Given the role of this type of Ca^{2+} channel in carrying large Ca^{2+} fluxes for transmembrane signaling, we compared the effect of flunarizine on ACh release to that of the selective blocker of the L-type Ca^{2+} channel, nifedipine [11]. We thus measured basal ACh release in the hippocampus of rats chronically treated either with nifedipine alone or with both PTZ and nifedipine. Moreover, since flunarizine was reported to competitively displace [^{3}H]spiperone binding to dopamine D_2 receptors [12], we measured hippocampal basal ACh output after long-term administration of (−)-sulpiride, a selective antagonist of D_2 receptors [13], either alone or in combination with PTZ.

MATERIALS AND METHODS

Male Sprague–Dawley CD rats (Charles River), with initial body masses of 200 to 220 g, were maintained under a 12-hr light, 12-hr dark cycle (light on 8 a.m. to 8 p.m.) at a temperature of 23 ± 2° and 65% humidity. Food and water were freely available, and the animals were acclimatized for >7 days before use. Animal care and handling throughout the experimental procedure were in accordance with the

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† *Abbreviations:* ACh, acetylcholine; and PTZ, pentylenetetrazole.

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TABLE 1. Effect of flunarizine, nifedipine, and (−)-sulpiride on expression of PTZ-induced kindling in rats

	Seizure score			
Treatment time (weeks)	PTZ	FLU + PTZ	NIF + PTZ	SULP + PTZ
1	0	0	0	0
2	0.85 ± 0.1	0.44 ± 0.1*	0†	1.35 ± 0.3
3	1.80 ± 0.1	0.97 ± 0.3†	0†	2.22 ± 0.2
4	2.94 ± 0.3	2.21 ± 0.2	1.8 ± 0.6	2.30 ± 0.2
6	3.13 ± 0.5	2.65 ± 0.5	2.7 ± 0.2	3.65 ± 0.2
8	4.90 ± 0.8	4.24 ± 0.7	4.20 ± 0.6	4.98 ± 0.3

Rats were treated three times a week for 8 weeks with pentylentetrazole (PTZ) (30 mg/kg, i.p.) or with both flunarizine (FLU) (7.5 mg/kg, i.p.), nifedipine (NIF) (7.5 mg/Kg i.p.), or (−)-sulpiride (SULP) (25 mg/Kg i.p.) and pentylentetrazole.

Data are mean ± SEM value of 20 rats per group.

* $P < 0.05$, † $P < 0.01$ versus PTZ-treated rats.

European Community Council Directive of 24 November 1986 (86/609/EEC). The experimental protocol was approved by the Animal Ethical Committee of the University of Cagliari.

Rats were housed five per cage and divided into four groups. Three times a week for 8 weeks, animals in each group received two intraperitoneal injections: tested drugs (flunarizine or nifedipine: 7.5 mg; (−)-sulpiride: 25 mg per kilogram of body mass, i.p.) or drug vehicle (physiological saline containing one drop of Tween 80 per 5 mL) and, 1 hr later, PTZ (30 mg/kg, i.p.) or PTZ vehicle (saline). Rats were observed for 15 min after each drug administration, and seizures were recorded according to the following scale: 0, no response; 1, ear and facial twitching; 2, 1–20 myoclonic jerks in 10 min; 3, >20 body jerks in 10 min; 4, clonic forelimb convulsions; 5, generalized clonic convulsions with episodes of rearing and falling down; and 6, generalized convulsions with tonic extension episodes. Three days after the last drug administration, rats were subjected to the microdialysis experiments.

For the microdialysis experiments, rats were anesthetized with chloral hydrate (0.4 g/kg, i.p.), and a dialysis tube with an outer diameter of 320 μm when wet (AN 69-HF; Hospal-Dasco), was implanted at the level of the dorsal hippocampus according to the Paxinos atlas (A −3.0 from the bregma, V −3.0 from the dura). Surgery was performed according to the transversal microdialysis technique described previously [14]. The dialysis tube, with a tungsten wire inside as a rigid support, was held directly in the micromanipulator of a stereotaxic instrument and inserted into the hippocampus, and the wire was not connected to the stainless cannula. This approach minimizes tissue damage and the glia reaction around the dialysis tube. Experiments were initiated 24 hr after implantation of the dialysis tube. The Ringer's solution used to perfuse the hippocampus contained 3 mM KCl, 125 mM NaCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23 mM $NaHCO_3$, 1.5 mM potassium phosphate buffer (pH 7.3), and 0.1 μM neostigmine. The extracellular concentration of ACh in samples collected every 20 min was determined by HPLC with electrochemical detection [15]. The average concentration of ACh in the last four samples was taken as 100%. The detection limit for ACh was 0.05 pmol per injection. At the end of experiments, correct implantation of dialysis tubes was verified by histology.

Data are presented as means ± SEM from 10 rats in terms of dialysate concentration. Comparisons between groups were performed by one-way analysis of variance (ANOVA) for repeated measures. Post hoc analysis was performed by Newman–Keuls test. A P value < 0.05 was considered statistically significant.

RESULTS

As expected [3], repeated administration of an initially subconvulsant dose of PTZ (30 mg/kg, i.p.) resulted in a progressive increase in the susceptibility to seizures to a maximum score of 4.9 ± 0.8 after 8 weeks (Table 1). The expression of PTZ-induced kindling was not prevented by administration of flunarizine (7.5 mg/kg, i.p.) 1 hr before each dose of PTZ (seizure score after 8 weeks, 4.2 ± 0.7). In these animals, the development of kindling in the first three weeks was significantly delayed compared to those treated with PTZ alone; however, the two groups reached the same score after four weeks (Table 1).

ACh release stabilized within 1 hr after the start of perfusion and did not change by more than 6 to 10% during the subsequent 10 hr. As previously shown [3, 4], 4 days after the last administration of long-term PTZ treatment, the basal concentration of ACh in the hippocampal dialysate was reduced by ~50% compared with that of vehicle-treated rats: 2.04 ± 0.5 vs 3.94 ± 0.3 pmol per 20-min sample; $P < 0.01$ (Fig. 1). Administration of flunarizine (7.5 mg/kg i.p.) before each PTZ injection prevented the decrease in basal ACh output in the hippocampus of kindled rats (3.75 ± 0.4 pmol per 20-min sample). In contrast, long-term treatment with flunarizine alone reduced basal ACh release by ~25% (3.02 ± 0.2 pmol per 20-min sample; Fig. 1), while acute administration of this drug increased hippocampal ACh release, the effect being significant (34 and 66% increase over the basal value) 10 and 20 min after the injection of 7.5 mg/kg (Fig. 2).

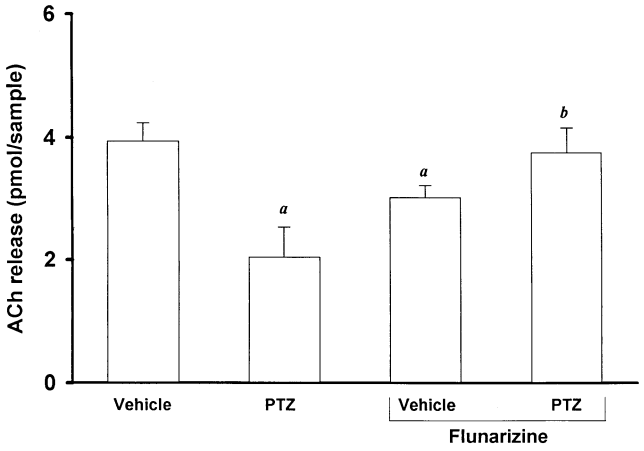

FIG. 1. Chronic administration of flunarizine prevented the decrease in hippocampal acetylcholine release induced by PTZ. Rats were treated three times for 8 weeks with vehicle, PTZ, flunarizine or PTZ, and flunarizine. Data are means ± SEM values from 10 rats and are expressed as percentage of basal values. [a]P < 0.01 versus vehicle-treated rats; [b]P < 0.01 versus PTZ-treated rats.

Long-term treatment with nifedipine (7.5 mg/kg i.p.) before each PTZ injection delayed the development of kindling: indeed, these animals did not show any behavioral activation by PTZ during the first three weeks of treatment (Table 1). Nifedipine prevented the decrease in basal acetylcholine output in the hippocampus of kindled rats (4.54 ± 0.3 pmol per 20-min sample), while chronic treatment of nifedipine alone failed to affect basal ACh release (3.91 ± 0.3 pmol per 20-min sample; Fig. 3). Finally, after chronic treatment with (−)-sulpiride (25 mg/kg i.p.), the baseline concentration of ACh in the dialyzate from the hippocampus of control animals was markedly higher (+50%) than that for rats chronically treated with vehicle (5.43 ± 0.4 vs 3.55 ± 0.2 pmol per 20-min sample). (−)-Sulpiride administered concomitantly with PTZ increased hippocampal ACh release to the same extent (5.22 ± 0.3 pmol per 20-min sample; Fig. 3). Moreover, the development of kindling was accelerated by this treatment (Table 1).

DISCUSSION

Our data demonstrate that, in rats, repeated administration of flunarizine together with PTZ prevents the decrease in hippocampal basal ACh release induced by PTZ alone. This antagonistic action was not due to a direct effect of flunarizine on cholinergic neurons because, although acute injection of this drug alone induced a dose-dependent increase in basal ACh release (data not shown), its long-term administration slightly reduced basal ACh release in control animals. Thus, chronic treatment with flunarizine appears to exert opposite effects in control and PTZ-treated rats. Given that a similar treatment prevents the impairment in learning induced by PTZ-induced kindling in rats [6], it is likely that the loss of cognitive function in such kindled rats results from the associated decrease in ACh release. Dysfunction of the septohippocampal cholinergic system is associated with learning and memory deficits in rats [16].

In spite of the anticonvulsant properties reported by acute flunarizine in different types of seizure activities [9, 17, 18], in this study flunarizine did not affect the expression of kindling. This finding is in accordance with previous papers [18, 19] showing that chronic flunarizine does not delay the development of generalized seizures by amygdala kindling. On the other hand, Becker and Grecksch [6] found that acutely administered flunarizine significantly suppressed the expression of PTZ-kindled seizures. Since flunarizine was administered 1 hr before each injection in both studies, the difference between the earlier study and ours may be due to the different protocols for kindling induction. In fact, Becker and Grecksch used a higher dose of PTZ (45 mg/kg), and the rats reached the kindled state after 10 injections, while we injected animals 24 times during the 8 weeks' treatment. It is possible that during this longer treatment animals developed tolerance to the anticonvulsant effect of flunarizine. However, previous findings [3] suggest that the level of basal ACh release is independent of the presence of motor seizures; in fact, the concomitant administration of abecarnil, a selective benzodiazepine receptor agonist, during kindling completely abolished the seizures elicited by PTZ while failing to prevent the decrease in basal release in hippocampal ACh.

The exact mechanism responsible for the antagonistic action of flunarizine on the PTZ-induced decrease in hippocampal ACh release remains to be determined. Kindling epileptogenesis is thought to result from an enhancement of glutamate-mediated excitation [20]. Thus, overstimulation of *N*-methyl-D-aspartate-sensitive glutamate receptors may result in decreased viability [21] and, ultimately, death of cholinergic cell bodies located in the medial septal nucleus. By blocking calcium influx mediated by various calcium

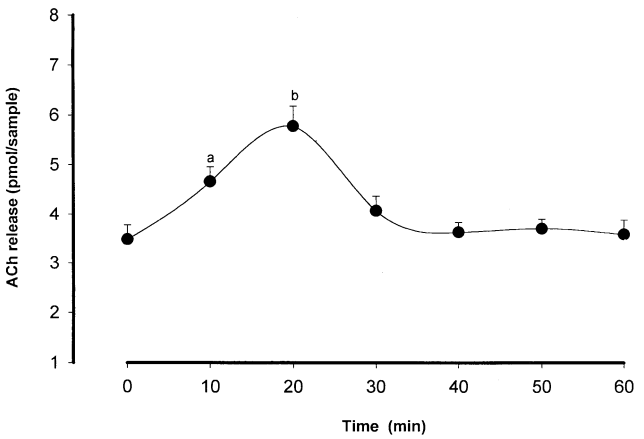

FIG. 2. Effect of acute administration of flunarizine on hippocampal acetylcholine release in control rats. Animals were injected with flunarizine at a dose of 7.5 mg/kg at time 0. Data are means ± SEM values for 10 rats and are expressed in pmol per 10-min sample. [a]P < 0.05; [b]P < 0.01 versus basal value.

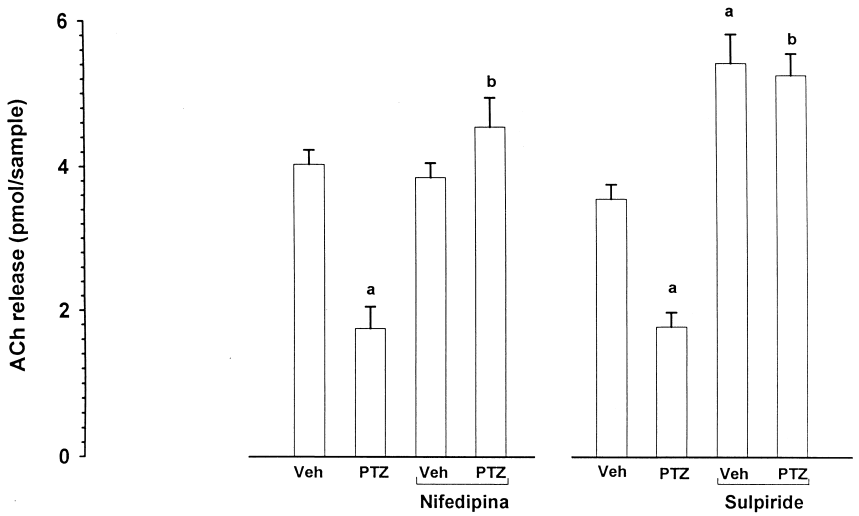

FIG. 3. Effects of chronic treatment with nifedipine and sulpiride on hippocampal acetylcholine release in control and in pentylenetetrazol chronically treated rats. Rats were treated three times a week for 8 weeks with vehicle (Veh), PTZ, nifedipine or (−)-sulpiride, or PTZ and nifedipine or (−)-sulpiride. Data are means ± SEM values from 10 rats and are expressed as a percentage of basal values. [a]P < 0.01 versus vehicle-treated rats; [b]P < 0.01 versus PTZ-treated rats.

channels (T-, L-, and N-type) and by veratridine-sensitive sodium channels, flunarizine might protect neurons against calcium overload and consequent injury or death [10]. Accordingly, it has been shown [22] that in cerebrocortical and cerebellar synaptosomes and cultured cerebellar granule cells, flunarizine inhibits KCl-evoked Ca^{2+} influx and exocytotic glutamate release in a concentration-dependent manner through antagonism at the L-type voltage-dependent Ca^{2+} channel. These authors also showed that flunarizine inhibits Ca^{2+}-independent cytoplasmatic release of glutamate through inhibition of Na^{2+} channels. The evidence that the dihydropyridine nifedipine, a selective blocker of L-type voltage-dependent Ca^{2+} channels, like flunarizine, antagonized the decrease in ACh release in kindled animals, suggests that the blockade of these channels is likely responsible for the protective action of flunarizine on cholinergic neurons. Accordingly, nifedipine improved performance in a rotary pursuit test of procedural learning in schizophrenic patients [23]. Since flunarizine blocks veratridine-sensitive Na^{+} channels [24], thereby reducing synaptic excitoxic neurotransmitter release, the contribution of this action to the protective effect of flunarizine on cholinergic neuron cannot be ruled out.

The antagonistic action of flunarizine on dopamine D_2 receptors [12] does not seem to play a role in the antagonistic action of flunarizine on the decrease in ACh release in kindled animals. This conclusion is suggested by the results obtained measuring ACh release in the hippocampus of rats chronically treated with (−)-sulpiride, a selective antagonist of D_2 receptors [13]. In fact, this treatment induced per se a significant increase in hippocampal ACh output. Thus, the enhanced ACh release measured in the group of rats treated with both (−)-sulpiride and PTZ might be due to a direct and selective action of sulpiride on D_2 receptor located on cholinergic neurons rather than an antagonistic action exerted by this drug on the effect of PTZ.

In conclusion, our observation that long-term treatment with flunarizine prevents the loss of cholinergic function in this experimental model of epilepsy, together with the demonstration that such treatment also reduces the associated deficit in learning capacity [6], suggests a potential therapeutic application for this drug in diseases characterized by an impairment in cognitive processes.

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