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Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals.

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Regulation of the Ecto-Nucleotidase Pathway in Rat Hippocampal Nerve Terminals*

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Ecto-nucleotidases play a pivotal role in terminating the signalling via ATP and in producing adenosine, a neuromodulator in the nervous system. We have now investigated the pattern of adenosine formation with different concentrations of extracellular ATP in rat hippocampal nerve terminals. It was found that adenosine formation is delayed with increasing concentrations of ATP. Also, the rate of adenosine formation increased sharply when the extracellular concentrations of ATP + ADP decrease below 5 μM, indicating that ATP/ADP feed-forwardly inhibit ecto-5′-nucleotidase allowing a burst-like formation of adenosine possibly designed to activate facilitatory A_{2A} receptors. Initial rate measurements of ecto-5′-nucleotidase in hippocampal nerve terminals, using IMP as substrate, showed that ATP and ADP are competitive inhibitors (apparent K_i of 14 and 4 μM). In contrast, in hippocampal immunopurified cholinergic nerve terminals, a burst-like formation of adenosine is not apparent, suggesting that channelling processes may overcome the feed-forward inhibition of ecto-5′-nucleotidase, thus favouring A_1 receptor activation.

KEY WORDS: Ecto-nucleotidases; ecto-5′-nucleotidase; ATP; adenosine; hippocampus; nerve terminals.

INTRODUCTION

Purines are well recognised as part of the chemical arsenal used in transmission and fine-tuning of transmission in the nervous system (1). There are two major players in this family: ATP and adenosine, the former being converted into the latter by an ecto-nucleotidase pathway that plays a critical 'go-between' role.

ATP is stored together with neurotransmitters in synaptic vesicles (reviewed in 2) and is released in a quantal (3), vesicular (4) and frequency-dependent manner (5,6). ATP can also be released from the stimulated post-synaptic component (7), from glial cells (8) and upon mechanical stimulation of various cell types (e.g. 9,10). Once released, ATP can act as neurotransmitter via activation of ionotropic P_{2X} receptors, as now established in the sympathetic and also in some parasympathetic systems (1). However, in the central nervous system, ATP has only been shown to act as a neurotransmitter in a few synapses (11). Instead, or in parallel, ATP can also act as a presynaptic modulator (12) and be involved in neuron-glia signalling (13) via either P_{2X} or metabotropic P_{2Y} receptors.

In contrast to the versatile role proposed for ATP in the nervous system, adenosine fulfils a neuromodulatory role. Adenosine can also be released as such through bi-directional non-concentrative adenosine transporters from nerve terminals, activated postsynaptic components or from glial cells (14). Adenosine mostly modulates synaptic transmission acting presynaptically to

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control neurotransmitter release (14). Adenosine also acts postsynaptically to hyperpolarise neuronal membrane potential (15), tightly controlling the functioning (e.g. 16) and rate of desensitisation of ionotropic receptors to excitatory neurotransmitters (17). Until a few years ago, it was commonly thought that adenosine was mainly an inhibitory neuromodulator, but it is now clear that the effects of adenosine, at least in relation to the control of neurotransmitter release, depend on the balanced activation of inhibitory A_1 receptors and of facilitatory A_{2A} receptors (14). The generally recognised role of A_1 receptor-mediated inhibition in the central nervous system mostly results from the greater density of A_1 receptors in the nervous system and to the experimental conditions generally used that favour the release of adenosine as such mostly from non-synaptic sources and that do not favour A_{2A} receptor function (discussed in 14,18). It has recently been proposed that one way for extracellular adenosine to preferentially activate either inhibitory or facilitatory receptors would depend on the manner in which extracellular adenosine was generated: release of adenosine as such through bi-directional non-concentrative adenosine transporters would favour A_1 receptor-mediated inhibition whereas formation of adenosine from the extracellular catabolism of ATP would favour A_{2A} receptor-mediated facilitation (14,19,20).

According to this proposal, the ecto-nucleotidase pathway would not only be designed to remove a released signalling molecule (ATP) but also to produce in a highly controlled manner another messenger molecule (adenosine) and contribute to purine salvage (21). The importance of ATP catabolism is strengthen by the numerous enzymatic activities that can fulfil this function either from the E-NTPase family (including the 'ecto-ATPase' and 'ecto-ATP diphosphohydrolase') and the ecto-phosphodiesterase/nucleotide pyrophosphatase (E-PNPP) family (22). At this stage, the lack of clear kinetic hallmarks and of pharmacological tools makes it difficult to identify in situ the molecular entities responsible for ATP catabolism, in particular because several of them appear to be co-expressed and co-located in the same cells (e.g. 23). Also, the lack of potent and selective inhibitors of extracellular ATP catabolism, makes it difficult to assess the functional role of ecto-ATPases in removing extracellular ATP [I will use the old terminology of ecto-ATPase to refer to any possible(s) molecular entity(ies) involved in extracellular ATP catabolism from now on].

Functionally, the ecto-nucleotidase pathway has mostly been related to its ability to form extracellular adenosine involved in the modulation of neurotransmitter release (14). In this respect, this pathway potentially allows to relate the amounts of released ATP to the activation of different adenosine receptors, based on the feed-forward activation of ecto-5′-nucleotidase (the enzyme that converts AMP into adenosine) by ATP and/or ADP. Thus, when the transient amounts of released ATP are high, AMP will have to accumulate due to the feed-forward inhibition of ecto-5′-nucleotidase and only when the levels of ATP and/or ADP decrease below the threshold level of inhibition of ecto-5′-nucleotidase will adenosine be formed in high transient amounts, possibly enough to trigger A_{2A} receptor-mediated facilitation (discussed in 14,18). This kinetic pattern has experimentally been verified in striatal nerve terminals (24) and is in agreement with the ability of α,β-methylene ADP, a competitive ecto-5′-nucleotidase inhibitor (25), to relieve a tonic A_{2A} receptor-mediated facilitation of neurotransmitter release (19,26). Indeed, the presence of α,β-methylene ADP causes a decreased formation of synaptically released adenosine (5,20,27,28). However, in some preparations, it was concluded that the presence of α,β-methylene ADP instead blunts inhibitory A_1 receptor activation (29–32). This may either be due to a lower release of ATP, that would only reach concentrations below the threshold of inhibition of ecto-5′-nucleotidase or to the existence of kinetic restrains of the activity of the ecto-nucleotidase pathway, like channelling processes (33,34), that would force a preferential activation of inhibitory A_1 receptors, in spite of a feed-forward inhibition of ecto-5′-nucleotidase (discussed in 33).

In this work, the kinetic pattern of ATP catabolism and adenosine formation in hippocampal nerve terminals was compared at different concentrations of ATP to try to estimate the threshold of inhibition of ecto-5′-nucleotidase by ATP and/or ADP. The kinetic parameters of ecto-5′-nucleotidase inhibition by ATP and ADP were then directly studied by carrying out initial rate measurements of ecto-5′-nucleotidase activity. Finally, the relevance of this feed-forward inhibition of ecto-5′-nucleotidase activity by ATP and ADP was investigated in hippocampal immunopurified cholinergic nerve terminals, where adenosine generated from the ecto-nucleotidase pathway activates A_1 receptors (32).

EXPERIMENTAL PROCEDURE

Preparation of Hippocampal Nerve Terminals. Hippocampal nerve terminals were prepared from male Wistar rats (6–9 weeks old) by a sucrose/Percoll purification method, as previously described (35).

When using hippocampal cholinergic nerve terminals, they were obtained by immunopurification from the Percoll purified nerve terminals using a goat anti-Chol1 anti-serum and rat IgG anti-goat monoclonal antibodies covalently coupled to a cellulose matrix (see 35,36).

Time Course Kinetic Experiments. Synaptosomes were resuspended and equilibrated at 37°C in 1 ml of Krebs/HEPES solution of the following composition (mM): NaCl 124, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, HEPES 26, glucose 10, pH 7.4. Aliquots of 100 μl were added to incubation vials with 600 μl of Krebs/HEPES solution and kept at 37°C. After 10 min incubation, the initial substrate, i.e. ATP (1–300 μM) was added. The kinetic protocols consisted of a 60 min incubation period with sample collection (50 μl) at 0, 1, 2.5, 5, 10, 15, 30, 45 and 60 min. The zero time was defined as the sample collected immediately after (approximately 5 s) addition of the initial substrate. Each collected sample was centrifuged (14,000 *g* for 10 s) and the supernatant (40 μl) was ice-stored for HPLC analysis (37). After the 60 min incubation, the synaptosomes were pelleted by centrifugation (14,000 *g* for 10 s). The medium was used to estimate if there was any released ecto-nucleotidase activity (exo-nucleotidases, see 22) and to quantify lactate dehydrogenase activity, an index of cellular disruption, which was always lower than 3% of total lactate dehydrogenase activity in the synaptosomes. The pelleted synaptosomes were homogenized in 200 μl of 2% (v/v) Triton X-100 to determine total lactate dehydrogenase activity and protein content (34,35).

The areas of the peaks obtained in the HPLC chromatograms were converted into concentrations using calibration curves for each substance (37). The concentrations of products in samples collected from the same batch of nerve terminals incubated without adding substrate (ATP) were subtracted for correction of spontaneous release. Plots of concentration of the substrate and products as function of time (progress curves) were constructed. Each progress curve was analysed by the following parameters: half-degradation time of the initial substrate; time of appearance of the maximum concentration of the first product; and concentration of substrate or any product remaining at the end of the experiment. These parameters were calculated as previously described (35) by fitting each curve with a third degree polynomium using the Raphson-Newton method. Unless otherwise stated, all values are presented as means ± SEM obtained in *n* experiments.

Initial Rate Measurement of Ecto-5′-Nucleotidase. Synaptosomes were resuspended and equilibrated at 37°C in 1 ml of Krebs/HEPES solution and aliquots of 10 μl were added to incubation vials with 590 μl of Krebs/HEPES solution and kept at 37°C. After 10 min incubation, the initial substrate, either AMP (1–300 μM) or IMP (1–300 μM), was added without or with the tested inhibitor ATP or ADP (1–100 μM). Samples (50 μl) were collected from the bath at 0, 0.5, 1 and 1.5 min. Each collected sample was centrifuged (14,000 *g* for 10 s) and the supernatant (40 μl) was ice-stored for HPLC analysis (37). After the 1.5 min incubation, the synaptosomes were pelleted by centrifugation (14,000 *g* for 10 s) and homogenized in 200 μl of 2% (v/v) Triton X-100 to determine the protein content (34,35). The areas of the AMP or IMP peaks obtained in the HPLC chromatograms were converted into concentration (37) and the initial rate of AMP or IMP catabolism was calculated upon linear regression of the decrease in the concentration of AMP or IMP with time. When studying the initial rate of extracellular catabolism of ATP and ADP, the experimental protocols were identical except that the initially added substrate was either ATP (1 μM–3 mM) or ADP (1–300 μM).

The apparent kinetic parameters (K_M and *V*) of ecto-5′-nucleotidase (measured as the extracellular catabolism of AMP or IMP) were obtained by non-linear fitting of the variation of the initial rate with the concentration of AMP or IMP to the logistic Henri-Michaelis-Menten equation. The apparent K_i of ATP and ADP inhibition of ecto-5′-nucleotidase activity were estimated with the use of a Dixon plot (38) and the type of inhibition assessed with a Cornish-Bowden plot (39).

RESULTS AND DISCUSSION

Extracellular ATP Catabolism in Hippocampal Nerve Terminals. Time course kinetic studies have the advantage of providing a general overview of the change in concentration of several pathway-related metabolites over time. But they have as a major disadvantage the difficulty in normalising results in between experiments, specially when intact cells or sub-cellular components are used, as is the case with the presently described studies in nerve terminals. Normalization by the amount of protein used is the only possible strategy of normalisation but different amount of nerve terminals will influence both the absolute amount of purine metabolites and the time-course of the appearance of their maximal concentrations. Thus, it was decided to present individual experiments that are considered representative of a series of experiments performed with different batches of nerve terminals prepared from different animals, as well as average parameter values.

Fig. 1 illustrates the time-dependent changes in the concentration of ATP, ADP, AMP and adenosine after addition at zero time of different concentrations of ATP (3–100 μM) to the same population of nerve terminals. The first conclusion to be drawn from these experiments is that the rate of extracellular catabolism of ATP is rapid for any of the tested concentrations of ATP with the amount of nerve terminals in these assays. The efficiency of the extracellular catabolism of ATP is so intense that, in the first time point collected between 5–10s after adding ATP, there is a marked decrease in the concentration of ATP with a concomitant formation of ADP and AMP. This is due to an efficient catabolism by the hippocampal nerve terminals since the initial concentration as well as the purity of ATP solutions added to the nerve terminals was always confirmed by HPLC and the total amounts of ADP + AMP + adenosine present in these solution was always less than 1.6% of the concentration of ATP.

An attempt was made to estimate the apparent kinetic parameters of extracellular ATP catabolism using a 10 times lower amount of nerve terminals. The rapid initial rate of catabolism was still observed in these conditions and this lead to an apparent K_M value of

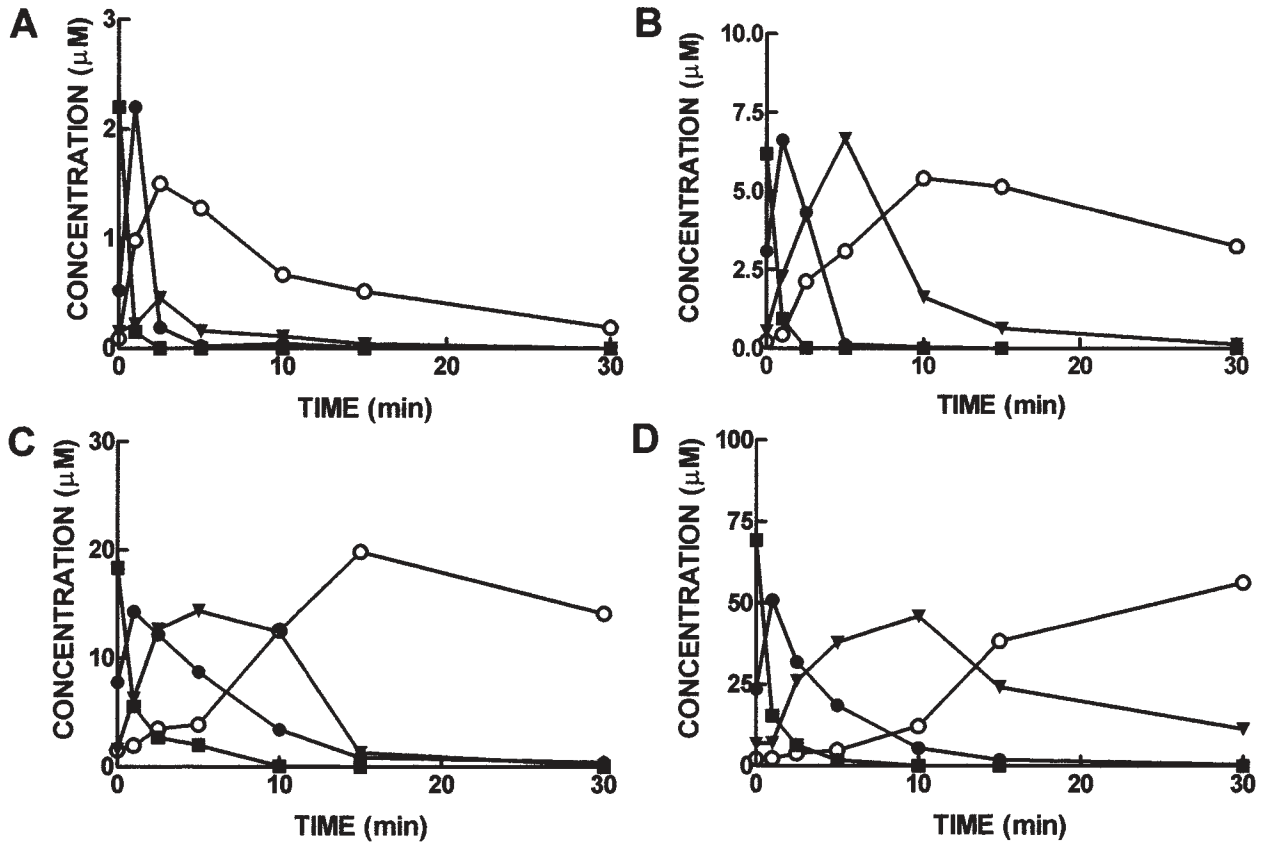

Fig. 1. Different time course kinetic pattern of ATP metabolism in rat hippocampal nerve terminals with different concentrations of extracellularly added ATP. ATP (3 μM in **A;** 10 μM in **B,** 30 μM in **C** and 100 μM in **D**) was added at zero time to rat hippocampal nerve terminals (696 μg protein) and samples were collected from the bath at the times indicated in the abscissa. Each collected sample was analysed by HPLC to separate and quantify ATP (■) and its metabolites, ADP (●), AMP (▼) and adesonine (○). The data were obtained from a single experiment representative of 12 similar experiments.

extracellular catabolism between 400–1200 μM, considerably higher than what is found in most systems (reviewed in 40). High K_M values of extracellular ATP catabolism were also found in rat ventricular myocytes, in pig arterial smooth muscle cells and in pig aorta endothelial cells and were justified by the hypothesis that the depletion of substrate at the cell surface is rate limiting for hydrolysis of ATP supplied from the bulk phase (41–43). Interestingly, ecto-ATPase was found to be located in caveolae (44), supporting the possible occurrence of channelling processes.

One issue that can only marginally be addressed with this type of studies is on the molecular entity responsible for the extracellular catabolism of ATP. Some authors favour the view that there are two main different entities, an ecto-ATPase and an ecto-ADPase (24,41–43) whereas others champion the idea that the extracellular catabolism of ATP and ADP are mainly due to an ecto-ATP diphosphohydrolase (45–47). The observed accumulation of ADP upon ATP catabolism (Fig. 1) is strongly suggestive of a main 'ecto-ATPase' activity with higher catalytic efficiency than an 'ecto-ADPase'-like activity, rather than an ecto-ATP diphosphohydrolase activity. To further explore this issue, the effect of β,γ-imido ATP on the extracellular catabolism of ATP and of ADP was tested. As illustrated in Fig. 2, it was found that β,γ-imido ATP (100 μM) inhibited more profoundly the extracellular catabolism of ATP than that of ADP. Since β,γ-imido ATP displayed a competitive-like behaviour in relation to inhibition to the extracellular catabolism of ATP (data not shown), these data can only be taken as an indication favouring the possibility that the major enzymatic activity responsible for extracellular ATP catabolism in rat hippocampal synaptosomes might be an ecto-ATPase rather than an ecto-ATP diphosphohydrolase. It should be added that besides E-NTPases, there should be an ecto-nucleotide pyrophosphatase (E-PNPP) activity

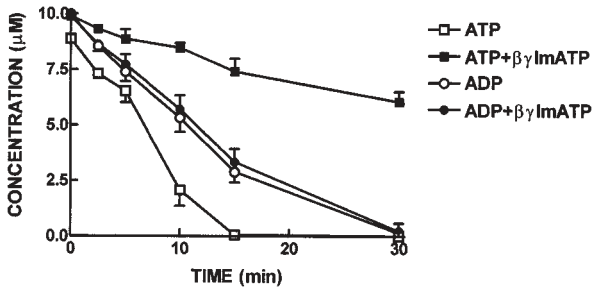

Fig. 2. Inhibition by β,γ-imido ATP of ATP and ADP extracellular catabolism in rat hippocampal nerve terminals. ATP (10 μM; □,■) or ADP (10 μM; ○,●) was added at zero time to rat hippocampal nerve terminals (62–104 μg protein) in the absence (open symbols) or in the presence (filled symbols) of β,γ-imido ATP (β,γ-ImATP, 100 μM) and samples were collected from the bath at the times indicated in the abscissa. Each collected sample was analysed by ion-pair reverse-phase HPLC (Cunha et al., 1998) to quantify ATP and ADP. The data are mean ± sem of 5 experiments.

present in hippocampal nerve terminals to understand the previously reported conversion of β,γ-substituted ATP analogues into AMP, without a measurable formation of extracellular ADP in rat hippocampal nerve terminals (34). However, in the absence of selective inhibitors, it is not yet possible to estimate the relative contribution of E-NTPases and E-PNPPases for extracellular ATP catabolism.

The Ecto-Nucleotidase Pathway in Rat Hippocampal Nerve Terminals. Fig. 1 also illustrates that there was a qualitatively similar pattern of extracellular metabolism of ATP into adenosine in hippocampal synaptosomes, with the different initial concentrations of ATP tested (3–100 μM). The decrease in the extracellular concentration of ATP was accompanied by an increase in the extracellular concentration first of ADP then of AMP and later of adenosine.

The formation of IMP was not detected in any experiment, indicating that ecto-AMP deaminase activity is not present in rat hippocampal nerve terminals, in contrast to what was reported in frog (48) and rat neuromuscular junctions (49) where this pathway acts as an adenosine shunt-like system to extracellularly metabolise adenine nucleotides (27).

Also, the decrease in ATP is continuous over time, indicating that a possible ecto-adenylate kinase activity (see 50,51) or ecto-nucleoside diphosphokinase activity (see 52), if present, do not appreciably contribute for the extracellular ATP catabolism in rat hippocampal nerve terminals, as observed in striatal cholinergic nerve terminals (24).

Another issue carefully investigated was the possible activity-dependent release of exo-nucleotidases that has been observed in different peripheral tissues (10,53). After 60 min incubation of rat hippocampal nerve terminals in the absence or in the presence of ATP (30 μM), there was no measurable activity able to metabolise ATP, ADP or AMP in the media after centrifugation at 14,000 *g* (n = 6). The same experiments were also carried out in rat hippocampal nerve terminals depolarised with 20 mM K^+ for period ranging from 2 min to 30 min (this period of incubation with 20 mM K^+ was not further extended, since a release of 10–15% of total lactate dehydrogenase already occurred after 30 min incubation). Again, under these conditions, there was a lack of measurable exo-nucleotidases (n = 2–3 for each of the 5 tested conditions). Thus, the release of exo-nucleotidases does not appear to occur from hippocampal nerve terminals, although it remains to be established if other synaptic partners, like the post-synaptic component or glial cells, may release exo-nucleotidases to the synapses in the central nervous system.

Different Pattern of Adenosine Formation According to the Initial Concentration of ATP in Hippocampal Nerve Terminals. In spite of being qualitatively similar, the time course pattern of appearance of AMP and of adenosine was different at different initial concentrations of ATP. Indeed, at low concentrations of ATP (3 μM; Figure 1A), there was a low accumulation of AMP (its peak extracellular concentration was 0.46 ± 0.04 μM, n = 16, i.e. nearly 15% of initially added ATP). As the concentration of initially added ATP was increased, the peak of AMP concentration was reached later (3.1 ± 0.7 min with 3 μM ATP, 4.8 ± 0.6 min with 10 μM ATP, 7.6 ± 1.1 min with 30 μM ATP and 9.8 ± 1.2 min with 100 μM ATP) and the maximal amount of AMP was also percentually higher (37% with 10 μM ATP, 51% with 30 μM ATP and 60% with 100 μM ATP) in the average of 12–18 experiments similar to these illustrated in Fig. 1.

The pattern of appearance of extracellular adenosine was also different with the different amounts of initially added ATP. Indeed, when ATP (3 μM) was used as initial substrate, the extracellular concentration of adenosine rose immediately from time zero until reaching a maximal concentration of 1.52 ± 0.08 μM after 2.9 ± 0.2 min (n = 16). However, as the concentration of initially added ATP was increased, there was an increasing time span before the concentration of adenosine started to increase. Thus, with 10 μM ATP, only after 2.5 min did the extracellular concentration of adenosine start to increase; with 30 μM ATP it took more than 10 min to see a change in the rate of extracellular adenosine formation and this lag-time was increased to more 15 min when 100 μM ATP was used

as initial substrate. This different lag time required to see changes in the extracellular concentration of adenosine is best illustrated by Fig. 3A that shows the change in the relation of products and 'substrates' of ecto-5′-nucleotidase with time (see references 27 and 48 for reasons to choose the ratio adenosine/ATP + ADP + AMP rather than adenosine/AMP to express ecto-5′-nucleatidase activity). The maximal concentration of adenosine reached with the different concentrations of ATP used as initial substrates was also percentually higher as the concentration of ATP increased. Thus, the maximal concentration of adenosine was 44% of initially added ATP with 3 μM ATP, it increased to 52% with 10 μM ATP, to 62% with 30 μM ATP and was not further increased (58%) with 100 μM ATP.

Although out of the scope of the present study, this increased accumulation of adenosine with increasing concentrations of ATP used as initial substrate is also suggestive of the existence of an high affinity but low capacity adenosine transport system. This does not invalidate the present conclusions based on the analysis of adenosine formation with different concentrations of initially added ATP since the rate of formation of adenosine from ecto-nucleotidases clearly overcomes the rate of removal of extracellular adenosine, as judged from the parallel lines shown in Fig. 3B.

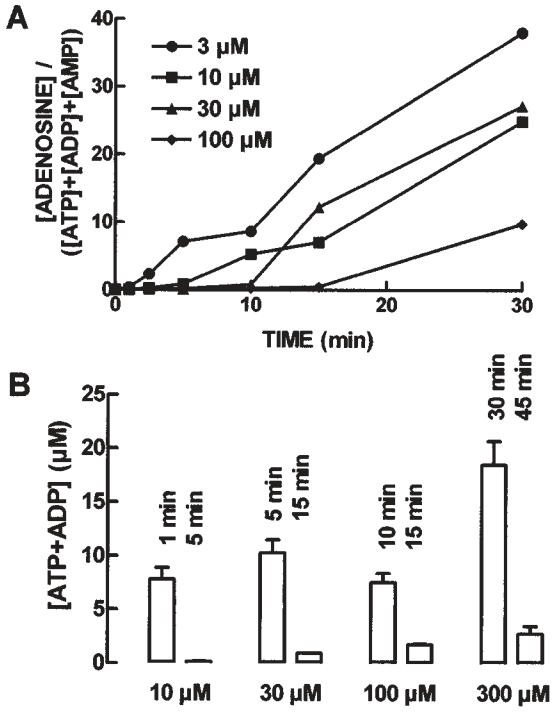

Fig. 3. Different pattern of ATP-derived adenosine formation with different concentrations of initially added ATP. In panel **A** is plotted the time-dependent change in the ratio of product/'substrates' of ecto-5′-nucleotidase during the time course kinetics of extracellular ATP (3 μM as ●; 10 μM as ■; 30 μM as ▲; and 100 μM as ◆) metabolism shown in Figure 1. Note that all the curves (except for 3 μM ATP) are biphasic, indicating that the rate of ATP-derived adenosine formation increases at certain time points during extracellular metabolism. In panel **B** are shown the concentrations of ATP+ADP at the time points before and after the change in the rate of ATP-derived adenosine formation shown in panel **A.** Note that there is a marked and near constant change in the extracellular concentration of ATP + ADP associated with the increase in the rate of ATP-derived adenosine formation.

Inhibition of Adenosine Formation with Increasing Concentrations of ATP in Hippocampal Nerve Terminals. One factor that may be responsible for this different pattern of adenosine formation with different concentrations of ATP used, is a feed-forward inhibition of ecto-5′-nucleotidase by ATP and/or ADP (54). To explore if there would be a threshold concentration of ATP and ADP below which the rate of adenosine formation would increase, the concentrations of ATP + ADP were determined at the time points before and after the change in the rate of adenosine formation occurred when using different concentrations of ATP as initial substrate. From the data shown in Fig. 3B, it is concluded that the concentration of ATP + ADP has to decrease below an average value of 5 μM to allow the bursting of extracellular adenosine formation.

Inhibition of Ecto-5′-Nucleotidase Activity by ATP and ADP in Hippocampal Nerve Terminals. In order to confirm the threshold concentration of ATP and of ADP above which ecto-5′-nucleotidase inhibition occurs, we conducted initial rate studies of ecto-5′-nucleotidase in rat hippocampal nerve terminals. Although there are apparently several isoforms of ecto-5′-nucleotidase responsible for the extracellular metabolism of AMP in rat hippocampal nerve terminals as judged from immunological studies (55), it is assumed that they all represent an ecto-5′-nucleotidase activity based on the inhibition by α,β-methylene ADP (34,35), possibly the only operational criteria to characterise an ecto-5′-nucleotidase activity (56). As shown in Fig. 4, AMP and IMP are metabolised equally well by hippocampal nerve terminals. The apparent kinetic parameters of extracellular AMP catabolism yield an apparent K_M of 10.9 μM (95% confidence interval: 6.2–15.7 μM, n = 6) and an apparent *V* of 0.91 ± 0.02 nmol/min/mg protein (n = 6) and these of extracellular IMP catabolism yield an apparent K_M of 14.4 μM (95% confidence interval: 8.6–20.2 μM, n = 6) and an apparent *V* of 0.93 ± 0.07 nmol/min/mg protein (n = 6). This similarity in the pattern of catabolism of IMP and AMP makes it reasonable to use IMP as a substrate to probe the pattern of ecto-5′-nucleotidase inhibition by ATP and ADP. This overcomes the problem of changing the substrate concentration when adding the inhibitors (ATP or ADP), due to the fast catabolism of ATP and

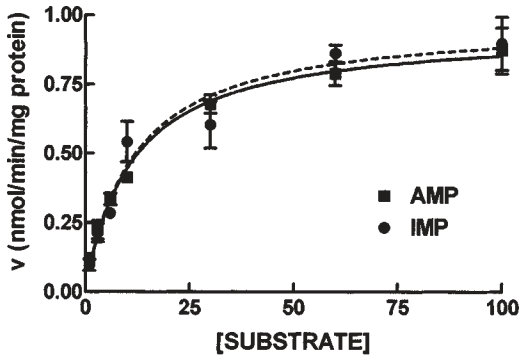

Fig. 4. Initial rate measurements of ecto-5′-nucleotidase activity in rat hippocampal nerve terminals using AMP (■, filled line) and IMP (●, dashed line) as substrates. AMP or IMP (0.3–100 μM) was added at zero time to rat hippocampal nerve terminals (47–78 μg protein) and samples were collected from the bath at 0, 0.5, 1 and 1.5 min to measure by HPLC the rate of disappearance of AMP or IMP. The data are mean ± sem of 6 experiments and the average data were adjusted by the Henri-Michaelis-Menten logistic equation. Note that AMP and IMP yield a nearly superimposed kinetic profile.

ADP in hippocampal nerve terminals. This rapid catabolism of ATP and ADP is also a problem for the determination of apparent inhibition constants, since the effective concentration of ATP and ADP will decrease sharply during the 1.5 min of the enzymatic assay. To minimise this problem, the nerve terminals were diluted 10-fold in the initial rate assays.

Fig. 5 illustrates the pattern of inhibition by ATP and ADP of extracellular IMP catabolism by rat hippocampal nerve terminals. The Dixon's plot illustrate in Fig. 5A and 5B indicate that ADP is a slightly more potent inhibitor of ecto-5′-nucleotidase [K_i = 4.3 μM (95% confidence interval: 1.7–6.8 μM), n = 5] than ATP [K_i = 14.1 μM (95% confidence interval: 7.8–20.5 μM), n = 5]. The corresponding Cornish-Bowden's plots shown in Figures 5C and 5D indicate that both ATP and ADP are competitive inhibitors of ecto-5′-nucleotidase in rat hippocampal nerve terminals,

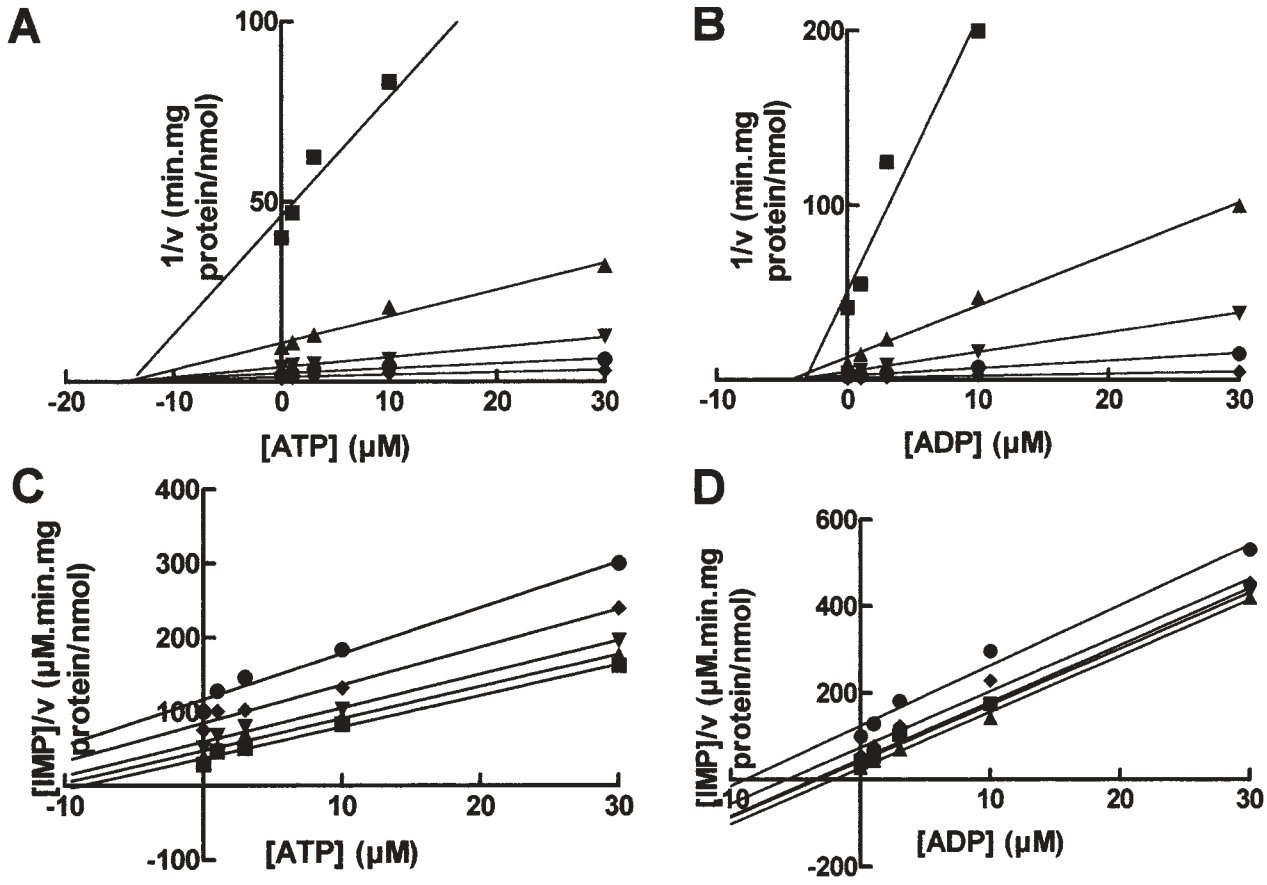

Fig. 5. Inhibition by ATP and ADP of ecto-5′-nucleotidase in rat hippocampal nerve terminals. IMP (1 μM as ■; 3 μM as ▲; 10 μM as ▼; 30 μM as ●; and 100 μM as ♦) was added at zero time to rat hippocampal nerve terminals (42–82 μg protein) in the absence or in the presence of either ATP or ADP (1–100 μM) and samples were collected from the bath at 0, 0.5, 1 and 1.5 min to measure by HPLC the rate of disappearance of IMP. Panels **A** and **B** show the Dixon's plots of inhibition of ecto-5′-nucleotidase by ATP **(A)** and ADP **(B),** indicating that ADP is slightly more potent than ATP to inhibit ecto-5′-nucleotidase. Panels **C** and **D** show Cornish-Bowden's plots that suggest a competitive pattern of inhibition of ecto-5′-nucleotidase by both ATP and ADP. The results are mean of 6 experiments.

given that the regression lines are nearly parallel (39). These findings are in agreement with these found for *Torpedo* nerve terminals where a competitive inhibition pattern by ATP and ADP of ecto-5′-nucleotidase was reported (57).

It should be noted that the determination of the kinetic parameters of ATP and ADP inhibition of ecto-5′-nucleotidase is subject to an error due to the metabolism of ATP and ADP during the determination of the initial rates of ecto-5′-nucleotidase. However, it can be excluded that the competitive inhibition of ecto-5′-nucleotidase by ATP and ADP might be due to AMP formation during the initial rate determinations, since the K_i of AMP as inhibitor of the extracellular catabolism of IMP is higher (K_i = 68–104 μM, n = 2) (data not shown).

Comparison of 'Ecto-ATPase', 'Ecto-ADPase' and Ecto-5′-Nucleotidase Specific Activities in Hippocampal Nerve Terminals. It is interesting to note that the values obtained for the K_i of ATP and ADP [14 μM and 4 μM, similar to the K_i values obtained for these compounds as ecto-5′-nucleotidase inhibitors, as reviewed in (56)] are in very good agreement with the threshold concentration of ATP and ADP required to relieve extracellular adenosine formation in rat hippocampal nerve terminals (5 μM). This suggests that a near 50% inhibition of ecto-5′-nucleotidase has a profound effect on the pattern of ATP-derived adenosine formation. This is only expectable if ecto-5′-nucleotidase is the enzyme with the lower activity in the pathway (i.e. 'rate-limiting step'). To test this prediction, the specific activity of the extracellular catabolism of ATP, ADP and AMP was compared in rat hippocampal nerve terminals. As illustrated in Table I the specific activity of 'ecto-ATPase' and 'ecto-ADPase' is more than 20–25 times higher than the specific activity of ecto-5′-nucleotidase (please keep in mind that this nomenclature does not imply the understanding of any particular molecular form of any of these enzymes). The data shown in Table I were obtained with initial concentration of ATP, ADP and AMP of 10 μM, but the relation between the specific activities of extracellular catabolism of ATP, ADP and AMP was observed at initial substrate concentrations of 3 μM and 100 μM (n = 3–6).

Table I. Comparison of the Specific Rate of Extracellular Catabolism of ATP, ADP, and AMP in Rat Hippocampal Nerve Terminals

	Specific activity (nmol/min/mg protein)
ATP hydrolysis (n = 3)	27.5 ± 0.9
ADP hydrolysis (n = 5)	18.4 ± 0.3
AMP hydrolysis (n = 5)	0.78 ± 0.05

ATP, ADP or AMP (10 μM) was added at zero time to rat hippocampal nerve terminals (87–145 μg protein) and samples were collected from the bath at 0, 0.5, 1, 1.5, 2, 3 and 5 min. Each collected sample was analysed by HPLC to separate and quantify ATP and its metabolites. The initial rates were calculated by fitting the initial decrease of concentration of the initially added substrate, and the specific activity was then normalised by the amount of protein in the assay. The presented specific activities are the mean ± sem of the n experiments.

The relation between ecto-nucleotidases activities appears to change in different preparations (reviewed in 40). Indeed, the ratio of extracellular catabolism of ATP:ADP:AMP was also high (from 40:30:1 to 60:20:10) in striatal cholinergic nerve terminals (24), cultured rat brain astrocytes (58) or in chromaffin cells (59), but was lower (from 4:3:1 to 2:1:1) in other preparations such as *Torpedo* nerve terminals (60) or rat cortical nerve terminals (61), Thus, it can be concluded that the relative density of each ecto-nucleotidase activity changes between tissues, as indicated by the nearly 2-fold spread in the specific activities of ecto-ATPases (33,45,61) and ecto-5′-nucleotidases (see references in 56) between preparations.

Extracellular ATP Catabolism in Hippocampal Cholinergic Nerve Terminals. It was previously described that ecto-5′-nucleotidase was enriched in hippocampal cholinergic nerve terminals (35). It was now tested if 'ecto-ATPase' was also enriched in hippocampal cholinergic nerve terminals by comparing the initial rate of extracellular catabolism of ATP, normalised by mg of protein, in whole hippocampal nerve terminals and in immunopurified cholinergic nerve terminals. The specific activity of extracellular catabolism of ATP (10 μM) in hippocampal cholinergic nerve terminals (measured during the initial 5 min of catabolism using 2.2–4.6 μg protein) was 146 ± 11 nmol/min/mg protein (n = 6), revealing a 5-fold enrichment when compared with the specific activity of extracellular catabolism of ATP (10 μM) in whole hippocampal nerve terminals (measured during the first 2.5 min of catabolism using 72–104 μg protein), which was 27.5 ± 0.9 nmol/min/mg protein (n = 3).

As was done with whole hippocampal nerve terminals, it was also tested if β,γ-imido ATP (100 μM) displayed a different efficiency to inhibit extracellular ATP and ADP catabolism in immunopurified cholinergic nerve terminals. As illustrated in Fig. 6, β,γ-imido ATP (100 μM) caused a similar inhibition of ATP and ADP catabolism (n = 3). Thus, in contrast to what was concluded in whole hippocampal nerve terminals (cf.

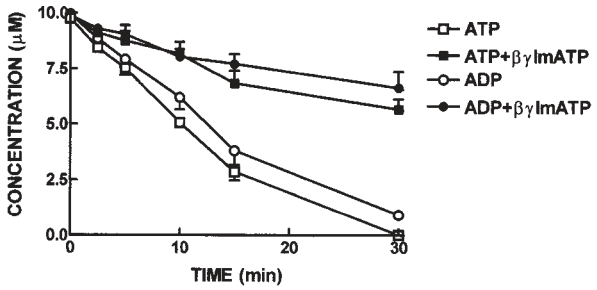

Fig. 6. Inhibition by β,γ-imido ATP of ATP and ADP extracellular catabolism in rat hippocampal cholinergic nerve terminals. ATP (10 μM; □,■) or ADP (10 μM; ○,●) was added at zero time to rat hippocampal nerve terminals (2.2–4.3 μg protein) in the absence (open symbols) or in the presence (filled symbols) of β,γ-imido ATP (β,γ-ImATP, 100 μM) and samples were collected from the bath at the times indicated in the abscissa. Each collected sample was analysed by ion-pair reverse-phase HPLC (Cunha et al., 1998) to quantify ATP and ADP. The data are mean ± sem of 3 experiments.

Fig. 2), it appears that in cholinergic nerve terminals the major enzymatic activity responsible for ATP catabolism might be an ecto-ATP diphosphohydrolase. It should also be added that β,γ-imido ATP (100 μM) was not catabolised, in a measurable manner, in cholinergic nerve terminals, indicating an apparent lack of an ecto-nucleotide pyrophosphatase activity.

Different Pattern of Adenosine Formation According to the Initial Concentration of ATP in Rat Hippocampal Cholinergic Nerve Terminals. In Fig. 7 is shown the time-course kinetic of purines metabolites identified upon addition of different initial concentrations of ATP (3 μM, 10 μM and 100 μM). This is one out of 4 similar experiments where the three concentrations of ATP were tested in the same batch of nerve terminals. It can be concluded from Fig. 7 that the pattern of ATP catabolism is qualitatively similar to that observed for whole hippocampal nerve terminals (cf. Fig. 1). However, there are quantitative differences mostly in relation to the pattern of AMP and adenosine formation. Thus, in contrast to whole hippocampal nerve terminals, in cholinergic nerve terminals, there is not an accumulation of AMP as the initial concentration of ATP is increased. The maximal peak of AMP concentration is reached at similar time points after addition of different initial concentrations of ATP (12.4 ± 0.7 min with 3 μM ATP, 12.8 ± 0.8 min with 10 μM ATP and 13.1 ± 0.9 min with 30 μM ATP). Also, the percentage of the maximal concentration of AMP in relation to the initial concentration of ATP is similar (28% with 3 μM ATP, 26% with 10 μM ATP and 21% with 30 μM ATP).

In relation to adenosine formation, the data shown in Fig. 7 clearly indicate the lack of appearance of a

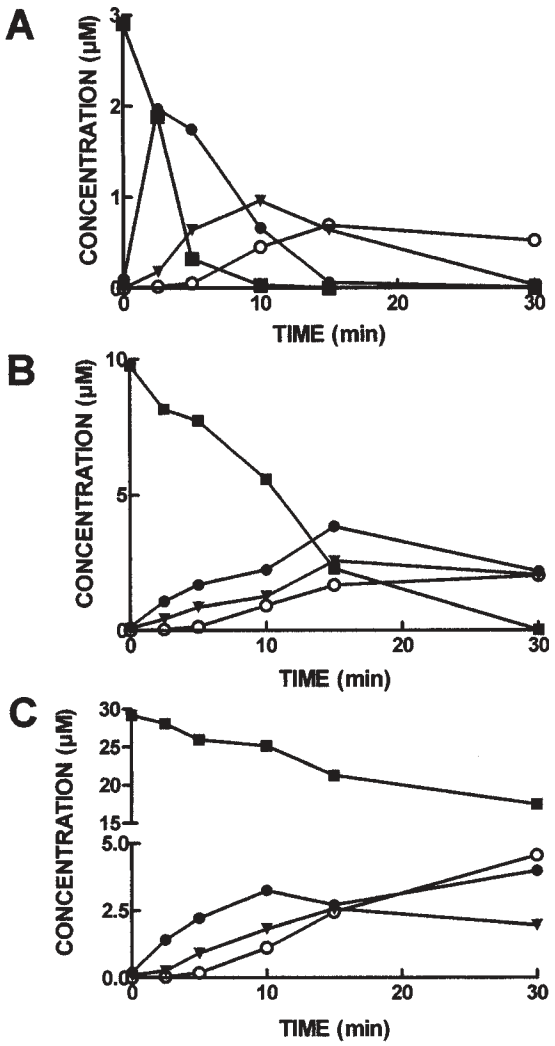

Fig. 7. Different time course kinetic pattern of ATP metabolism in rat hippocampal cholinergic nerve terminals with different concentrations of extracellularly added ATP. ATP (3 μM in **A;** 10 μM in **B;** and 30 μM in **C**) was added at zero time to rat hippocampal immunopurified cholinergic nerve terminals (2.8 μg protein) and samples were collected from the bath at the times indicated in the abscissa. Each collected sample was analysed by HPLC to separate and quantify ATP (■) and its metabolites, ADP (●), AMP (▼) and adenosine (○). The data were obtained from a single experiment representative of 4 similar experiments.

delayed burst-like formation of adenosine as the initial concentration of ATP is increased. The rate of formation of extracellular adenosine is similar throughout the time course kinetic experiments, irrespective of the initial concentration of added ATP. This maintenance of the patterns of AMP and adenosine formation at different concentrations of initially added ATP in cholinergic nerve terminals, in contrast to what occurred in whole nerve terminals, cannot be attributed to the use

of different amounts of protein in each of the assays. In time course kinetic studies, changing the amount of protein in the assay only changes the temporal pattern of variation of the pathway metabolites, not the relation between the pattern of formation between each of them. Thus, this maintenance of AMP and adenosine formation at different concentrations of initially added ATP is indicative of a lack of effective feed-forward inhibition of ecto-5′-nucleotidase by ATP and ADP in cholinergic nerve terminals.

This could possibly be a consequence of the much higher relative enrichment in ecto-5′-nucleotidase (42-fold) than of 'ecto-ATPase' (5-fold) when immunopurifying the cholinergic nerve terminals, so that it would require a much more profound inhibition of ecto-5′-nucleotidase to be able to make this step rate-limiting in the ecto-nucleotidase cascade. Alternatively, one can argue that there is a channelling organization between ATP/ADP catabolism and ecto-5′-nucleotidase. This would match the observation that the extracellular concentration of AMP in the bio-phase does not increase, as would be predicted based on the amounts of ADP metabolised, and there is a proportionally larger formation of the product of the channelling process, adenosine. This possible channelling organisation would be in accordance with the existence of a feed-forward inhibition of ecto-5′-nucleotidase by ATP and ADP that would not have an appreciable impact on the flow through the entire pathway.

CONCLUDING REMARKS

The data presented in this work indicate that the regulation of adenosine production from released ATP is different in different nerve terminals populations. Indeed, in whole hippocampal nerve terminals, there is a dominant feed-forward inhibition of ecto-5′-nucleotidase that initially decreases the efficiency of adenosine formation, but, once the levels of ATP + ADP decrease below the threshold of inhibition of ecto-5′-nucleotidase, there is a delayed burst-like formation of adenosine. In contrast, in hippocampal cholinergic nerve terminals, the preferential delivery of AMP to ecto-5′-nucleotidase outweighs feed-forward inhibition of ecto-5′-nucleotidase and the net effect is a rapid and near-linear formation of adenosine irrespective of the extracellular concentration of ATP.

This pattern of extracellular ATP-derived adenosine formation might be dependent on the balance between the two modes of control of ecto-5′-nucleotidase activity: feed-forward inhibition by ATP/ADP and channelling of AMP to ecto-5′-nucleotidase. Feed-forward inhibition of ecto-5′-nucleotidase allows to relate the pattern of adenosine formation to the amounts of released ATP, and the amounts of released ATP in glutamatergic synapses of the hippocampus are exponentially related to the frequency of firing (5,6). Thus, when low amounts of ATP are released, one expects the rapid activation of A_1 receptors to refrain excessive presynaptic facilitation (34,62,63). However, high frequencies of stimulation of hippocampal glutamatergic circuits trigger synaptic plasticity phenomena (64). In these high-frequency stimulation conditions, extracellular adenosine is mostly formed by the ecto-nucleotidase pathway from the massive amounts of released ATP (5,34). The levels of adenosine reached extracellularly, if directed to A_1 receptor activation, would be enough to nearly block synaptic transmission (34), and adenosine would fulfil a role opposite to that required in a situation of activity-dependent facilitation. It is precisely in this situation that the feed-forward inhibition of ecto-5′-nucleotidase by the high amounts of ATP released will cause a delay in adenosine formation thus overcoming immediate A_1 receptor activation. Furthermore, the delayed formation of large amounts of adenosine may favour A_{2A} receptor activation (19). Thus, adenosine would be fulfilling a role in accordance with the physiological needs, i.e. to contribute for facilitation of synaptic transmission, possibly through an A_{2A} receptor-induced A_1 receptor desensitisation (65). As previously discussed, the effect of changing the amount of enzyme in a time course kinetic assay is to distend or contract the x-axis (66). In synapses, ATP may be released in high concentrations (see 18) mostly because of the small volume of the synaptic cleft. But this released ATP faces high densities of ecto-nucleotidases (the same enzymatic densities as these measured in the 600 μl assays in the present study). Thus, it is expected that the burst-like formation of adenosine may occur much faster than observed in the present in vitro assays, making this controlled ATP-derived extracellular adenosine potentially more relevant for synaptic plasticity phenomena that occur in a millisecond rather than in a minute scale.

The situation is different in hippocampal cholinergic nerve terminals where preferential AMP delivery to ecto-5′-nucleotidase causes a near-linear formation of ATP-derived adenosine. In such conditions, one does not expect a delay in adenosine formation, nor a burst-like formation of adenosine. Thus, the kinetic characteristics of the ecto-nucleotidase pathway would mainly conduct to an A_1 receptor-mediated inhibition of acetylcholine release over a wide range of frequency stimulation. This

might not be inadequate in this preparation, since I am not aware of long-term plasticity in cholinergic synapses, only short-term plasticity phenomena (67). Interestingly, it is precisely in cholinergic synapses that inhibition of ecto-5′-nucleotidase blunts tonic A_1 rather than A_{2A} receptor activation (29,31,32). And, according to Redman and Silinsky (31), the channelling properties of the ecto-nucleotidase pathway at the level of ecto-5′-nucleotidase would be ideally suited to relate the activity-dependent ATP release (3) with a short-term frequency-dependent depression of neuromuscular transmission (Wedinski's inhibition). Acetylcholine release from hippocampal nerve terminals is controlled both by A_1 and A_{2A} receptors (68), and endogenous adenosine can activate both A_1 and A_{2A} receptors (69,70). It is evident that, in contrast to glutamatergic synapses, the ecto-nucleotidase pathway that equips cholinergic nerve terminals is not endowed with kinetic characteristics to select A_{2A} versus A_1 receptor activation. Other, still undetermined factors may be responsible for determining A_1/A_{2A} receptor-mediated control of acetylcholine release. In this respect, an elegant study has shown that A_{2A} receptor facilitation of acetylcholine release from hippocampal slices occur at endogenous extracellular concentrations of adenosine lower than these required to activate A_1 receptors (70), indicating that the intrinsic efficiency of A_1/A_{2A} receptors is different in cholinergic and glutamatergic synapses.

In summary, the present work provides evidences for different patterns of regulation of extracellular ATP-derived adenosine formation that provide a logical candidate explanation for the different roles of ATP-derived adenosine in the control of glutamatergic and cholinergic synapses in the hippocampus. However, it should be stressed that this study was only carried in nerve terminal preparations and one needs to know the relative contribution for extracellular adenosine formation of postsynaptic components and of glial cells to understand the relation between adenosine formation and neuromodulation at a synapse. Also, the example of hippocampal cholinergic nerve terminals makes it clear that the characteristics of the ecto-nucleotidase pathway are only one of the variables involved in the control of adenosine modulation of neurotransmitter release, as previously discussed (14).

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