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Action of suramin upon ecto-apyrase activity and synaptic depression of *Torpedo* electric organ

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1 The role of ATP, which is co-released with acetylcholine in synaptic contacts of *Torpedo* electric organ, was investigated by use of suramin. Suramin [8-(3-benzamido-4-methylbenzamido)naphthalene-1,3,5-trisulphonic acid], a P_2 purinoceptor antagonist, potently inhibited in a non-competitive manner the ecto-apyrase activity associated with plasma membrane isolated from cholinergic nerve terminals of *Torpedo* electric organ. The K_i was 30 μM and 43 μM for Ca^{2+}-ADPase and Ca^{2+}-ATPase respectively.

2 In *Torpedo* electric organ, repetitive stimulation decreased the evoked synaptic current by 51%. However, when fragments of electric organ were incubated with suramin the evoked synaptic current declined by only 14%. Fragments incubated with the selective A_1 purinoceptor antagonist, DPCPX, showed 5% synaptic depression.

3 The effects of suramin and DPCPX on synaptic depression were not addictive. Synaptic depression may thus be linked to endogenous adenosine formed by dephosphorylation of released ATP by an ecto-apyrase. The final effector in synaptic depression, adenosine, acts via the A_1 purinoceptor.

4 ATP hydrolysis is prevented in the presence of suramin. It slightly increased (20%) the mean amplitude of spontaneous miniature endplate currents. The frequency distribution of the amplitude of spontaneous events was shifted to the right, indicating that ATP, when not degraded, may modulate the activation of nicotinic acetylcholine receptors activated by the quantal secretion of acetycholine.

Keywords: Neuromuscular junction; purinoceptors; adenosine; quantal release; ATP-ase; ADP-ase; neurotransmitter release; acetylcholine; miniature endplate currents

Introduction

The physiological role of ATP as a neurotransmitter has been widely studied (Zimmermann, 1994). Recently a direct function of ATP in nociception has been demonstrated (Chen *et al.*, 1995; Lewis *et al.*, 1995). Earlier studies pointed out that ATP is a cotransmitter at neuromuscular junctions (Silinsky, 1975). ATP is stored, together with acetylcholine, in the synaptic vesicles from the cholinergic nerve terminals of the electric organ of *Torpedo marmorata* (Dowdall *et al.*, 1974); upon stimulation both ATP and acetylcholine are released into the synaptic cleft (Meunier *et al.*, 1975; Morel & Meunier, 1981). During synaptic activity, the release of large amounts of ATP from postsynaptic sources has also been reported in this model (Israel *et al.*, 1976).

It has been shown that extracellular ATP modulates ionic conductances, interacts with P_2 type purinoceptors promoting an excitatory cell response (Bean, 1992) and can serve as a substrate of several extracellular enzymatic activities. It has also been shown that extracellular ATP regulated the nicotinic acetylcholine receptor (Eterovic *et al.*, 1990; Carlson & Raftery, 1993) derived from the cholinergic synapse of *Torpedo* electric organ.

Adenosine, a degradation product of ATP, is an important inhibitory neuromodulator acting presynaptically through P_1 purinoceptors (Zimmermann, 1994). In *Torpedo* electric organ, adenosine has been shown to inhibit acetylcholine release (Muller *et al.*, 1987). Thus the extracellular metabolism of nucleotides may affect those synaptic phenomena regulated by ATP and its metabolites.

The enzymes responsible for ATP breakdown are nucleotidases whose active site faces the extracellular space (Grondal & Zimmermann, 1986). In synaptosomes isolated from *Torpedo* electric organ, ecto-apyrase activity (ATP-diphosphohydrolase) has been described that unspecifically hydrolyses tri and diphosphate nucleotides to the corresponding monophosphonucleotide (Sarkis & Saltó, 1991). Ecto-apyrase is believed to be part of an enzymatic chain together with 5' nucleotidase, which converts AMP to adenosine, for the complete hydrolysis of ATP to adenosine (Richardson *et al.*, 1987).

The pharmacology of ecto-apyrase does not match that found in different membrane associated-ATPases because usual ATPase inhibitors do not affect ecto-apyrase even at very high concentrations (Knowles *et al.*, 1983). No specific inhibitor of apyrase activity has been described to date. Apyrase inhibition is of particular interest in the study of the physiological importanct of extracellular hydrolysis of nucleotides in the regulation of synaptic transmission.

Suramin is used as a therapeutic agent in different pathological processes, like trypanosomiasis, AIDS, and metastatic carcinomas (Voogd *et al.*, 1993). Suramin has also been used as an antagonist of P_{2X} and P_{2Y} types of purinoceptor (Hoyle, 1990), and thus it has been commonly used for pharmacological analysis of the role of ATP in neurotransmission. On the other hand the inhibitory effect of suramin on ecto-ATPase activity has recently been reported (Hourani & Chown, 1989; Meghji & Burnstock, 1995; Ziganshin *et al.*, 1995; Beukers *et al.*, 1995). In the present paper we examine the inhibitory effect of suramin on ecto-apyrase from a presynaptic plasma membrane (PSPM) preparation of *Torpedo* electric organ. The suramin-mediated inhibition allowed us to study the effect of endogenous ATP on the cholinergic synaptic activity.

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Methods

Animals and isolation of presynaptic plasma membranes

Torpedo marmorata specimens were caught in the Mediterranean sea and maintained alive in artificial sea water. Electric organs were dissected out under anaesthesia (tricaine, 0.33 g l^{-1} of sea water) (MS22, Sandoz, Switzerland) and immersed in physiological elasmobranch solution for electrophysiological analysis that contained in mM: NaCl 280, KCl 3, $MgCl_2$ 1.8, $CaCl_2$ 3.4, glucose 5, sucrose 100, urea 100, HEPES/NaOH 5, $NACO_3H$ 5, pH 7.0 and for the isolation of PSPM the solution was Tris-HCl 10 mM buffer pH 7 containing 1 mM EDTA. PSPM were isolated from the electric organ according to Morel *et al.* (1985).

Determination of apyrase activity and protein determination

Apyrase activity was calculated by simultaneous determinations of Ca^{2+}-ATPase and Ca^{2+}-ADPase activities in Tris-HCl 30 mM pH 7.7 buffer containing NaCl 150 mM, KCl 5 mM, $CaCl_2$ 6.5 mM, EDTA 5 mM and 1 mM of the corresponding nucleotide. Under these conditions 5'-nucleotidase is inactive (Sarkis & Saltó, 1991).

Protein concentration (from 1 to 5 μg) and incubation times were chosen to ensure linearity of the velocity of product formation with a final hydrolysis of 2–3% of substrate. The reaction was stopped by the addition of trichloracetic acid 5% (w/v) concentration. Free phosphate was determined by the method of Lanzetta *et al.* (1979) with KH_2PO_4 as a reference standard. Blanks were determined by incubation of parallel samples in the same buffer lacking Ca^{2+}.

One unit of enzyme activity is defined as the amount of enzyme that releases 1 nmol Pi min^{-1} under the conditions specified above. Data are mean ± standard deviation of at least three experiments.

Electrophysiological methods

Sections of the electric organ 5 mm thick, containing around 15 prisms, were dissected maintaining thin nerve branches of electric nerve. Fragments were rinsed in the elasmobranch physiological solution and placed in parallel in different solutions containing, suramin, DPCPX, or both at the indicated concentrations. These drugs were diluted in the saline solution containing $CaCl_2$, 3.4 mM. Control slices were maintained in the calcium solution. All fragments, treated and non-treated, were incubated for 12 h at 4°C and left for 1 h at room temperature before starting stimulation. This incubation time ensures complete diffusion of the drugs into the tissue. The electric organ is still active even at 48 h after its excision. Amplitude and frequency of spontaneous synaptic events do not change during this incubation time.

Electrical stimulation was performed with a suction electrode. Maximal responses were obtained by applying pulses of 0.1 to 1 ms of supramaximal voltage every 2 min. Fragments were left for 15 min without any stimulus before starting experimental excitation. Electrical responses were recorded through a low resistance (300 KOhm) borosilicate pipette filled with elasmobranch solution and amplified by a high input impedance amplifier (GeneClamp 500, Axon Instruments) digitalized through a TL-1 interface and pClamp software (Axon Instruments) in a compatible PC. Signals were analysed by WCP program (Dr Dempster, Strathclyde University, Scotland). Since the electrical response depends on the size and topology of the fragments, results were normalized with respect to the first discharge of each fragment. Spontaneous quantal release was performed as previously described by Muller & Dunant (1987) and Cantí *et al.* (1994). M.e.p.c.s. recorded as potential changes of a focal extracellular electrode connected to Geneclamp 500 and digitalized as above, were monitored on a Tektronix 5110 oscilloscope and recorded in parallel on a VCR tape recorder (Biologic). Signals were analysed by WCP program as described. The mean of several hundreds of spontaneous quanta were compared between treated and non-treated sample by *t* test for independent data. The difference of amplitude distributions of m.e.p.c.s. of non-treated and suramin-treated electric organ fragments was analysed by the Kolmogorov-Smirnov test.

Materials

Suramin (Germanin) was a gift from Bayer (Germany). ATP and ADP were purchased from Sigma (U.S.A.). DPCPX (8-cyclopentyl-1,3-dipropylaxanthine) was from RBI (U.S.A.). All the other reagents were analytical grade.

Results

Effect of suramin on apyrase activity

In PSPM there was calcium-dependent nucleotidase activity that fulfilled the criteria established for apyrase activity in a *Torpedo* synaptosomal preparation, as previously shown (Sarkis & Saltó, 1991). Apyrase hydrolyses both ATP and ADP in a calcium- or magnesium-dependent manner, and the effect of suramin on calcium-dependent hydrolysis of these two substrates was measured. Apyrase activity was maximally inhibited by 72 ± 6% at 100 μM suramin and 77 ± 1% at 300 μM. The saturation effect of suramin began at 70 μM. Suramin reversibly inhibited the apyrase activity associated with PSPM since the inactivation was reversed after washing the membranes that had been exposed to suramin.

The Lineweaver-Burk plot (Figure 1), demonstrates a non-competitive interaction of suramin with the enzyme, since the K_m values did not vary, while maximal Ca^{2+}-ATPase and Ca^{2+}-ADPase velocities decreased as suramin concentration increased. Similar results were obtained when the competitor used was ADP instead of ATP. K_i values, obtained from Dixon plots, were 43 μM and 30 μM for Ca^{2+}-ATPase and Ca^{2+}-ADPase activities respectively.

Effect of suramin upon synaptic depression

Fragments of electric organ, when stimulated through a nerve branch, can generate an electrical response of several hundreds of millivolts. The topological characteristic of the electric tissue hinders the diffusion of molecules (Dunant *et al.*, 1987) and it takes a long time to test drugs in a continuous perfusion bath because the penetration of the drugs into the deep parts of the organ cannot be monitored. We avoided this problem by incubating fragments of electric organ overnight.

The electric discharge of the electric organ is very sensitive to the frequency of stimulus, and to maintain a stable signal the nerve trunk must be stimulated at a rate of 1 pulse every 2 min. When fragments of electric tissue were stimulated repetitively in the presence of calcium there was a decrease in the amplitude by 51.6 ± 15.9% ($n = 4$) of the initial electrical discharge. In four different experiments, when the fragments were incubated with 500 μM suramin, for 12 h (Figure 2), the depression was significantly reversed: the final amplitude of the electrical response was 85.8 ± 12.5% of the initial signal ($P < 0.05$). The synaptic depression was also prevented when the fragments were pre-incubated with the A_1 purinoceptor antagonist, DPCPX at 0.5 μM; the final amplitude was 94.5 ± 5.0% of the initial discharge ($P < 0.05$). The effects of suramin and DPCPX (Figure 2) were not additive, suggesting that they modulated synaptic activity at the same molecular level.

Effect of suramin on spontaneous synaptic activity

To test the impact of suramin on synaptic activity we also recorded the spontaneous activity of fragments of electric organ that had been treated with suramin for 12 h. Suramin at

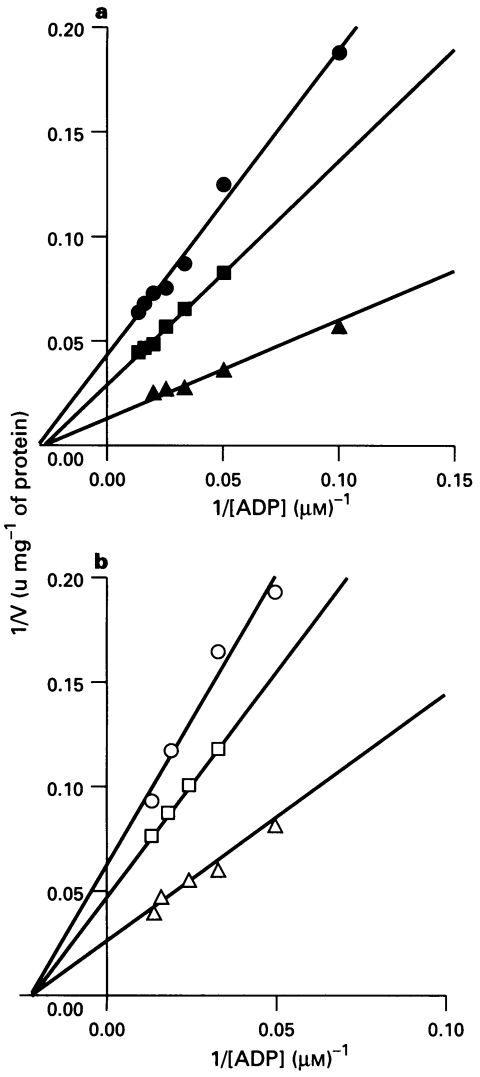

Figure 1 Inhibition of apyrase activity by suramin. Apyrase activity was measured as calcium-dependent ATPase and ADPase activities. Lineweaver-Burk plots showing non-competitive inhibition of ATPase (a) and ADPase (b) activities by suramin. Presynaptic plasma membrane fraction at a protein concentration of 1 mg ml^{-1} was preincubated for 5 min with 100 μM (○, ●), 30 μM (□, ■) of suramin or with buffer (△, ▲). Then ATP or ADP was added at different concentrations and free phosphate resulting from Ca^{2+}-ATPase or Ca^{2+}-ADPase activities was measured. Ca^{2+}-ATPase (solid symbols), Ca^{2+}-ADPase (open symbols).

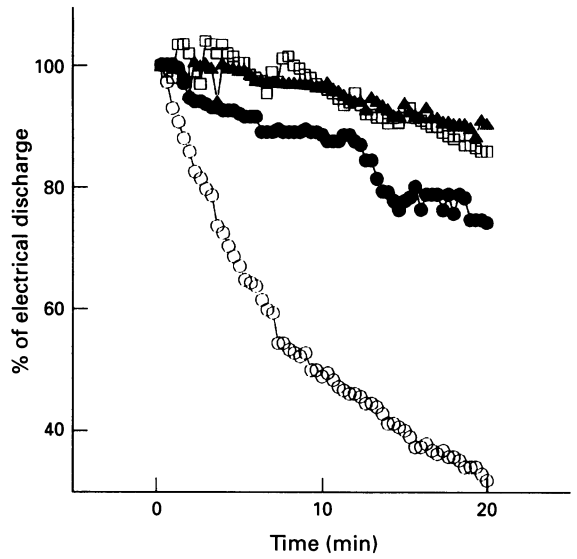

Figure 2 Effect of suramin on synaptic depression. Electrical discharge reflects the synaptic activity in the electric organ of *Torpedo*. Fragments of the electric organ were stimulated at 0.05 Hz, suramin was applied at 500 μM: (○) non-treated control fragments, (●) suramin-treated fragments; (□) framents incubated with DPCPX (0.5 μM); (▲) fragments treated with suramin 500 μM and DPCPX (0.5 μM). This figure represents one of four different experiments.

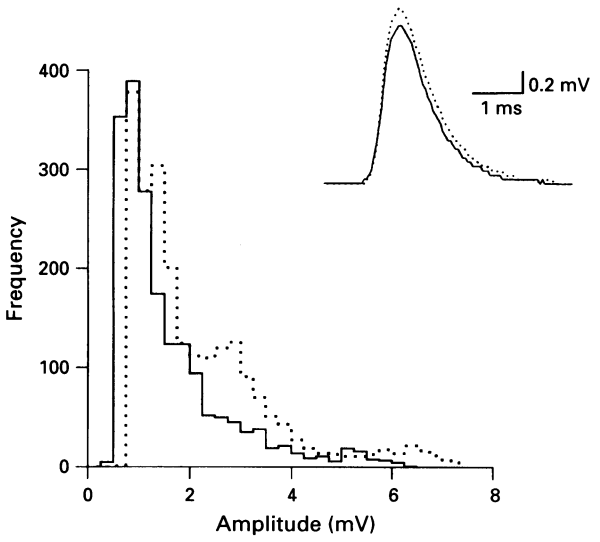

Figure 3 Effect of suramin upon spontaneous synaptic events. Histogram showing the effect of suramin on m.e.p.cs amplitude. Control condition: continuous histogram line. Suramin (500 μM): dotted histogram outline. Suramin slightly shifts the amplitude distribution towards higher values. Histogram plots are different at $P<0.001$ (K-S test, $n=3$ different experiments). Inset superimposed average traces of 300 m.e.p.cs from control tissue (continuous line) and 300 m.e.p.cs from suramin-treated tissue (dotted line), average traces are from a single representative experiment and show a slightly higher amplitude for m.e.p.cs recorded with suramin than under control conditions.

500 μM increased the amplitude of m.e.p.cs and induced a slight shift towards higher amplitudes of m.e.p.cs. Figure 3 shows a histogram plot comparing the amplitude distribution of m.e.p.c.s. According to Kolmogorov-Smirnov test, suramin treatment had a significant effect on the distributions of m.e.p.cs ($P<0.001$). Mean amplitude ± s.e. for m.e.p.cs recorded in untreated tissue was 1.6 ± 0.06 mV (1897 m.e.p.cs, from three different experiments), whereas for the suramin-treated electric organ it was 2 ± 0.03 (2233 m.e.p.cs from three different experiments). These differences were significant ($P<0.05$). The inset of Figure 3 shows the average of m.e.p.cs in treated and non-treated conditions.

Discussion

The efficient breakdown of extracellular ATP to other nucleotides has obscured the characterization of cholinergic synaptic responses to extracellular ATP. Here we present evidence that suramin can inhibit ecto-apyrase activity associated with the plasma membrane of cholinergic nerve terminals in a non-competitive way. Sodium azide and fluoride inhibit apyrase to some extent when used at high concentrations (20 mM) (LeBel *et al.*, 1980; Knowles *et al.*, 1983; Battastini *et al.*, 1991), while suramin can significantly inactivate apyrase when used in the low micromolar range. Suramin inhibits ecto-ATPase activity from urinary bladder (Hourani &

Chown, 1989), blood cells (Beukers *et al.*, 1995) and endothelial cells (Meghji & Burnstock, 1995) and also that from *Xenopus* oocytes (Ziganshin *et al.*, 1985). Our results show that suramin is one of the most potent inhibitors of neural ecto-apyrase activity reported to date.

The *Torpedo* electric organ is homologous with the neuromuscular junction, and repetitive stimulation results in synaptic depression. Adenosine exerts an inhibitory action on the evoked activity of *Torpedo* electric organ (Muller *et al.*, 1987). Extracellular ATP can also inhibit synaptic activity when it is degraded to adenosine (Israel *et al.*, 1980). However, there is no direct evidence that endogenous ATP affects synaptic modulation.

The potent inhibitory effect of suramin on ecto-apyrase activity allowed the evaluation of the role of both ATP and ecto-apyrase in synaptic depression. Both suramin and the A_1 purinoceptor selective antagonist, DPCPX (Haleen *et al.*, 1987) reversed synaptic depression. Our results show that suramin inhibited 75% of apyrase activity; similarly, synaptic depression was not completely reversed. The fact that the effect of these substances was not additive suggests that they act through related mechanisms. Consequently, we suggest that ATP released by the electric organ during stimulation is responsible for synaptic depression when it is degraded to adenosine by ecto-apyrase and 5'-nucleotidase. This degradation pathway is the only source of adenosine in the synaptic cleft, since it cannot be released from pre- or postsynaptic sources in *Torpedo* electric organ (Solsona *et al.*, 1990). Adenosine is directly responsible for synaptic depression acting presynaptically through A_1 purinoceptors.

The observed depression of synaptic activity by endogenous adenosine fits well with the adenosine-mediated synaptic depression described recently in the frog neuromuscular junction by inhibiting ecto-5'-nucleotidase (Redman & Silinsky, 1994). However, in contrast to that report, we should emphasize that we have inhibited the initial step of the dephosphorylation cascade of released ATP. In other experimental models, the effect of ATP is enhanced by suramin; in guinea-pig vas deferens suramin inhibits the extracellular breakdown of ATP (Bailey & Hourani, 1994; 1995). Similarly, in guinea-pig ileum, suramin may protect exogenous ATP from degradation (Barajas-López *et al.*, 1993). The inhibition of ecto-ATPase by suramin could explain the removal of neuromuscular block production by pancuronium (Henning *et al.*, 1992; 1993) because it would prevent the formation of adenosine which would inhibit basal acetycholine release. The same mechanism may account for the facilitation effect of suramin on hippocampal potentials (Wieraszko, 1995).

Since suramin prevents ATP degradation, we used it to measure the effect of endogenous ATP on the size of spontaneous quantal events. Suramin does not interact with nicotinic acetylcholine receptors (Evans *et al.*, 1992; Nakazawa, 1994). Therefore, the small increase in the amplitude of spontaneous events in the presence of suramin reported here is not a consequence of its direct interaction with acetylcholine receptor. It may reflect the contribution of ATP released with acetylcholine to generate a miniature endplate current. The inhibitory action of suramin upon ecto-apyrase activity would increase the concentration of extracellular ATP and, in turn, the probability of interaction of ATP with the nicotinic acetylcholine receptor. Eterovic *et al.* (1990) demonstrated that ATP increases by 20% the acetylcholine current induced in *Xenopus* oocytes expressing nicotinic ACh receptor mRNA from *Torpedo* electric organ; in addition extracellular ATP interacts with subunits of the nicotinic acetylcholine receptor (Carlson & Raftery, 1993). It has been demonstrated that ATP can open nicotinic acetylcholine receptors in cultured myocytes (Igussa, 1988); moreover Fu (1994) found that ATP can increase the response of muscle fibres to applied acetylcholine.

In conclusion, suramin is an efficient non-competitive ecto-apyrase inhibitor at the cholinergic synapses of the electric organ of *Torpedo* and it prevents the degradation of ATP in the synaptic cleft, thus preventing the formation of adenosine and eventually preventing synaptic depression. Consequently, suramin may be used to understand the role of ATP in synaptic activity and to characterize the ecto-apyrase molecule associated with the plasma membrane of cholinergic nerve terminals.

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