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Effects of Suramin on Hippocampal Apyrase Activity and Inhibitory Avoidance Learning of Rats

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BONAN, C. D., R. ROESLER, J. QUEVEDO, A. M. O. BATTASTINI, I. IZQUIERDO AND J. J. F. SARKIS. *Effects of suramin on hippocampal apyrase activity and inhibitory avoidance learning of rats.* PHARMACOL BIOCHEM BEHAV **63**(1) 153–158, 1999.—The action of suramin on apyrase activity in hippocampal synaptosomes and its effects on retention of inhibitory avoidance learning were evaluated. Suramin, a P_2-purinoceptor antagonist, significantly inhibited in a noncompetitive manner the ATP and ADP hydrolysis promoted by apyrase in hippocampal synaptosomes of adult rats. The Ki values obtained were 72.8 and 109 μM for ATP and ADP hydrolysis, respectively. Intrahippocampal infusion of suramin (0.01, 0.1, 1, and 10 μg) immediately posttraining, in a dose-dependent effect, significantly reduced the response latency during the retention test applied 24 h after the rats received step-down inhibitory avoidance training. The amnesic effects promoted by suramin probably occur by its antagonist action on hippocampal P_2-purinoceptors and NMDA receptors. In view of the fact that ATP-metabolizing enzymes and P_2-purinoceptors have similar binding domains, these results suggest that suramin can either alter ATP degradation and/or block purinergic neurotransmission. © 1999 Elsevier Science Inc.

Suramin ATP diphosphohydrolase Apyrase Memory Inhibitory avoidance task

SINCE the purinergic nerve hypothesis defining extracellular ATP as a neurotransmitter was proposed (7), there has been growing interest in its role in synaptic transmission (8). ATP is known to exert potent effects on the central nervous system, where it can act as a neurotransmitter or as a modulator regulating the activity of other transmitter substances (28,31). It has been shown that exogenously applied ATP can influence cell excitability (12,13) and, at low nanomolar concentrations, it is able to induce long-lasting enhancement of the population spikes recorded from hippocampal slices, called long-term potentiation (LTP) (33).

Extracellular ATP evokes responses through two subclasses of P_2-purinoceptors, P_{2x} and P_{2y}. The P_{2x} subclass has been shown to be coupled to ligand-gated Ca^{2+}-permeable channels, whereas the P_{2y} has been considered a G-protein–linked subclass. These P_2-purinoceptors have been subdivided into several subtypes based on agonist potencies (35), although the pharmacological characterization of these receptors is hampered by the absence of selective and potent antagonists (3).

Moreover, ATP as well as other nucleotide agonists for the P_2-purinoceptors, are subject to degradation by ectonucleotidases (38). Breakdown of ATP by one of these enzymes, ATP diphosphohydrolase (Apyrase, ATPDase, EC 3.6.1.5), is the first step towards complete ATP dephosphorylation yielding adenosine. Adenosine can bind to P_1 receptors, and in turn, is taken up and inactivated. It has been reported that extracellular ATP is hydrolyzed by an apyrase in synaptosomes of the peripheral and central nervous systems (2,30). Furthermore, rat and mouse brain ecto-apyrase cDNAs were isolated, and it has been suggested that there might be a single copy of the ecto-apyrase gene (32). An important function of this enzyme is its participation in an enzyme chain, together with 5′-nucleotidase, to control the availability of ligands (ATP, ADP, AMP,

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and adenosine) for both nucleotide and nucleoside receptors, and consequently, the duration and extent of receptor activation (38). Recently, in our laboratory, we observed that a memory task (step-down inhibitory avoidance task) can alter apyrase and 5′-nucleotidase activities in hippocampal synaptosomes of rats. The modulation of these enzyme activities can be involved in specific biochemical events related to memory acquisition (5).

Among a variety of P_2 ligands, suramin has been used as a nonselective antagonist of P_{2x} and P_{2y} purinoceptors (15,35). It has also been suggested that suramin can interact with NMDA receptors probably as an antagonist (26). It blocked ATP-mediated fast synaptic transmission (13) and antagonized ATP-activated currents in PC12 cells (25). However, suramin is able to facilitate synaptic responses recorded from hippocampal slices (34). The action of suramin involves various other biological effects. Suramin has been reported to have inhibitory effects on the ecto-apyrase of *Torpedo* electric organ (22) and on the ecto-ATPase activity from several sources (3,14,23,37).

In the present investigation, we demonstrate the effect in vitro of suramin on apyrase activity from hippocampal synaptosomes of rats.

Because our recent studies indicate that apyrase activity participate in the mechanisms of memory acquisition (5) and suramin is able to facilitate synaptic responses, inducing LTP (34), we evaluate the effects of intrahippocampal infusion of suramin on retention of a learning task, the step-down inhibitory avoidance task. The actions of suramin on extracellular ATP degradation and its possible effects on mechanisms of memory are discussed.

MATERIALS AND METHODS

Subjects

Male Wistar rats (age, 70–90 days; weight, 220–260 g) from our breeding stock were housed five to a cage, with water and food ad libitum. The animal house was kept on a 12-h light/dark cycle (lights on at 0700 h) at a temperature of 23°C.

Subcellular Fractionation

The rats were killed by decapitation, and their hippocampi were removed and gently homogenized in 5 vol of an ice-cold medium consisting of 320 mM sucrose, 0.1 mM EDTA, and 5.0 mM HEPES, pH = 7.5, with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as described previously (24). Briefly, 0.5 ml of the crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000 × g for 20 min with the same ice-cold medium to remove the contaminating Percoll and the synaptosome pellet was resuspended to a final protein concentration of approximately 0.5 mg/ml. The material was prepared fresh daily and maintained at 0–4°C throughout preparation.

Enzyme Assays

The reaction medium used to assay apyrase activity was essentially as described previously (2), and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM TRIS-HCl buffer, pH 8.0, in a final volume of 200 μl. The concentrations of suramin [8-(3-benzoamido-4-methylbenzamido) naphtalene-1,3,5,-trisulfonic acid] used in the assay were in the 12–150-μM range. The enzyme preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37°C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM, and stopped by the addition of 200 μl 10% trichloroacetic acid. The samples were chilled on ice for 10 min and 100-μl samples were taken for the assay of released inorganic phosphate (Pi) (10). Incubation times and protein concentration were chosen to ensure the linearity of the reactions. Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in duplicate. Protein was measured by the Coomassie Blue method (6), using bovine serum albumin as standard.

Surgery

Animals were implanted under thionembutal anesthesia (30 mg/kg, ip), with bilateral 30-g cannuale aimed 1.0 mm above the CA1 area of the dorsal hippocampus (18,19,36). Coordinates for the guide cannulae were A −4.3, L 4.0, V 2.0 (27). Histological verification of cannulae placement was as previously described (18,19,36). Two to 24 h after the end of the behavioral experiments, a 0.5 μl infusion of 4% methylene blue was applied through the infusion cannulae. The brains were removed and conserved in a 10% formalin solution, and cannula placement was verified. Only data for animals considered to have cannulae correctly placed in the desired area were submitted to final analysis (Fig. 3). The placements were correct in 90% of the animals.

Behavioral Procedures

Three to 5 days after surgery, animals were submitted to the behavioral procedures. Training and test session procedures were as described in several previous reports (18,19,36). A 50 cm wide, 25 cm high, 25 cm deep acrylic box was used. The left extreme of the box was occupied by a 7 cm wide, 3 cm high formica platform. The rest of the floor was a grid of parallel 0.1 cm caliber steel bars spaced 1 cm apart. In the training sessions, the animals were gently placed on the platform and their latency to step down with the four paws on the grid was measured. On stepping down, animals received a 0.4 mA, 60 Hz footshock for 2 s, and were withdrawn from the box. The test session was procedurally identical except that the footshock was omitted. Training–test interval was 24 h. A ceiling of 180 s was imposed in the test session, i.e., animals with retention latencies higher than 180 s were computed with this value. Test session step-down latency was used as a measure of retention (18,19,36).

Drug Infusion Procedure

Immediately after training in the task, a cannula was fitted into the guide cannula. The infusion cannula was connected by a polyethylene tube to a microsyringe (18,19,36). The tip of the infusion cannula protruded 1.0 mm beyond that of the guide cannula and was, therefore, aimed at the CA1 region of the dorsal hippocampus. Animals were infused with 0.5 μl saline (0.9% NaCl), or different doses of suramin (0.01, 0.1, 1.0, or 10.0 μg) dissolved in 0.5 μl saline. The solutions injected were brought to pH 7.4 with 0.1 N phosphate buffer.

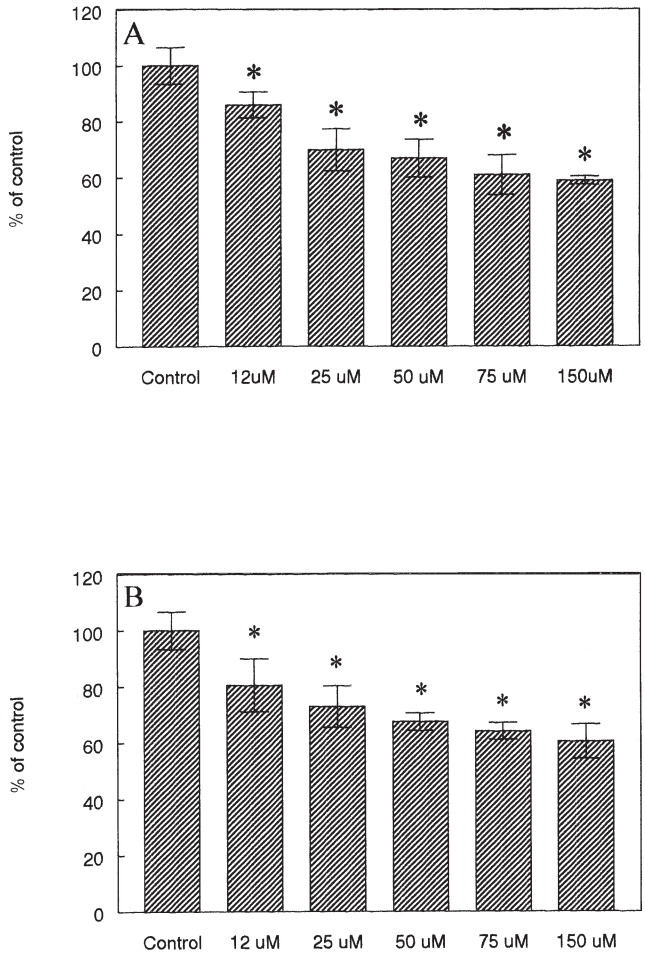

FIG. 1. Effect of suramin on ATP (A) and ADP (B) hydrolysis by the apyrase activity in synaptosomes from hippocampus of rats. Bars represent the mean ± SD of four experiments. The control ATPase and ADPase actvities (100% = no suramin added) measured were 153.02 ± 9.95 nmol Pi × min^{-1} × mg^{-1} protein and 44.62 ± 3.0 nmol Pi × min^{-1} × mg^{-1} protein, respectively. *$p < 0.05$.

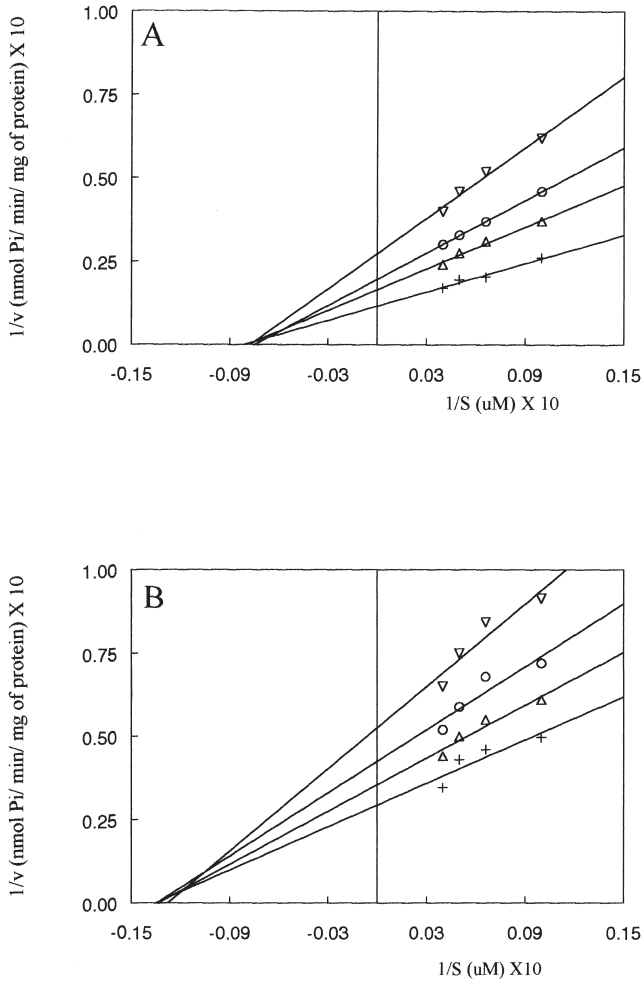

FIG. 2. Kinetic analysis of the inhibition of apyrase by suramin in hippocampal synaptosomes of rats. The graphs show double-reciprocal plots of apyrase activity for ATP (A) and ADP (B) concentrations (0.1–0.25 mM) in the absence (+) and in the presence of 15 μM (△), 45 μM (○) and 75 μM (▽) suramin. All experiments were repeated at least four times and similar results were obtained. Data presented were from individual experiments.

Statistical Analysis

The statistical analysis used in the in vitro experiments was one-way analysis of variance (ANOVA). Training and test sessions step-down latency differences among groups were evaluated by one-way Kruskal–Wallis analysis of variance, followed by a Mann–Whitney test when necessary. Comparisons between training and test sessions latencies within each group were made by a Wilcoxon test.

RESULTS

The effect of suramin on apyrase activity (ATP and ADP hydrolysis) was measured in synaptosomes from the hippocampus of adult rats. A significant inhibition of apyrase activity (16 and 20% for ATP and ADP, respectively) in relation to control (no suramin added = 100% of activity) was observed at a concentration of 12 μM. The inhibition observed was concentration-dependent, with the apyrase activity markedly reduced in the presence of 150 μM suramin (41 and 40% for ATP and ADP in relation to control activity, respectively) (Fig. 1A and 1B).

The kinetics of the interaction of suramin with apyrase activity in synaptosomes from hippocampus was also determined (Fig. 2). The Lineaweaver-Burk double-reciprocal plot was analyzed over the range of ATP and ADP concentrations as substrates (0.1–0.25 mM) in the absence and presence of suramin (15–75 μM). The data indicated that the inhibition of ATP and ADP hydrolysis by suramin was noncompetitive, because the K_m values did not change significantly, while maximal ATPase and ADPase velocities decreased with increasing suramin concentrations (Fig. 2A and 2B). The K_i values (inhibition constant) were determined from Dixon plots (plots not shown). The K_i values obtained were 72.8 ± 7.1 μM for ATP (means ± SD, $n = 4$) and 109 ± 10.6 μM for ADP (means ± SD, $n = 4$).

In the present study, we also evaluated the effect of intrahippocampal infusion of suramin on retention of an aversive learning task, the step-down inhibitory avoidance task. The results for the inhibitory avoidance task are shown in Fig. 3. There were no significant differences among groups in train-

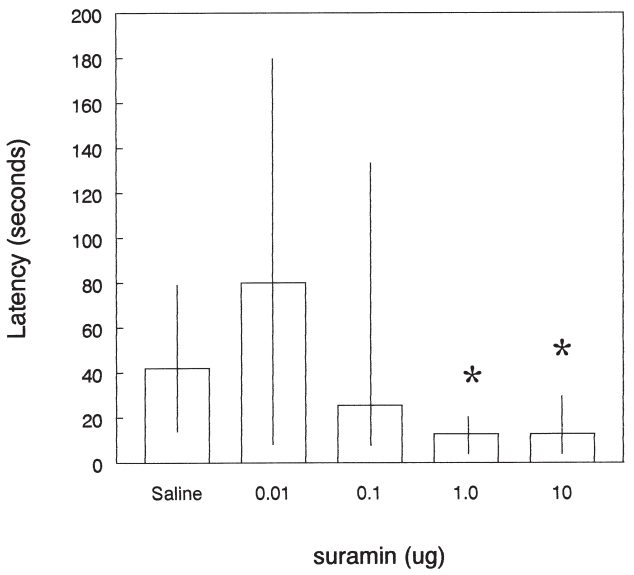

FIG. 3. Effect of intrahippocampal infusion of suramin (0.01, 0.1, 1.0, and 10.0 μg/side) on retention of step-down inhibitory avoidance task. Data are median (interquartile range) of retention test step-down latencies. n = 10–15 animals per group. *Significantly different from the saline group ($p < 0.05$) by the Mann–Whitney U-test.

ing session latencies (Kruskal–Wallis analysis of variance, $p > 0.10$), but there was a significant difference in retention test latencies among groups (Kruskal–Wallis analysis of variance, $p < 0.05$). Groups treated with suramin at doses 0.01 and 0.1 μg did not show significant difference when compared to the saline group (Mann–Whitney test, $p > 0.10$).

Groups treated with suramin at doses 1.0 and 10.0 μg showed a significant impairment of retention when compared with the saline group (Mann–Whitney test, $p < 0.01$). There was a significant difference between training and test session performances in the vehicle group, 0.01 and 0.1 μg suramin groups (Wilcoxon test, $p < 0.01$), but not in the suramin 1.0 and 10.0 μg-treated groups (Wilcoxon test, $p > 0.05$). The results show that immediate posttraining intrahippocampal infusions of suramin impaired inhibitory avoidance retention in a dose-dependent response.

DISCUSSION

In the present investigation, we showed that suramin can inhibit the apyrase activity in hippocampal synaptosomes of rats. Inhibitory effects of this compound on ATP breakdown have been previously described. Suramin inhibits ecto-ATPase activity from guinea pig urinary bladder (14), endothelial cells (23), blood cells (3), *Xenopus* oocytes (37), and can also inhibit ecto-apyrase activity from *Torpedo* electric organ (22). In agreement with the reports indicated above that suramin inhibits ATP breakdown in a noncompetitive manner (3,14,22), our results showed a similar mechanism of inhibition on apyrase activity in rat hippocampal synaptosomes. The K_i values obtained for inhibition of apyrase by suramin were of the micromolar order. These results agree with the K_i values obtained for the inhibition of this enzyme in *Torpedo* electric organ (22). However, we suggest that hippocampal apyrase (K_i = 72.8 and 109 μM for ATP and ADP, respectively) is less sensitive to suramin than *Torpedo* ecto-apyrase (K_i = 43 and 30 μM for ATP and ADP, respectively) (22). To our knowledge, this is the first report about suramin as an inhibitor of ATP diphosphohydrolase activity in synaptosomes from central nervous system (hippocampus). The usefulness of suramin as an inhibitor of hippocampal apyrase is questionable, because it is itself a P_2-purinoceptor antagonist (15). It has been suggested that ATP-metabolizing enzymes and P_2-purinoceptors have similar binding domains, thus suramin can either alter ATP degradation and/or block purinergic neurotransmission (16). Because of its inhibitory effect on ATP degradation, the potency of suramin as an antagonist can be understimated if it is tested against agonists (ATP, ADP, AMP) that are substrates for ectonucleotidases (11).

Recently, our laboratory has showed that inhibitory avoidance learning inhibits ectonucleotidase activities in hippocampal synaptosomes of rats (5). Because suramin was able to facilitate synaptic responses (34), and this drug acts as an apyrase inhibitor, we evaluated the effect of this drug on the retention of step-down inhibitory avoidance task. The administration of suramin could potentiate the apyrase inhibition observed in the acquisition of inhibitory avoidance task (5), increasing the ATP levels and improving the retention of this memory task. On the basis of these considerations, the amnesic effect of suramin was unexpected. Our results show that suramin can impair the retention of step-down inhibitory avoidance task, in a dose-dependent way, when infused intrahippoccampally at doses of 1 and 10 μg. Independently of the question whether there is an involvement of apyrase activity in this condition, an important consequence of intrahippocampal infusion of suramin is the amnesic effect. This condition can be produced by interactions between different systems upon which suramin can exert its effects, including P_2-purinoceptors, NMDA receptors, and ATP-metabolizing enzymes. Suramin is an apparent exception to the list of drugs that affect LTP or LTP-like phenomena and memory in a similar way (20), because it facilitated synaptic responses in hippocampal slices (34), but when given into the hippocampus was amnesic. The different effects of suramin reinforce the concept that, due to its broad spectrum of biological properties, the actions of this drug significantly depend on the system studied (34).

A NMDA receptor-mediated Ca^{2+} increase in hippocampal neurons has been implicated in formation of memory of the inhibitory avoidance learning (20). It is also known that the induction of LTP in postsynaptic neurons requires a rise in intracellular Ca^{2+} concentration ([Ca]i), which is thought to be mainly mediated by glutamate via *N*-methyl-D-aspartate (NMDA) glutamate receptors (4). Stimulation of P_{2x}-purinoceptors by ATP leads to a rise in [Ca]i and suramin, with its antagonist action, decrease Ca^{2+} influx, which could impair the synaptic efficiency (17). Furthermore, it has been shown that suramin can also antagonize *N*-methyl-D-aspartate (NMDA) receptors (26). As suramin blocks P_{2x}-receptors and NMDA receptors, the impairing effect of this drug in memory may be related to a blockade of Ca^{2+} influx through these receptor channels. As hippocampal LTP also depends on NMDA receptors activation and increased Ca^{2+} influx, our results are controversial with the facilitatory effect of suramin on induction of hippocampal LTP (34). Our results showed a dissociation, rather than a correlation, between LTP and memory.

The dissociation observed between the LTP studies and the behavioral data could be related to LTP hippocampal saturation. A different approach to study whether LTP is essential for learning is try to saturate all synapses in the hippocam-

pus by electrical stimulation, assuming that the upregulation of all synapses should make learning impossible. This prediction arises from the idea that neural networks store information as a specific distribution of synaptic strengths that would be disrupted by saturation of their capacity for LTP (1). The first studies have showed that after saturation, animals were not able to learn a spatial task very well, while animals in which hippocampus was stimulated but LTP was allowed to decay were not impaired (9). It was observed in the LTP studies that the magnitude of suramin effect was concentration dependent (34). At 12 μM, the effect became saturated, and a increased in the suramin concentration did not enhance the magnitude of the population spike (34). On the basis of these considerations, it is possible to suggest that, in the doses used in the behavioral studies, the suramin-induced potentiation could result in maximal expression of LTP in the entire population of relevant synapses (saturation), which could produce significant learning and memory deficits.

Another important action of suramin is its interaction with protein kinase C (PKC). It has been shown that PKC can be inhibited or activated by suramin in a type-dependent manner by multiple mechanisms (21). The chain of events starting with Ca^{2+} increase and PKC activation, followed by protein synthesis, may account for a response amplification not necessarily related to LTP but otherwise involved in memory (20,29). It is probable that compounds able to alter one of these events can promote changes in synaptic efficiency.

Accordingly, in this study we have shown the action of suramin, a P_2-purinoceptor antagonist, on the apyrase activity and on the retention of a learning task, the step-down inhibitory avoidance task. Probably the amnesic effects of suramin is related to its antagonist action on P_2-purinoceptors, NMDA receptors and/or to its inhibitory effects on ATP degradation. Further investigation is required to determine the exact biochemical mechanism involved in the amnesic effect, and whether there is a relationship between apyrase inhibition and the induction of amnesia by suramin.

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