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Inhibition of ATP-diphosphohydrolase (apyrase) of *Torpedo* electric organ by 5′-*p*-fluorosulfonylbenzoyladenosine

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Abstract

It has been shown previously that ATP is released into extracellular space from pre- and postsynaptic sources in peripheral synapses. The extracellular metabolism of ATP is likely to affect nucleotide- and nucleoside-mediated regulation of neurotransmission. The enzymes responsible for ATP breakdown are nucleotidases whose active site faces the extracellular space. ATPase and ADPase Ca^{2+}-dependent activities were characterized in presynaptic plasma membrane preparation from the electric organ of *Torpedo*. Features described were in accordance with the presence of an ATP-diphosphohydrolase (apyrase EC 3.6.1.5) in this fraction. Active site studies using the affinity label 5′-fluorosulfonylbenzoyladenosine were performed on *Torpedo* apyrase. ATPase and ADPase Ca^{2+}-dependent activities were inhibited with 5′-fluorosulfonylbenzoyladenosine. From this study it is concluded: (1) 5′-fluorosulfonylbenzoyladenosine binds specifically to the active site of apyrase. (2) Divalent cations accelerate the apyrase inactivation rate. (3) Divalent cations are not required for the binding of either the substrate or the inhibitor to the active site. (4) The apyrase active site is more specific for highly phosphorylated nucleotides. The results presented may be extrapolated to apyrases from other sources. The importance of this enzyme and its regulation are discussed.

Keywords: Active site; Cholinergic; Apyrase; 5′-*p*-Fluorosulfonylbenzoyladenosine; (*Torpedo*)

1. Introduction

The importance of extracellular nucleotides in the regulation of neurotransmission has been established. ATP is packaged and released in a calcium-dependent manner together with several neurotransmitters like acetylcholine, in both the peripheral and the central nervous system [1–3]. Once released, ATP may interact with P_2 purinoreceptors [4,5], serves as a substrate for ecto-protein kinases [6,7] or be degraded to adenosine via ecto-nucleotidases. Adenosine is one of the most important inhibitory modulators of synaptic transmission in both central and peripheral nervous systems, acting presynaptically through P_1 purinoreceptors [8–10]. Ecto-nucleotidase activities have been described in synaptosomal preparations either from the central [11,12] or the peripheral nervous system [13,14]. These enzymes may participate in the control of extracellular ATP level in the synaptic cleft and hence in the control of purinergic neuromodulation.

In several synaptosomal fractions, including those from *Torpedo* electric organ, there is evidence for the presence of an ecto-ATPdiphosphohydrolase [15]. ATPdiphosphohydrolase (Apyrase EC 3.6.1.5), is a general designation for enzymes that hydrolyse β- and γ-bonds of diphospho- and triphosphonucleosides [16]. Several features that differentiate these enzymes from other ATP hydrolyzing activities have been specified [13,17]. A number of apyrases have been purified, [18–20] but little is known about their amino acid sequence, structure or substrate binding site. Enzyme inhibitors have been used successfully to describe enzyme active sites; however, no specific or efficient inhibitor for apyrases has been reported so far.

5′-Fluorosulfonylbenzoyladenosine (5′FSO_2BzA) is considered to be an analogue of ADP and ATP [21]. The sulfonyl fluoride moiety may be located in a position

Abbreviations: 5′FSO_2BzA, 5′-*p*-fluorosulfonylbenzoyladenosine; Me_2SO, dimethylsulfoxide; PSPM, presynaptic plasma membrane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-β-aminoethyl ether; α,β,Me-ATP, α,β-methyleneadenosine 5′triphosphate.

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analogous to the terminal phosphate of ATP and can act as an electrophilic agent in covalent reactions with several amino acids. Thus, this reagent is especially suitable for a specific modification of the purine nucleotide binding sites of proteins. Several ATPases, including apyrases, have been shown to bind 5'FSO_2BzA [22,23]. Hence, 5'FSO_2BzA may be able to modify the *Torpedo* apyrase active site covalently and to inhibit its activity. Studies of the active site using affinity analogues of adenosine nucleotides have yet to be performed on apyrases.

Torpedo marmorata electric organ is especially appropriate for the study of apyrase in the peripheral nervous system, since large amounts of ATP coming from pre- and postsynaptic sources have been reported under stimulatory conditions [24,25]. Moreover, adenosine depresses acetylcholine release in *Torpedo* nerve endings [26,27].

In the present communication, we demonstrate the presence and main features of apyrase activity in a presynaptic plasma membrane (PSPM) preparation from the electric organ of *Torpedo marmorata*. The effect of the irreversible inhibitor 5'FSO_2BzA on apyrase Ca^{2+}-dependent activity in PSPM preparation is also characterized. The study of the kinetics of inactivation of *Torpedo* apyrase by 5'FSO_2BzA may help to understand the general mechanism of action of apyrases.

2. Materials and methods

The following products were obtained from Sigma chemical Company (St. Louis MO, USA): 5'FSO_2BzA, adenosine, AMP, ATP, ADP, GTP, GDP, ITP, IDP, p^1,p^5-di(adenosine-5')pentaphosphate, α,β-Me-ATP, levamisole, 2-mercaptoethanol. Ouabain was purchased from Boehringer Mannheim (Germany) and 2-glycerophosphate, NaN_3 and NaF from Merck (Germany). All general laboratory reagents were of the purest grade available.

2.1. Animals and obtaining of PSPM

Torpedo marmorata specimens were caught in the Mediterranean sea and maintained alive in artificial sea water. Electric organs were dissected out under anaesthesia (0.33 g tricaine/l of sea water) (MS222, Sandoz, Basel, Switzerland) and immersed in Tris-HCl 10 mM buffer (pH 7) containing 1 mM EDTA. PSPM were isolated from the electric organ according to Morel et al. [28]

2.2. Enzyme assays and protein determination

Ca^{2+}-nucleotidase activity was determined in 30 mM Tris-HCl (pH 7.7) buffer containing 150 mM NaCl, 5 mM KCl, 6.5 mM $CaCl_2$, 5 mM EDTA and 1 mM of the corresponding nucleotide. Under these conditions 5'nucleotidase activity has been shown to be inactive [15]. Furthermore, this incubation medium ensures that Ca^{2+} but no Mg^{2+} nucleotidase activity is measured. When ATP was used as a substrate and Mg^{2+} as the cosubstrate, ouabain 1 mM was included in the incubation medium to avoid detection of Na/K ATPase.

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% (mass/V) final concentration. Free phosphate was determined by the method of Lanzetta et al. [29] with KH_2PO_4 as a reference standard. Blanks were determined by incubation of parallel samples in the same buffer lacking Ca^{2+} and/or Mg^{2+}.

Na/K ATPase activity (EC 3.6.1.37) [30] was assessed in Tris-HCl 30 mM (pH 7.7) buffer containing 150 mM NaCl, 5 mM KCl, 5 mM EDTA, 6.5 mM $MgCl_2$ and 1 mM ATP. Blanks for Na/K ATPase activity were determined in the same buffer but in the presence of 1 mM ouabain. Na/K ATPase activity was calculated as the difference between the ouabain-insensitive and ouabain-sensitive activity.

5'Nucleotidase (EC 3.1.3.5) [31] was evaluated in 30 mM Tris-HCl (pH 7.7) buffer containing 150 mM NaCl, 5 mM KCl, 6.5 mM $MgCl_2$ and 1 mM AMP. Blanks for 5'nucleotidase activity were determined in the same buffer in the presence of 5 mM EDTA and the absence of $MgCl_2$.

One unit of enzyme activity is defined as the amount of enzyme that releases 1 nmol Pi/min at 23°C under the conditions specified above. Data are mean ± standard deviation from at least three experiments.

Protein was determined by the method of Bradford [32] using bovine serum albumin as standard.

2.3. Reaction of 5'-p-fluorosulfonylbenzoyladenosine with presynaptic plasma membrane Ca^{2+}-ATPase and Ca^{2+}-ADPase activities

Enriched presynaptic plasma membrane fraction at a protein concentration of 0.5 mg/ml was incubated at 30°C with 5'FSO_2BzA in the presence and in the absence of several nucleotides.

PSPM were diluted in 10 mM Tris-HCl (pH 7.4) containing 1% (v/v) Me_2SO. Inclusion of the organic solvent increased the solubility of 5'FSO_2BzA.

During the reaction, aliquots were withdrawn at given time intervals, diluted 8-fold with Tris-HCl (pH 7.4) buffer at 4°C and immediately pelleted by centrifugation at 10 000 × g. Under such conditions the probability of additional reaction between the reagent and the protein was insignificant. Membranes were washed three times in the same buffer to remove unbound reagent. ATPase and ADPAse activities were then assayed.

Semilogarithmic plots of V/V_0 versus time were linear. V and V_0 are specific activities at a given time either in the presence or absence of 5'FSO_2BzA (control conditions).

Data are mean ± standard deviation from at least three

experiments. Statistical differences between groups were established using nonparametric Kurskal-Wallis (analysis of variance) and Mann-Whitney test.

3. Results

3.1. Nucleotidase activities associated with presynaptic plasma membranes

The PSPM fraction was extremely rich in 5'-nucleotidase and Na/K ATPase activities, 74.7 ± 11 and 412 ± 54 (U/mg of protein) respectively. Ca-dependent nucleotidase activity was also highly represented in this fraction.

Ca^{2+}-dependent nucleotidase activities from PSPM fraction were characterized. Michaelis-Menten constants for the hydrolysis of ATP and ADP in the presence of supramaximal concentrations of $CaCl_2$ were (μM) 46 ± 9 and 48 ± 10 respectively. Maximal velocities (U/mg of protein) calculated were 47 ± 9 and 34 ± 5 respectively. In the presence of supramaximal concentrations of $MgCl_2$, maximal velocities of 70 ± 14 for ATPase and 43 ± 7 for ADPase were found. No additive effect was observed when the activity was measured in the presence of both $CaCl_2$ and $MgCl_2$ together with ATP (49 ± 7 U/mg protein) or ADP (50 ± 10 U/mg protein), suggesting that a single enzyme may be activated by either Ca^{2+} or Mg^{2+} independently.

A mixed-substrate kinetic approach was performed in order to identify a possible association of two separate ATPase and ADPase activities. PSPM were incubated in a medium containing 1 mM ATP (5 mM $CaCl_2$), 1 mM ADP (5 mM $CaCl_2$) or 1 mM ATP + 1 mM ADP (10 mM $CaCl_2$). In the simultaneous presence of both substrates the rate of P_i production (in U/mg of protein) of 38 ± 6 was very close to the arithmetic mean (36) of those activities obtained with ADP (32 ± 8) and ATP (40 ± 13) when incubated individually. An additive effect would be expected if two separate enzymes were present.

Table 1
Calcium-dependent nucleotidase activity in presynaptic plasma membranes

Substrate	Relative velocity
ATP	1
ADP	0.66
GTP	0.67
GDP	0.64
CTP	1.26
CDP	0.67
ITP	0.99
IDP	0.85
AMP	n.d
2-glycerophosphate	n.d

PSPM nucleotidase activity was assayed under stimulatory conditions, i.e., Tris-HCl (pH 7.7) containing 6 mM, $CaCl_2$, 5 mM EDTA and 1 mM of the indicated nucleotide as described in Section 2. The relative velocity is calculated as the nucleotide/ATP hydrolysis ratio. Data represent the average of three experiments. n.d., not detected.

Table 2
Effect of various inhibitors in Ca^{2+}-ATP and Ca^{2+}-ADP hydrolysis by presynaptic plasma membranes

Inhibitor	% of activity remaining	
	Ca^{2+}-ATPase	Ca^{2+}-ADPase
None	100	100
NaN_3 (20 mM)	62	41
NaN_3 (1 mM)	93	92
NaF (20 mM)	62	62
NaF (1 mM)	93	90
2-Mercaptoethanol (5 mM)	94	89
Levamisole (1mM)	98	97
p^1,p^5-Di(adenosine-5')pentaphosphate (10 μM)	98	95
Ouabain (1 mM)	89	96

Values of 100% of nucleotidase activities refer to 46.7 and 36.5 U/mg of protein for ATP and ADP substrates respectively. All the inhibitors tested were preincubated 5 min with PSPM. Remaining activities were calculated as a percentage of control activity. Data represent the average of three experiments.

Ca^{2+}-dependent hydrolysis of several nucleotides tri-, di- and monophosphate was analyzed (Table 1). Little substrate hydrolysis specifity was observed, suggesting low specificity for the type of nucleoside. No free phosphate was detected when AMP was used as a substrate. 2-Glycerophosphate hydrolysis was not detected, indicating that non-specific phosphatases are poorly represented in the subcellular fraction of PSPM.

Results shown in Table 2 summarize the effect of several known ATPase inhibitors on PSPM Ca^{2+}-dependent ATPase and ADPase activities. At concentrations of N_3Na and NaF sufficient to inhibit mitochondrial ATPase and alkaline phosphatase respectively, no effect was observed on ATPase or ADPase from PSPM. When we increased sodium azide concentration up to 20 mM, an inhibition of 37% and 58.5% was observed for ATPase and ADPase Ca^{2+}-dependent activities respectively. When 20 mM NaF was used, 37% of the inhibitory effect on both activities was found. The amount of free phosphate detected when ADP is used as a substrate is not altered in the presence of p^1,p^5-di(adenosine-5')pentaphosphate, an inhibitor of adenylate kinase [33], suggesting that no ADP was hydrolysed with the participation of an adenylate kinase and ATPase association. Lanthanum, a (Ca^{2+}/Mg^{2+}) ATPase inhibitor [34], also had no effect on ATPase and ADPase activities from PSPM. The alkaline phosphatase inhibitor levamisole [35] and 2-mercaptoethanol did not modify ATPase or ADPase activities in our preparation. Ouabain, a specific inhibitor of Na^+/K^+ ATPase [36] did not have any effect on Ca^{2+}-dependent ATPase or ADPase activities. However, it should be noted that only 39% of the Mg^{2+}-dependent ATPase was ouabain-insensitive in our preparation.

3.2. Reaction of 5'FSO_2BzA with ADPase and ATPase activities

The kinetics of inhibition of nucleotidase activity in the presence of calcium by 5'FSO_2BzA were analyzed. Since several ATPases may be present in PSPM, ADP was chosen as the most specific substrate to study apyrase activity. Therefore the inhibitory effect of 5'FSO_2BzA on ADPase activity was studied first.

ADPase activity of PSPM, at a protein concentration of 0.5 mg/ml, was inactivated upon incubation with 5'FSO_2BzA at 30°C in Tris-HCl 10 mM (pH 7.5) buffer.

The time course of Ca^{2+}-dependent ADPase activity in the presence of 5'FSO_2BzA is presented in Fig. 1. Inactivation of ADPase activity reached its maximal effect of 75% inhibition after 90 min incubation with 1 mM 5'FSO_2BzA. No further decrease in ADPase activity was observed when incubation was prolonged for up to 120 min or when reagent concentration was increased up to 2 mM.

The decrease in Ca^{2+}-ADPase activity obeyed pseudo-first order kinetics. A plot of the natural logarithm of the residual enzyme activity as a function of the reaction time (Fig. 2), allowed for the calculation of the pseudo-first order constant (K_{obs}) from the slope at every reagent concentration, using the equation:

$$-\ln(V/V_0) = K_{obs.}t$$

At 1 mM 5'FSO_2BzA, an apparent first order rate constant of 0.0924 ± 0.012 min^{-1} was calculated. To establish a reversible binding of the reagent to the enzyme prior to covalent modification, the K_{obs} at different 5'FSO_2BzA concentrations were calculated [37]. The plot of the first-order rate constants as a function of 5'FSO_2BzA concentration between 0.1 and 2 mM exhibited saturation kinetics (Fig. 3). The double reciprocal plot of these data (Fig. 3, inset), based on the equation:

$$1/K_{obs.} = 1/K_2 + K_i/K_2 \cdot [I]$$

gave a value of K_i of 1.1 ± 0.2 mM, which represented the concentration of the reagent giving half maximal inactivation rate. The initial formation of a reversible complex between the reagent and the enzyme is demonstrated, since the line does not pass through the origin showing positive intersection on the abscissa ($P \leq 0.05$, $n = 4$) [37]. Gradual development of irreversible inhibition is reported by a K_2 of 0.175 ± 0.03 min, which illustrates the maximal rate of inhibition at saturating concentrations of the reagent.

The inhibition of ATPase activity by 5'FSO_2BzA was also examined. The kinetics of inactivation was very similar to that described for ADPase activity (data not shown). Calculated K_{obs} at 1 mM 5'FSO_2BzA concentration was 0.115 ± 0.027 min − 1 and a K_i of 2 mM was found. The nearly identical behavior of both activities in the presence of the reagent supports the idea that most of the Ca^{2+}-dependent nucleotidase activity measured was due to a single enzyme i.e., apyrase.

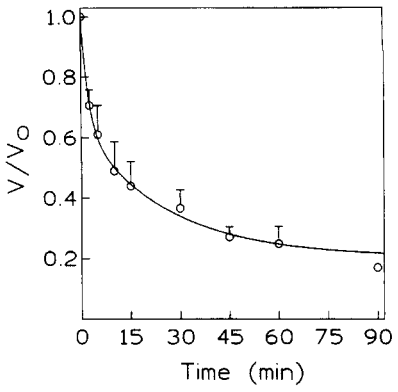

Fig. 1. Time course of Ca^{2+}-ADPase activity inhibition by 1 mM. 5'FSO_2BzA. PSPM at a protein concentration of 0.5 mg/ml were incubated in the presence and in the absence (control) of the reagent at 30°C in Tris-HCl 10 mM (pH 7.4) buffer containing 1% (v/v) Me_2SO. At the indicated times an aliquot was removed and the activity was assayed as described in Section 2. Residual activity was calculated from the ratio of the measaured enzymatic activity for the reaction at the indicated time (V), to the measured enzyme activity for the corresponding control reaction (V_0). The kinetic of inactivation follows an exponential decay. Each point represents the average of at least 3 determinations, and the error bars show the standard deviation.

3.3. Effect of substrates and other reagents on inactivation by 5'-p-fluorosulfonylbenzoyladenosine

We tested the specificity of 5'FSO_2BzA-mediated inhibition by measuring the ability of several ligands to prevent the 5'FSO_2BzA inhibition of putative apyrase activity (Table 3). Different reagents were incubated together with 5'FSO_2BzA over the time period (60 min) required to block about 75% of the activity.

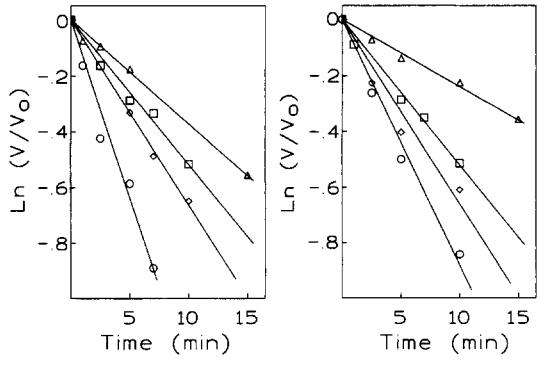

Fig. 2. Representative experiment of determination of the pseudo-first order rate constant from the decreased observed Ca^{2+}-ATPase velocity (left panel) and Ca^{2+}-ADPase velocity (right panel). PSPM were incubated at different concentrations of 5'FSO_2BzA: 0.25 mM (triangles), 0.5 mM (squares), 0.75 mM (diamonds), 1 mM (circles). Residual activity was determined as described in the legend to Fig. 1. The rate constant is determined from the slope of the natural logarithm of the residual enzyme activity plotted against time.

Table 3
Effect of potential protecting agents on inactivation of Ca^{2+}-dependent nucleotidase activity by 5'-FSO_2BzA

Additions to reaction mixture	Percent residual activity	
	Ca^{2+}-ATPase	Ca^{2+}-ADPase
None	30	28
Adenine nucleotides		
ATP (10 mM)	100	102
ADP (10 mM)	101	105
AMP (10 mM)	97	100
Adenosine (10 mM)	58	55
ATP (10 mM)+Ca^{2+} (10 mM)	108	107
ATP (10 mM)+Mg^{2+} (10 mM)	100	99
ADP (10 mM)+Ca^{2+} (10 mM)	107	105
ADP (10 mM)+Mg^{2+} (10 mM)	106	98
Divalent cations		
Ca^{2+} (10 mM)	28	26
Mg^{2+} (10 mM)	26	26
Chelators		
EDTA (2 mM)+ EGTA (2 mM)	35	27
P2x-agonists		
α,β-Me ATP (1 mM)	108	97
Suramin (10 mM)	85	63

PSPM Ca^{2+} dependent nucleotidase activity was inactivated with 1 mM 5'-FSO2BzA at 30°C, in Tris-HCl 10 mM (pH 7.4) buffer containing 1% (v/v) Me_2SO. Potential competitors were added together with 5'-FSO2BzA. Aliquots were removed after 1 h incubation and residual enzymatic activity was assayed. Percentage of residual activity was calculated by comparing the activity under the different treatments with the activity under control conditions; i.e., in the absence of possible competitors. Adenosine and adenosine derivatives were incubated together with EDTA and EGTA (2 mM) when used as protective reagents. Values are the average of one least of three experiments.

Both ATP and ADP were able to displace 100% of 5'FSO_2BzA inhibitory effect when used at a final concentration of 10 mM in the presence of EDTA and EGTA chelators. This result suggested that divalent cations may not be necessary for substrate recognition of the active site, but may be needed for both ATP and ADP hydrolysis. This could be extrapolated to the structural analogue 5'FSO_2BzA since the presence of EDTA and EGTA chelators removing trace concentrations of divalent cations in the incubation medium did not modify the degree of inhibition of apyrase.

Table 4
Effect of different reagents on rate constant for inactivation by 5'-5'FSO_2BzA

Ligand added to the reaction mixture	$K_{obs.}$ ($\times 10^{-3}$) min^{-1}	
	Ca^{2+}-ATPase	Ca^{2+}-ADPase
None	91	103
Nucleotides		
ATP (10 mM)	0	0
ADP (10 mM)	0	0
ATP (0.2 mM)	55	58
ADP (0.2 mM)	65	64
Divalent cations		
Ca^{2+} (10 mM)	154	153
Mg^{2+} (10 mM)	123	153

PSPM were incubated with 1 mM 5'FSO_2BzA in the presence and absence of different ligands. Aliquots were removed in the course of the reaction and inactivation rate constant was calculated as described in Fig. 3.

Divalent cations (10 mM) alone did not elicit any protection from 5'FSO_2BzA inactivation (Table 3); however, the inactivating reaction was 48% faster in the presence of high concentrations of $CaCl_2$ and $MgCl_2$. A similar result was obtained when ATPase inhibition reaction was evaluated (Table 4).

Other purine and pyrimidine nucleotides di- and triphosphate, which are natural substrates of the broad

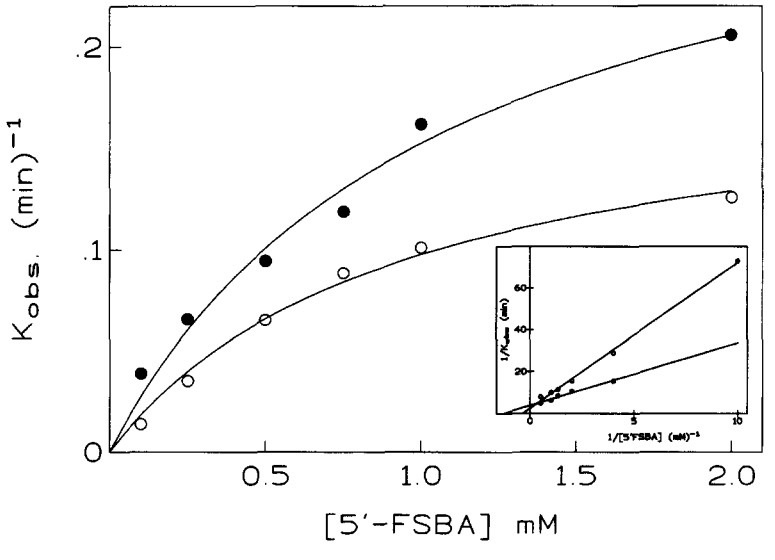

Fig. 3. Pseudo-first order rate constants of inactivation of Ca^{2+}-ATPase (closed circles) and Ca^{2+}-ADPase (open circles) as a function of 5'FSO_2BzA concentration. K_{obs} were determined at different 5'FSO_2BzA concentrations as described in the legend to Fig. 1. The inset is a double reciprocal plot of these data. From this plot K_i and K_3 are calculated.

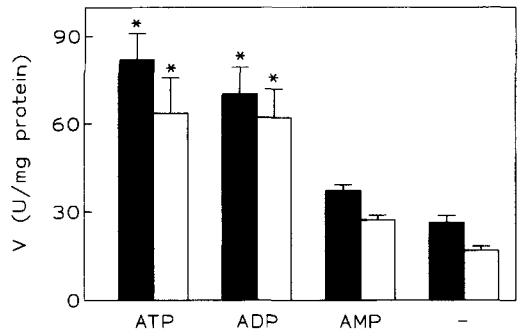

Fig. 4. Relative ability of nucleotides in protecting from 5'FSO_2BzA inhibition of Ca^{2+}-ATPase (filled bars) and Ca^{2+}-ADPase (open bars). PSPM at a protein concentration of 0.5 mg/ml were incubated with 1 mM 5'FSO_2BzA together with the indicated nucleotide at 0.5 mM final concentration. After 1 h incubation, samples were extracted, pelleted and washed as indicated in Section 2. Residual activity was then determined. Each bar is the mean and the standard deviation of three determinations. Asterisks indicate significant differences with control activities, i.e., in the absence of competing nucleotides. ($P \leq 0.05$, $n = 4$).

non-specific nucleotidase activity, i.e., ITP, IDP, GTP, GDP, UTP and UDP, were also able to prevent 5'FSO_2BzA inhibition of ATPase and ADPase activities when used at 10 mM in the presence of EDTA and EGTA chelators (data not shown).

α,β-Methylene ATP, a P_2 purinergic receptor agonist, was able to protect apyrase 5'FSO_2BzA-mediated inhibition at a concentration of 1 mM. Suramin, another P_2 receptor agonist, is also known to inhibit several ATPases reversibly [38,39]. This reagent prevented most of 5'FSO_2BzA-mediated inhibition.

AMP 10 mM also displaced 100% ATPase and ADPase inhibition, while adenosine, at the same concentration, only partially protected nucleotidase from the effect of 5'FSO_2BzA, suggesting that the active site may have more affinity for nucleotides than nucleosides. AMP is not a substrate but a product of apyrase activity. A possible inhibitory effect of the reaction product on apyrase was also examined. ATPase and ADPase activities were 50% inhibited by 10 mM AMP.

The relative ability of adenosine nucleotides to prevent 5'FSO_2BzA effect was studied. Both ADP and ATP were able to protect apyrase inhibition significantly at 0.3 mM ($P \leq 0.05$, $n = 4$), while higher concentrations of AMP were necessary for that purpose. Fig. 4 illustrates the ability of adenosine nucleotides to displace the 5'FSO_2BzA effect at 0.5 mM.

4. Discussion

The relevance of ecto-apyrases in the regulation of neurotransmission is an unsolved problem in neuronal systems. However, it has been shown recently that all the presynaptic depression observed in frog neuromuscular junction is due to the release of adenosine derivatives [10]. This could be extrapolated to the electric organ of *Torpedo*, considered to be homologous to the neuromuscular system. In this preparation high extracellular ATP levels coming from pre- and postsynaptic sources have been detected, which may be substrates for ecto-apyrase. The adenosine resulting from ecto-5'nucleotidase activity is believed to be the effector in retroinhibitory control of transmitter release [27]. Full understanding of the purinergic regulation of neurotransmission relies partially on the comprehension of the mechanisms of action of apyrase.

The specific Ca^{2+}-dependent ATPase and ADPase activities found in PSPM preparation are 5 times higher than those reported in the synaptosomal fraction [15]. The ratio of ATP/ADP Ca^{2+}-dependent hydrolysis of 1.3 is kept constant from synaptosomal preparation to PSPM, indicating that both activities copurified in this fraction. Therefore, the enzymatic activities that we have measured may represent the ecto-apyrase activities previously reported in isolated nerve terminals.

In the present study, calcium was chosen as cosubstrate since it is probably one of the physiological activators in the synaptic cleft and because our results suggested the presence of several Mg^{2+}-ATPases. ADPase activity was 15% higher active when the cosubstrate used was Mg^{2+}, while ATPase activity was 33.5% increased even in the presence of ouabain. Thus Mg^{2+} may activate other ATPases.

The low K_m values obtained for Ca^{2+}-ATP and for Ca^{2+}-ADP are in close agreement with K_m of other synaptosomal ecto-nucleotidase characterizations [13,40]. Similar K_m values for both substrates are characteristic of apyrases [18].

In PSPM, we have ruled out the possibility that apyrase activity measured under our assay conditions is the consequence of different enzyme combinations. The association of an ATPase and an ADPase is excluded by the mixed substrate experiment, which is a classical procedure for the characterization of apyrase enzymes [15,18,41,42]. Additionally non-specific phosphatases are negligible since no activity could be detected using 2-glycerophosphate as a substrate.

In summary, in PSPM preparation from *Torpedo* electric organ there is at least one nucleotidase activity that fulfils the typical characteristics described for apyrase enzyme: low substrate specificity toward purine and pyrimidine nucleotides, activation by either Ca^{2+} or Mg^{2+} as a cosubstrate, low K_m for the nucleoside di- and triphosphate and lack of inhibition by typical inhibitors of other ATPases except for high concentrations of fluoride and azide [13,17]. Moreover, we have shown that calcium-dependent nucleotidase activity determined under our assay conditions in PSPM fraction is due to apyrase activity. Thus the effect of 5'FSO_2BzA was measured on Ca^{2+}-ATPase and Ca^{2+}-ADPase activities.

5'FSO_2BzA behaves as a specific site-directed reagent in our preparation since the analysis of its reaction accom-

plishes two necessary criteria [21]. First, the rate of inactivation plotted as function of the reagent concentration rather shows saturation kinetics, providing kinetic evidence for the initial formation of a reversible complex prior to covalent irreversible inactivation. Second, the presence of natural purine nucleotides decreases the rate of inactivation by the reagent when used at low concentrations; higher concentrations of the nucleotides and reversible inhibitors totally abolish the inactivation rate.

Several proposals can be made both about the mechanism of hydrolysis and the active site of the enzyme from the analysis of 5′FSO_2BzA-mediated inhibition and displacement experiments. Divalent cations, which are necessary for the hydrolysis of nucleotides di- and triphosphate, are not needed for the recognition and binding of the substrate to apyrase active site. Natural substrates have been shown to displace 5′FSO_2BzA-mediated inhibition even in the presence of divalent cations chelators such as EDTA and EGTA. Additionally, 5′FSO_2BzA covalently modifies apyrase in the presence of both EDTA and EGTA, supporting the preceding suggestion. Our conclusions agree with the results obtained in ecto-ATPase characterization from mammalian synaptosomes [11].

Ca^{2+} and Mg^{2+} did not modify the maximal degree of inhibition of apyrase after 1 h incubation with 5′FSO_2BzA; however, these ions significantly increased the inactivation rate. These results are consistent with those obtained in studies on the active site of cyclic AMP-dependent protein kinase [43]. Those authors suggested the possibility that the cations formed complexes with 5′FSO_2BzA that were more potent inhibitors of the enzyme. This could be extrapolated to the natural substrate, as it has been previously shown that metal ions can form complexes with the purine ring as well as phosphate groups of nucleotides [44]. Otherwise metal cations could also form complex with the enzyme, improving the binding of both the reagent and the substrate to the active site.

According to the protection experiments, the active site seems to exhibit greater specifity for highly phosphorylated nucleotides. At high concentrations, adenosine is not able to displace 100% of 5′FSO_2BzA effect, while AMP is able to do so. Thus the monophosphate moiety is sufficient to protect from the effect of 5′FSO_2BzA. Moreover, ATP and ADP significantly displace the effect of 5′FSO_2BzA at a similar concentration, while AMP protects only at higher concentrations.

We have demonstrated that 5′FSO_2BzA is an effective inhibitor of apyrase activity compared with specific ATPase inhibitors. In the future, 5′FSO_2BzA may be a useful tool to identify the purine nucleotide site of *Torpedo* apyrase.

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