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Homo- and heteroexchange of adenine nucleotides and nucleosides in rat hippocampal slices by the nucleoside transport system

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1 Here, we investigated how nucleotides and nucleosides affect the release of tritiated purines and endogenous adenosine 5′-triphosphate (ATP) from superfused rat hippocampal slices.

2 ATP elicited concentration-dependent [^{3}H]purine efflux from slices preloaded with [^{3}H]adenosine. High-performance liquid chromatography analysis of the effluent showed that the tritium label represented the whole set of adenine nucleotides and nucleosides, and ATP significantly increased the outflow of [^{3}H]ATP.

3 Adenosine 5′-diphosphate, adenosine, uridine, uridine 5′-triphosphate, α,β-methylene-ATP and 3′-*O*-(4-benzoylbenzoyl)-ATP were also active in eliciting [^{3}H]purine release. Adenosine (300 μM) also evoked endogenous ATP efflux from the hippocampal slices.

4 Reverse transcription-coupled-polymerase chain reaction analysis revealed that mRNAs encoding a variety of P2X and P2Y receptor proteins are expressed in the rat hippocampus. Nevertheless, neither P2 receptor (i.e. pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid, 30 μM, suramin, 300 μM and reactive blue 2, 10 μM), nor adenosine receptor (8-cyclopentyl-1,3-dipropylxanthine, 250 nM and dimethyl-1-propargylxanthine, 250 nM) antagonists modified the effect of ATP (300 μM) to evoke [^{3}H]purine release.

5 The nucleoside transport inhibitors, dipyridamole (10 μM), nitrobenzylthioinosine (10 μM) and adenosine deaminase (2 – 10 U ml^{-1}), but not the ecto-adenylate kinase inhibitor diadenosine pentaphosphate (200 μM) significantly reduced ATP-evoked [^{3}H]purine efflux.

6 In summary, we found that ATP and other nucleotides and nucleosides promote the release of one another and themselves by the nucleoside transport system. This action could have relevance during physiological and pathological elevation of extracellular purine levels high enough to reverse the nucleoside transporter.

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Abbreviations: ADP, Adenosine 5′-diphosphate; AMP, Adenosine 5′-monophosphate; AP-5, D(-)-2-amino-5-phosphonopentanoic acid; Ap_5A, diadenosine pentaphosphate; ATP, Adenosine 5′-triphosphate; BzATP, 3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione-disodium; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EFS, electrical field stimulation; α,β-methylene-ATP, α,β-methylene-adenosine triphosphate; NBTI, nitrobenzylthioinosine; PPADS, pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid; RB2, reactive blue 2; RT – PCR, reverse transcription-coupled-polymerase chain reaction; TTX, tetrodotoxin

Introduction

Adenosine 5′-triphosphate (ATP) is an important extracellular messenger in the hippocampus. In previous studies, we (Cunha *et al*., 1996) and others (Wieraszko *et al*., 1989) have demonstrated the stimulation-dependent release of endogenous ATP from *in vitro* hippocampal slices upon low- and high-frequency electrical stimulation. Extracellular ATP – activating P2X receptors located in the postsynaptic sites (Rubio & Soto, 2001) – takes part in the excitatory transmission and synaptic plasticity in CA1 (Pankratov *et al*., 1998, 2002) and CA3 regions (Mori *et al*., 2001) and regulates the release of glutamate and GABA *via* $P2X_7$ receptors expressed on the presynaptic sites (Sperlágh *et al*., 2002). Extraneuronal ATP is inactivated by the ectonucleotidase enzyme cascade which in turn gives rise to the formation of extracellular adenosine acting as a neuroprotective modulator, inhibiting neuronal discharge and modulating the release of various transmitters *via* A_1 and A_{2A} receptors respectively (Cunha *et al*., 1994; Cunha & Ribeiro, 2000; Cunha, 2001a; Masino *et al*., 2002). In line with this concept, the frequency-dependent contribution of ATP to extracellular adenosine levels (Cunha *et al*., 1996) and the rapid and highly effective *in situ* formation of adenosine from pressure-ejected ATP has been shown in earlier studies (Dunwiddie *et al*., 1997). Moreover, it has been proposed that even the metabolically stable γ-phosphorus ATP analogues undergo localized catabolism to adenosine and a 'channeling'

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mechanism exists which directs nucleotide-derived adenosine to the vicinity of A_1-adenosine receptors (Cunha *et al*., 1998). The final step of this inactivation process is the uptake of adenosine into nerve terminals *via* ENT1 equilibrative nucleoside carrier which is driven by the concentration gradient of adenosine, and clearing up the extracellular space from biologically active purines. On the other hand, the involvement of the same transporter has been implicated in the release process of adenosine under different conditions, especially under energy deprivation (cf. Lloyd *et al*., 1993; Latini & Pedata, 2001); when metabolic stores are depleted, the intracellular free adenosine concentration is increased, which could reverse the direction of the transporter and elicit a massive release of adenosine into the extracellular space. The homo- or heteroexchange is another well-known mechanism, whereby a transmitter could reverse the direction of the transporter dedicated to its uptake and elicit an additional release of itself or another transmitter. This phenomenon has been described and characterized for other transmitters bearing specific transport systems, such as glutamate, GABA (Schwartz, 1982; Bernath & Zigmond, 1988; Wu *et al*., 2001) and monoamines (Vizi *et al*., 1985, 1986) and has been shown to be an important mechanism during pathological conditions and in the effect of a number of drugs acting at central targets (Vizi, 2000). Nevertheless, the possibility that ATP or adenosine could utilize such kind of mechanism and interact at the level of the release process has not been directly investigated in the central nervous system.

Here, we show that ATP and other purines, including adenosine, each promote the release of the other and themselves by homo- and/or heteroexchange *via* the nucleoside carrier. Additional purine outflow by this mechanism may contribute to extracellular purine levels during various physiological and pathological stimuli causing sustained elevation of extraneuronal adenosine levels sufficiently high to trigger the reversal of the ENT1 transporter.

Methods

All studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and use of Laboratory animals and were approved by the local Animal Care Committee of the Institute of Experimental Medicine (Budapest, Hungary).

[^{3}H]purine release experiments

[^{3}H]purine release experiments were carried out according to the technique described in our previous studies (Juranyi *et al*., 1999; Sperlagh *et al*., 2000). Male Wistar rats (140 – 160 g, Gedeon Richter Ltd, Budapest, Hungary) were decapitated under light CO_2 anesthesia and the brain was quickly put into ice-cold Krebs' solution oxygenated with 95% O_2 and 5% CO_2. Both hippocampi were rapidly dissected and 400-μm-thick slices were cut transversely with a McIwain tissue chopper and were loaded with [2-^{3}H]adenosine (0.37 MBq ml^{-1}, 1.55 TBq mmol^{-1}; Amersham International plc, Little Chalfont, U.K.) for 45 min at 37°C in 1 ml modified Krebs' solution (in mmol l^{-1}: NaCl 113; KCl 4.7; $CaCl_2$ 2.5; KH_2PO_4 1.2; $MgSO_4$ 1.2; $NaHCO_3$ 25; glucose 11.5, pH 7.4) containing ascorbic acid (30 μM) and Na_2EDTA (100 μM) gassed with 95% O_2 and 5% CO_2. After loading, the slices were rinsed three times with 6 ml Krebs' solution, transferred to polypropylene tissue chambers, and superfused continuously with 95% O_2- and 5% CO_2-saturated Krebs' solution (37°C, flow rate: 0.7 ml min^{-1}) for 60 min. The effluent was collected in 3-min fractions during the subsequent experimental period. Preparations were subjected to (1) electrical field stimulation (EFS1, EFS2) with the following parameters: 25 V, 2.5 ms, 10 Hz, 360 shocks or (2) 3-min perfusion of agonists (ATP, adenosine 5′-disphosphate (ADP), adenosine, uridine-5′-disphosphate (UDP), uridine-5′-triphosphate (UTP), 3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate (BzATP), α,β-methylene-adenosine triphosphate (α,β-methylene-ATP), uridine). (3) In some experiments, a continuous ATP perfusion was applied with or without electrical stimulation. Drugs and treatments (pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS)), suramin, reactive blue 2 (RB2), 3,7-dimethyl-1-propargylxanthine (DMPX), 6-cyano-7-nitroquinoxaline-2,3-dione-disodium (CNQX), D(-)-2-amino-5-phosphonopentanoic acid (AP-5), tetrodotoxin (TTX), dipyridamole, nitrobenzylthioinosine (NBTI), diadenosine pentaphosphate (Ap_5A), adenosine deaminase (ADA), verapamil, glibenclamide) were applied 10 min before the sample collection period and continuously thereafter except Ca^{2+}-free Krebs' solution supplemented with 1 mM EDTA and Na^+-free Krebs' solution supplemented with equimolar choline chloride, which were applied 60 min before the start of the sample collection period and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) which was preperfused 45 min before ATP application. At the end of the experiments, tissues were homogenized in 0.5 ml of 10% trichloroacetic acid for 30 min. Aliquots (500 μl of the superfusate and 100 μl of the tissue supernatant) were added to 2 ml of scintillation cocktail. The released [^{3}H]purines were counted by a liquid scintillation counter (Packard Tricarb 1900) and the radioactivity was expressed as Bq g^{-1} or as a percentage of the amount of radioactivity in the tissue at the sample collection time (fractional release).

The amount of [^{3}H]purine outflow, released by EFS or agonists (evoked release) was calculated by the area-under-the-curve (AUC) method, that is, by the subtraction of the basal tritium outflow from the outflow of [^{3}H]purines, measured in the effluent after stimulation.

High-performance liquid chromatography (HPLC) analysis

High-performance liquid chromatography (HPLC) separations were performed on a 3 μm Supelcosil-C18-AT (150 × 4.0 mm) analytical column with gradient working mode at ambient temperature, as previously described (Jurányi *et al*., 1999). The mobile phase A was 50 mM potassium phosphate buffer (pH 5.55), the B component was methanol; the gradient profile was linear to 25% (v/v) of B for 45 min time of analysis. The effluent was monitored with a UV detector at 254-nm wavelength. Samples (1.2 ml) were enriched and injected on a guard column in stripping mode. Radioactivity was determined by liquid scintillation counting. The amount of [^{3}H]adenosine triphosphate ([^{3}H]ATP), [^{3}H]adenosine diphosphate ([^{3}H]ADP), [^{3}H]adenosine monophosphate ([^{3}H]AMP), [^{3}H]adenosine, [^{3}H]inosine, [^{3}H]hypoxanthine and [^{3}H]xanthine in the [^{3}H]label was calculated from the total outflow of [^{3}H]purines expressed in dpm.

ATP determination by luciferin–luciferase assay

ATP released from the tissues was assayed using the luciferin–luciferase technique, as previously described (Sperlagh *et al.*, 1995). Briefly, 100-μl aliquots of the samples were added to 40 μl of ATP assay solution (ATP monitoring reagent, BioOrbit, Turku, reconstituted in 5 ml of sterile water) and the luminescence was measured with a BioOrbit Luminova luminometer (BioOrbit, Turku, Finland) for 5 s. Before the experiments, a standard calibration curve was prepared from different concentrations of ATP ranging from 10^{-7} to 10^{-11} M. A linear correlation was observed between the light emission and the ATP concentration ($r = 0.997$). The actual ATP levels in the samples were calculated by a double log curve-fitting program and expressed in pmol.g^{-1} wet weight. The Krebs' solution, with or without added drugs, was assayed for background ATP level. The agonist-evoked ATP release (S1) was calculated by subtracting the resting release, measured during the prestimulation period, from the release during, and after drug application using the area-under-the-curve (AUC) method.

Reverse transcription-coupled-polymerase chain reaction (RT–PCR) amplification of different P2X and P2Y receptor mRNAs

Rat hippocampi were collected in liquid nitrogen. Total RNA from tissue samples was extracted with TRIZOL Reagent according to the protocol provided by the supplier (Life Technologies, Rockville, MD, U.S.A.). DNA contamination of RNA preparations was eliminated by treatment with RNase-free DNase (Promega, Madison, WI, U.S.A.) as described in Current Protocols (4.14), followed by phenol–chloroform extraction and ethanol precipitation. RNA (1 μg) was reverse-transcribed with SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technologies, Rockville, MD, U.S.A.) using random hexamer primers. Aliquots of the first-strand cDNA template were amplified using 0.4 μM forward and reverse primers and 2 U of Taq DNA Polymerase (Promega, Madison, WI, U.S.A.). Primers for amplification of different forms of p2x and p2y receptor cDNAs are summarized in Table 1.

The conditions for amplification were as follows: initial denaturation at 95°C 5′, hot start at 80°C then 94°C 1′, 59°C 1′ and 72°C 1′ for 40 cycles, with a final extension at 72°C 5′. PCR products were analysed by agarose gel electrophoresis. To verify the identity of the amplified PCR products, DNA fragments were subcloned into PGEM T-Easy cloning vector (Promega, Madison, WI, U.S.A.) followed by cycle sequencing.

Materials

The following chemicals were used: [^{3}H]adenosine, (Amersham, Little Chalfont, U.K.) ATP, ADP, adenosine 5′-

Table 1 Primer sets for RT–PCR amplification of different P2X and P2Y purinoceptors

	GenBank accession	*Position*	*Sequence*	*Fragment size* (bp)
p2×1	NM_012997	F248 R730	5′-CTT TGA ATA TGA CAC TCC CCG-3′ 5′-GGG ATC TTG TCA TCC ACC TC-3′	482
P2×2	U14414	F79 R502	5′-GAC TAC GAG ACG CCT AAG GTG-3′ 5′-TGG AGT CCC CAT GGT AAT AGG-3′	423[a]
p2×3	NM_031075	F220 R843	5′-CAA GTC GGT GGT TGT GAA GAG-3′ 5′-ACA TCC CCT ACC CTC AAG ATG-3′	623
p2×4	X91200	F288 R888	5′-TCC AGC TCA GGA GGA AAA CTC-3′ 5′-GCC AGG AGA CAC ATT GTG TTC-3′	600
p2×5	X92069	F668 R1126	5′-ACC TGT GAG ATC TTT GCT TGG-3′ 5′-TTT CAT CAG GTC ACG GAA CTC-3′	462
p2×6	X97376	F451 R1078	5′-TCC TGG TAA CCA ACT TCC TTG-3′ 5′-CGG ATC CCA TAG ACT TTG AGC-3′	627
p2×7	NM_019256	F2649 R3248	5′-CTG AAA CTG CAG GAG ACG AAC-3′ 5′-TGT GCT CTT ACC CAC TGA ACC-3′	599
p2y1	U22830	F1226 R1622	5′-TGT CAC CTG CTA CGA CTC C-3′ 5′-GTC TCC TTC TGA ATG TAT CTC C-3′	404
p2y2	NM_017255	F922 R1553	5′-GCC CGC TAT GCC CGC CG-3′ 5′-GAG TCA TCA CTG CTG ATC GAC-3′	631
p2y4	NM_031680	F719 R1116	5′-GAC TGT ATC GAC CTT TGC CAG-3′ 5′-AGA GAA GGT GCT GTC TTG GTG-3′	397
p2y6	D63665	F654 R1175	5′-ACC TGC TGT ATG CCT GTT CAC-3′ 5′-CTG CTA CCA CGA CAG CCA TAC-3′	519
β-actin	X03765	F531 R1029	5′-AGC TGA GAG GGA AAT CGT GC-3′ 5′-GAT GGA GGG GCC GGA CTC AT-3′	499

[a]More than one splice variant, the theoretically smallest fragment indicated.

monophosphate (AMP), adenosine, ADA, dipyridamole, inosine, NBTI, TTX, α,β-methylene-ATP, BzATP, Ap_5A, UDP, UTP (all from Sigma, St Louis, MO, U.S.A.), AP-5, CNQX, DPCPX, DMPX, glibenclamide, PPADS, verapamil (RBI, Natick, MA, U.S.A.), suramin (Bayer, Leverkusen, Germany) and RB2 (Aldrich, Steinheim, Germany). All solutions were freshly prepared on the day of use.

Statistics

All data were expressed as means ± s.e.m. of n observations. The statistical analysis was made by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test (multiple comparisons), or Student's t-test (pairwise comparisons). P values less than 0.05 were considered statistically significant.

Results

Effect of EFS and ATP on the release of [^{3}H]purines

The radioactivity uptake of the tissue slices after 45 min preincubation with [^{3}H]adenosine and 60 min preperfusion was $8.031 \pm 0.93 \times 10^5$ Bq g^{-1}. The tritium label taken up by the tissue contained [^{3}H]ATP, [^{3}H]ADP, [^{3}H]AMP, [^{3}H]adenosine, [^{3}H]inosine and [^{3}H]hypoxanthine, and its majority was in the form of nucleotides indicating that tritiated adenosine was readily incorporated into tissue nucleotide pools (Table 2). At the beginning of the sample collection period, the basal tritium efflux measured in a 3 min sample was 0.34 ± 0.03% ($n = 12$) of tissue tritium content, which remained relatively constant during the subsequent sample collections. EFS (EFS1, 25 V, 10 Hz, 360 shocks) increased the outflow of [^{3}H]purines: the

Table 2 Composition of the radioactivity in the hippocampal slices after preincubation with [^{3}H]adenosine ($n = 4$)

	[^{3}H]adenosine (%)
[^{3}H]ATP	62.61 ± 3.448
[^{3}H]ADP	13.075 ± 0.875
[^{3}H]AMP	9.565 ± 2.333
[^{3}H]Adenosine	6.325 ± 1.094
[^{3}H]Inosine	7.080 ± 0.810
[^{3}H]Hypoxanthine	2.169 ± 0.787

The radioactivity content of the tissue supernatant was analysed by HPLC-UV technique after 45 min incubation with [^{3}H]adenosine and 60 min preperfusion with Krebs' solution. Then the tissues were homogenized in 0.5 ml of 10% trichloroacetic acid for 30 min and kept at −20°C until analysis. The results are expressed as a percentage of the total radioactivity reincorporated in the tissue.

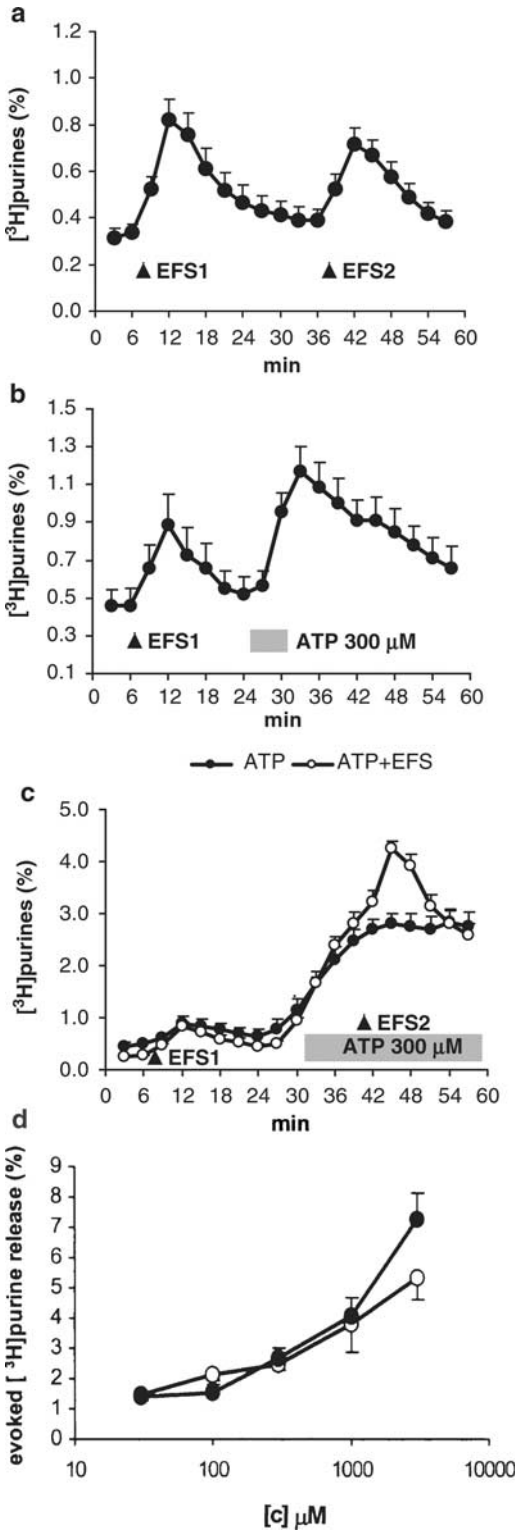

Figure 1 EFS- (a – c) and nucleotide-induced (b – d) release of [^{3}H]purines from rat hippocampal slices. Slices were superfused with Krebs' solution for 60 min and then subjected to EFS (a, EFS1 and EFS2; 25 V, 10 Hz, 2.5 ms, 360 shocks) or to a 3-min (b,d) or continuous (c) ATP or ADP (300 μM) perfusion, as indicated. The tritium content of the perfusate samples was measured by liquid scintillation spectrometry and was expressed as fractional release (%, for calculation see Methods), as a function of time. (c) Continuous ATP perfusion (filled circles) elicited an even higher increase in tritium efflux than the maximal increase detected in the case of 3-min exposure of the same concentration. When electrical stimulation and continuous ATP perfusion were applied together (open circles), their effect was clearly additive. (d) Concentration dependence of the effect of ATP (filled circles) and ADP (open circles) to induce [^{3}H]purine efflux. Hippocampal slices were superfused and subjected to ATP or ADP application in different concentrations indicated on the abscissa ranging from 30 μM to 3 mM according to the experimental protocol shown in (b). The net tritium release evoked by the nucleotides was calculated by the AUC method and expressed in fractional release (%). Data represent the means ± s.e.m. of four to eight identical experiments.

electrical stimulation-evoked release was 1.23±0.16% ($n=12$) in control experiments (Figure 1a). The second stimulation period (EFS2) gave rise to a smaller increase in the tritium outflow (0.96±0.17%, $n=12$), resulting in an EFS2/EFS1 ratio of 0.75±0.13 ($n=12$). The majority of stimulation-evoked [^{3}H]purine efflux was inhibited by the sodium channel inhibitor TTX (1 μM), and was largely extracellular Ca^{2+}-dependent (see Figure 6a).

Superfusion of the preparations with ATP for 3 min (300 μM) caused a transient increase in the efflux of [^{3}H]purines (Figure 1b) which peaked 6 min after ATP administration and then gradually declined and returned to the baseline level in the following 30 min. The net release evoked by ATP (300 μM), which was 2.66±0.36% (Figure 1b, $n=12$), was comparable to that evoked by field stimulation and was concentration-dependent between 0.03 and 3 mM (Figure 1d). Within this range, a clear maximal response was not obtained and therefore EC_{50} values could not be calculated. When the slices were exposed to a continuous ATP perfusion, the tritium efflux was permanently increased to even a higher level, than the maximal increase in case of 3 min exposure of the same concentration of ATP (300 μM) (Figure 1c). The effect of EFS and ATP (300 μM) was clearly additive under this condition (Figure 1c).

In order to explore the composition of [^{3}H]purines released by electrical stimulation and ATP, HPLC analysis of the tritium label was performed in the samples collected under resting conditions and during the peak of EFS- and ATP (300 μM)-evoked [^{3}H]purine outflow. The effluent in all samples contained [^{3}H]ATP, [^{3}H]ADP, [^{3}H]AMP, [^{3}H]adenosine, [^{3}H]inosine, [^{3}H]xanthine and [^{3}H]hypoxanthine (Figure 2a). The amount of [^{3}H]ATP significantly increased in the samples collected during the peak of electrical stimulation- and ATP-evoked increase in total tritium efflux indicating a net release of ATP in response to these stimuli (Figure 2a). When the individual components of the tritium efflux were expressed as the percentage of total tritium label, no significant difference was detected in the composition of the effluent collected under resting, EFS-and ATP-stimulated conditions indicating that different stimuli (EFS, ATP) release [^{3}H]purines from a homogenous pool (Figure 2b). To show whether an ecto-adenylate kinase activity is involved in the effect of ATP to increase the amount of [^{3}H]purines in the effluent, the ecto-adenylate kinase inhibitor AP_5A (200 μM, Yegutkin *et al*., 2002) was used. The basal purine efflux was significantly higher in the presence of AP_5A (1.52±0.14%, $n=4$, $P<0.01$) indicating that it in itself elicited tritiated purine outflow, but it did not inhibit [^{3}H]purine efflux evoked by ATP (300 μM); the ATP-evoked [^{3}H]purine efflux was 2.55±0.66% in the presence of AP_5A ($n=4$, $P>0.05$).

Involvement of purine receptors in the effect of ATP

The capability of other nucleotides and nucleosides to induce [^{3}H]purine release from hippocampal slices was also examined and compared to the effect elicited by an identical concentration of ATP (300 μM) (Figure 3). Among naturally occurring purines, ADP and adenosine were similarly effective to ATP at inducing [^{3}H]purine efflux, whereas pyrimidine nucleotides and nucleosides, such as UTP and UDP and uridine were less efficient (Figures 1d and 3). The metabolically stable analogue of ATP (α,β-methylene-ATP) and benzoylbenzoylATP appeared to be less effective than ATP at inducing [^{3}H]purine efflux (Figure 3).

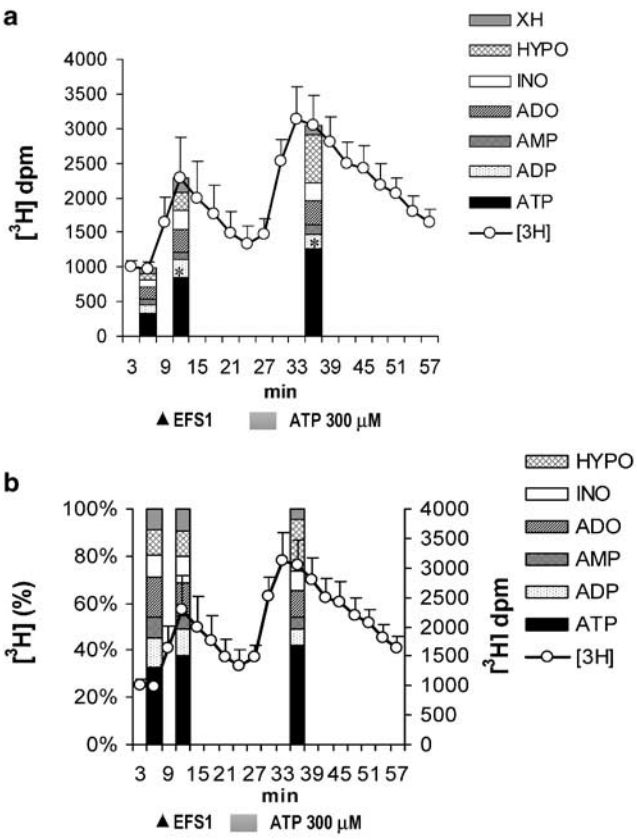

Figure 2 The composition of the tritium label in the effluent collected under basal conditions, and during the peak of EFS (EFS1) and after ATP (300 μM) application. Experiments were performed according to the protocol shown in Figure 1b and total tritium content in the samples is illustrated by open circles. The tritium content of selected samples was analysed by the HPLC-UV technique and expressed in dpm (a) or as a percentage of the total tritium content of the sample (b) shown on vertical bars. The following compounds were found in the tritium label: [^{3}H]ATP (ATP), [^{3}H]ADP (ADP), [^{3}H]AMP (AMP), [^{3}H]adenosine (ADO), [^{3}H]inosine (INO), [^{3}H]hypoxanthine (HYPO) and [^{3}H]xanthine (XH). Note that the amount of [^{3}H]ATP is significantly increased in response to electrical stimulation and ATP. Data represent the means±s.e.m. of four to eight identical experiments. Asterisks indicate significant differences from values measured in the basal effluent (*$P<0.05$).

The effect of adenosine on the efflux of endogenous ATP from rat hippocampal slices was also examined by the luciferin–luciferase assay. Using an identical experimental protocol to that applied in [^{3}H]purine release experiments, 3-min perfusion of adenosine (300 μM) released a substantial amount of endogenous ATP, determined by the luciferin–luciferase assay (Figure 4); the net release evoked by 300 μM adenosine was 10.3±1.67 pmol g^{-1} ($n=12$).

ATP might act on different subtypes of transmembrane P2 receptors, or, after sequential breakdown to adenosine, on adenosine receptors to elicit purine release. Whereas the expression of A_1 and A_{2A} adenosine receptors is well established in the hippocampus, the available data on the expression of P2 receptor subtypes in the hippocampus are limited. Therefore, to examine the involvement of various subtypes of purine receptors in the effect of ATP, mRNA expression of P2X and P2Y receptor subtypes was studied

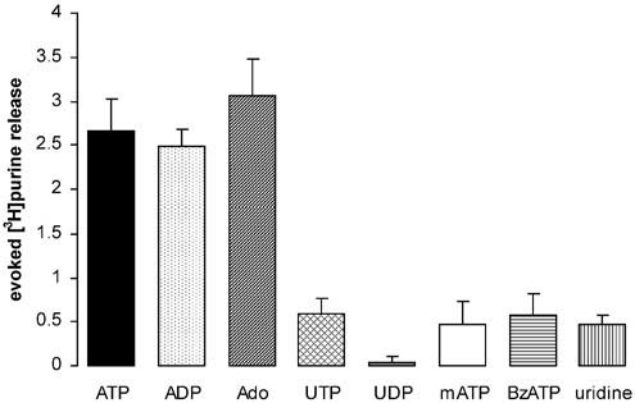

Figure 3 Effect of different nucleotides and nucleosides on the release of [^{3}H]purines from hippocampal slices. Tissues were superfused and subjected to 300 μM ATP, ADP, adenosine (Ado), UTP, UDP, α,β-methylene-ATP (mATP), benzoylbenzoylATP (BzATP) or uridine application according to the protocol shown in Figure 1b. The net tritium release evoked by drugs was calculated by the AUC method and expressed in fractional release (%). Data represent the means $\pm$ s.e.m. of six to eight identical experiments.

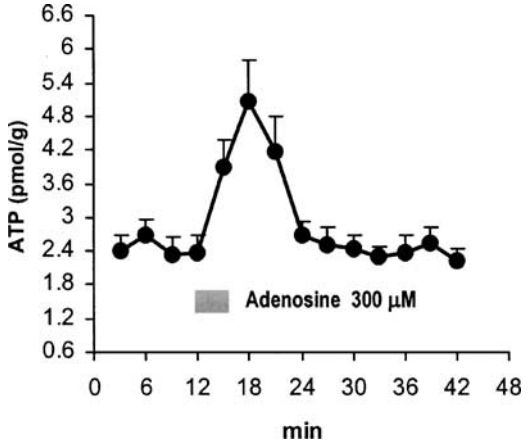

Figure 4 Effect of adenosine (300 μM) on the endogenous ATP efflux from rat hippocampal slices. Preparations were superfused with Krebs' solution and then challenged to 3-min adenosine (300 μM) perfusion, as indicated by the horizontal bar. The ATP content in the superfusate samples was determined by the luciferin – luciferase assay and expressed in pmol g^{-1} wet weight ($n = 12$).

first. Using primers specific for cDNAs of most of the cloned subtypes of P2X and P2Y receptors, RT – PCR analysis showed that mRNAs encoding a variety of P2X and P2Y receptor subtypes are present in rat hippocampus (Figure 5a), including $P2X_{1,2,3,4,5,6,7}$ and $P2Y_{1,2,4,6}$. Therefore, to study the involvement of these subtypes in the effect of ATP, ATP-evoked [^{3}H]purine release was examined in the presence of P2 receptor antagonists. However, PPADS (30 μM), suramin (300 μM) and RB2 (20 μM), P2-receptor antagonists with wide subtype selectivity, did not significantly affect either the resting tritium outflow (data not shown), or the [^{3}H]purine efflux evoked by ATP (30 μM – 1 mM) (Figure 5b). Furthermore, DPCPX, the A_1-selective antagonist (250 nM), and DMPX (250 nM), a nonselective adenosine receptor antagonist, also acting on A_{2A} receptors, were ineffective in modifying the resting and ATP-evoked [^{3}H]purine outflow (Figure 5b).

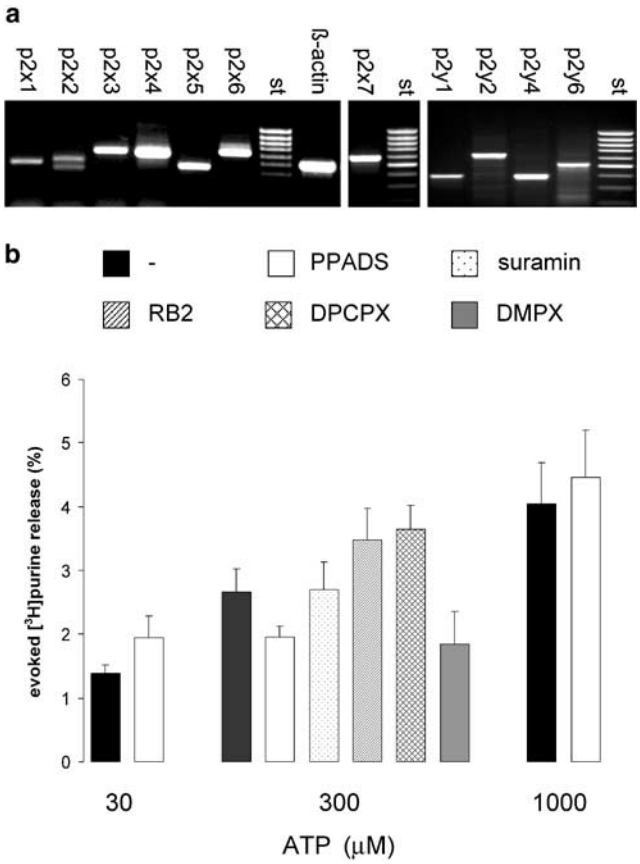

Figure 5 Involvement of various purine receptors in the effect of ATP to elicit the efflux of [^{3}H]purines. (a) RT – PCR indicates expression of a variety of P2X and P2Y receptor subtypes in the rat hippocampus. Total RNA sample from the hippocampus was reverse transcribed and amplified by PCR using primers specific to different purinoceptors. Amplification of β-actin was used as an internal control. A 100 bp ladder (Fermentas, Vilnius, Lithuania) was used to identify PCR fragment sizes. The amplified bands correspond to the calculated size for all P2X and P2Y specific amplification products. Their veracity was confirmed by sequencing the amplified PCR products. The gels shown are representative of at least three independent experiments. (b) Effect of different P1 and P2 receptor antagonists on the ATP-evoked efflux of [^{3}H]purines from rat hippocampal slices. Slices were superfused with Krebs' solution and subjected to ATP (30 – 300 μM) application according to the protocol shown in Figure 1b in the absence (black bars) or presence of PPADS (30 μM), suramin (300 μM), RB2 (20 μM), DPCPX (250 nM) or DMPX (250 nM). Antagonists were superfused 10 min before ATP application and, thereafter, except DPCPX which was preperfused 45 min before ATP application. The net tritium release evoked by ATP was calculated by the AUC method and expressed in fractional release (%). None of the antagonists significantly affect the basal outflow of [^{3}H]purines. ATP concentrations are indicated on the abscissa. Data show the means $\pm$ s.e.m. of six to six identical experiments.

The underlying mechanism of ATP-evoked [^{3}H]purine release

Since ATP cannot pass through the intact cell membrane and no convincing evidence was found for the involvement of a particular purine receptor in ATP-evoked [^{3}H]purine efflux, another underlying mechanism was searched whereby ATP and other purines could elicit tritiated and endogenous purine efflux. To this end, the effects of different drugs and treatments

on EFS- and ATP-evoked efflux of [^{3}H]purines were compared. Among the various treatments, only glibenclamide ($0.70\pm0.07\%$, $n=6$, $P<0.01$), Ca^{2+}-free conditions ($0.77\pm0.04\%$, $n=6$, $P<0.01$) and ADA ($0.59\pm0.15\%$, $n=4$, $P<0.05$) changed significantly the resting tritium efflux. In the presence of the NMDA receptor antagonist AP-5 (50 μM), and the non-NMDA receptor antagonist, CNQX (10 μM), EFS- and ATP-(300 μM) induced [^{3}H]purine release were unchanged, indicating that excitatory transmission does not play a role in these processes under our experimental conditions (Figure 6a). On the other hand, inhibition of action potential propagation by TTX (1 μM) and Ca^{2+}-free conditions strongly diminished EFS-evoked [^{3}H]purine outflow, whereas ATP-evoked [^{3}H]purine outflow was not affected, indicating that the two types of release are mediated in different ways and ATP-induced [^{3}H]purine release is non-exocytotic in nature (Figure 6a). Similarly, replacement of Na^+ in the perfusing solution with choline chloride inhibited EFS-evoked [^{3}H]purine outflow, but did not affect ATP-evoked [^{3}H]purine efflux (Figure 6a). In contrast, the nucleoside transport inhibitors dipyridamole (10 μM) and NBTI (10 μM) significantly decreased ATP-evoked release, but did not modify EFS-evoked release (Figure 6b). Cooling the bath temperature to 12°C which is the cut-off temperature between vesicular and carrier-mediated transmitter release (Vizi & Sperlágh, 1999) also diminished ATP-evoked [^{3}H]purine efflux ($S_{12°C}$: $0.57\pm0.07\%$, $n=6$, $P<0.01$). On the other hand, verapamil and glibenclamide, inhibitors of some ATP-transporting ABC proteins were without effect on ATP-evoked [^{3}H]purine efflux (data not shown). Since the ectoATPase activity is high in the hippocampus, the question arises as to whether ATP itself initiates purine release or is catabolized first to adenosine and thereby elicits further efflux. To address this question, the effect of ADA (2 U ml^{-1}) was tested at a concentration that was reported to effectively convert adenosine to inosine by deamination (Cunha *et al*., 1998). ADA (2 U ml^{-1}) significantly decreased the effect of ATP indicating that the effect of ATP is partly mediated by adenosine (Figure 6b). Increasing the concentration of ADA to 10 U ml^{-1}, although did not completely eliminate ATP-induced [^{3}H]purine efflux, further attenuated the effect of ATP.

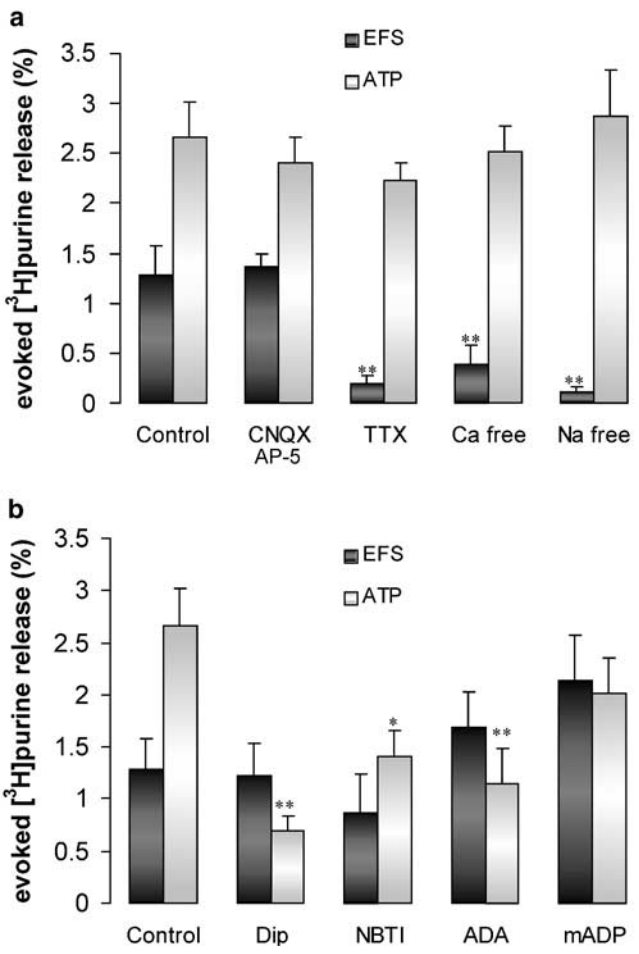

Figure 6 The underlying mechanism of EFS and ATP (300 μM)-evoked efflux of [^{3}H]purines from rat hippocampal slices. (a) Effect of the blockade of NMDA and non-NMDA-type glutamate receptors by CNQX and AP-5 (50 – 10 μM, CNQX-AP5), TTX (1 μM), Ca^{2+}-free Krebs' solution supplemented with 1 mM EGTA (Ca-free) and Na^+-free Krebs' solution supplemented with equimolar choline chloride (Na-free). (b) Effect of dipyridamole (Dip, 10 μM), NBTI (10 μM) and ADA (2 – 10 U ml^{-1}). The preparations were superfused and subjected to EFS (10 Hz, 2.5 ms, 360 shocks) or ATP application according to the protocol shown in Figure 1b. CNQX, AP-5, TTX dipyridamole, NBTI and ADA were superfused from 10 min before the start of the sample collection period and thereafter, whereas Ca^{2+}- and Na^+-free conditions were applied from 60 min before the sample collection and thereafter. The net tritium release evoked by EFS (dark bars) and ATP (light bars) was calculated by the AUC method and expressed in fractional release (%). Data represent the means ± s.e.m. of four to six identical experiments. Asterisks indicate significant differences from respective controls (*$P<0.05$,**$P<0.01$).

Discussion and conclusions

The principal finding of this study is that naturally occurring nucleotides and nucleosides, including ATP, ADP and adenosine, promote the outflow of tritium-labelled purines and endogenous ATP from the *in vitro* rat hippocampal slice. We used [^{3}H]adenosine to label purine stores, which have been proved to be a reliable model to study adenine nucleotide and adenosine release in tissue preparations (Cunha *et al*., 1996; Juranyi *et al*., 1999; Sperlagh *et al*., 2000). Furthermore, with this method, the amount of exogenously added adenine nucleotides and nucleosides can be separated from their counterparts released from the tissue. When the composition of the tritium label was analysed by HPLC, ATP, ADP, AMP adenosine, inosine, hypoxanthine and xanthine were present in the effluent. In our experiments, using higher frequency, but shorter stimulation intervals, a higher proportion of [^{3}H]nucleotides was detected in the effluent during EFS, than in other studies (Pedata *et al*., 1990; Cunha *et al*., 1996). These findings are consistent with the observation that there is a correlation between the amount of total [^{3}H]purine efflux and the proportion of [^{3}H]adenosine in the total (Lloyd *et al*., 1993), and that, at high-frequency stimulation, the contribution of extracellular nucleotides as a source of adenosine is higher (Cunha *et al*., 1996).

The composition of the tritium label in the effluent collected during the peak of ATP-evoked tritium efflux was similar to that found during resting conditions and electrical stimulation, suggesting that purines were released from a homogenous pool. Furthermore, the amount of [^{3}H]ATP was significantly increased in the effluent, collected during the peak of

ATP- and EFS-induced increase in the efflux of tritium, indicating a net release of ATP. This was confirmed when hippocampal slices were challenged by adenosine with an identical application protocol – ATP could not be used in this case because it interacts with the assay system – and the amount of endogenous ATP increased in the effluent. This finding, and the sensitivity of the ATP-evoked [^{3}H]purine efflux to low temperature and transporter inhibitors, makes it unlikely that [^{3}H]purine outflow by ATP and other analogues is due to the simple displacement of the surface-bound radioactive label. Although our observations do not exclude the existence of an ecto-adenylate kinase system in the hippocampus, similar to that described recently on cultured endothelial cell (Yegutkin *et al*., 2002), tritiated purine efflux could not be explained by this mechanism because a net increase in the level of extracellular radiolabelled purine compounds was detected, whereas an ecto-adenylate kinase activity should change the relative amount of AMP, ADP and ATP in the total tritium label. Furthermore, [^{3}H]purine efflux by ATP was not inhibited by Ap_5A, the inhibitor of the ecto-adenylate kinase enzyme. These observations are clear indications of a genuine release process, and the similar chemical nature of the releaser and the released compounds implies the homo- or heteroexchange as the underlying mechanism.

Therefore, attempts were made to identify the macromolecular pathway responsible for ATP-induced [^{3}H]purine efflux. EFS-induced [^{3}H]purine efflux was TTX sensitive and partly Ca^{2+}-dependent, indicating that the primary trigger of EFS-induced release is the voltage-dependent activation of sodium channels and subsequent activation of Ca^{2+} channels at the nerve terminal membrane, features characteristic of vesicular exocytosis. Although the composition of [^{3}H]purine efflux was similar, ATP-evoked efflux was unaffected by Ca^{2+}-free conditions and TTX; therefore, carrier-mediated release was taken into account as a possible mechanism. Among various membrane transporters, those that are able to transport nucleotides and nucleosides, ABC transporters (Schwiebert, 1999) and nucleoside transporters (Thorn & Jarvis, 1996; Cass *et al*., 1999), seemed to be potential candidates for the underlying carrier. Nucleoside transporters form two different families: the CNT transporters which function in a concentrative way, and utilize an Na^+ as a cotransport (Ritzel *et al*., 2001), and ENT transporters, which function in an equilibrative way and independent of Na^+. Dipyridamole and NBTI, the specific inhibitors of ENT1-type nucleoside transporter (Cass *et al*., 1999), strongly diminished ATP-evoked [^{3}H]purine outflow. Therefore, ENT1 equilibrative nucleoside transporter could be involved in this effect, which is in line with the observation that the effect of ATP was temperature dependent. This finding corroborates with the distribution of this transporter in the central nervous system, including the hippocampus, and its role in the transmembrane carriage of extracellular nucleosides (Glass *et al*., 1996; Anderson *et al*., 1999). The observation that adenosine was more efficient than uridine in eliciting [^{3}H]purine efflux is also consistent with the involvement of the ENT1 transporter, which has higher affinity for adenosine than for other substrates (Yao *et al*., 1997). In contrast, substitution of extracellular Na^+ with choline chloride inhibited EFS-, but not ATP-evoked [^{3}H]purine outflow; therefore CNT transporters are unlikely to mediate the ATP-evoked release. Inhibitors of ABC transporters, such as glibenclamide and verapamil, also did not affect significantly ATP-evoked [^{3}H]purine release.

Since presynaptic P2 receptors have been shown to be involved in the facilitation of the release of other transmitters such as acetylcholine (Sperlágh & Vizi, 1991), noradrenaline (Sperlágh *et al*., 2000), GABA and glutamate (Sperlágh *et al*., 2002), we also examined the possible involvement of purine receptors in the effect of ATP to promote [^{3}H]purine efflux in the hippocampus. Although, in early studies, it was proposed that P2 receptor subtypes that expressed in the hippocampus of the rat is limited to a few subtypes (Kidd *et al*., 1995; Collo *et al*., 1996), we performed a systematic RT – PCR approach to reveal the expression of known subtypes of P2X and P2Y receptors. RT – PCR analysis showed that mRNAs encoding almost all molecularly identified subtypes of P2 receptor family are expressed in the hippocampus. Nevertheless, the pharmacological analysis did not confirm the involvement of any of these in the ATP-evoked [^{3}H]purine release, because (1) adenosine, ATP and adenosine were all almost equieffective in eliciting [^{3}H]purine efflux, which is not characteristic of any of the cloned purinoceptors, (2) PPADS, suramin and reactive blue2, P2 receptor antagonists with wide subtype selectivity, did not affect [^{3}H]purine outflow evoked by ATP, (3) α,β-methylene-ATP which is a potent agonist on several subtypes of P2X receptors showed low efficacy, and (4) uridine nucleotides, such as UDP and UTP, agonists of pyrimidine-sensitive subtypes of P2Y receptors were relatively ineffective, the latter observation excluding a major involvement of $P2Y_2$, $P2Y_4$ and $P2Y_6$. Although P2X receptors can also function in heteromeric assemblies, the pharmacological profile found in our experiments differed also from all of the known phenotypes of heteromeric P2X receptors, that is from $P2X_2/P2X_3$, $P2X_1/P2X_5$, $P2X_2/P2X_6$, $P2X_4/P2X_6$ and $P2X_1/P2X_2$ combinations (North & Surprenant, 2000; Khakh *et al*., 2001; Brown *et al*., 2002; North, 2002). Furthermore, as DPCPX, the selective A_1-receptor antagonist, and DMPX, the nonselective A_{2A} antagonist, did not affect the response, the involvement of adenosine A_1 and A_{2A} receptors seems also unlikely. Consequently, unless the response is mediated by a yet unknown purinoceptor, it appears that purine release evoked by ATP and other purines is mediated by a non receptor-mediated mechanism.

Although nucleoside transporters are usually characterized by nucleoside transport ability, the same transporter expressed in erythrocytes has been shown in the transmembrane movement of nucleotides, including ATP (Bergfeld & Forrester, 1992). Therefore, the question arises as to whether ATP itself causes this effect or it is necessary first to be broken down to adenosine. In our experiments, ADA, the enzyme which converts adenosine to inosine by deamination, substantially decreased ATP-evoked [^{3}H]purine release, indicating that extracellular adenosine formation and subsequent transport by the ENT1 nucleoside transporter are crucial for the action of ATP.

We tentatively propose, therefore, that adenosine nucleosides, and perhaps to a minor extent nucleotides, are taken up by ENT1 nucleoside transporter and reverses its direction eliciting further purine release from the cytoplasm (Figure 7a, b). It seems feasible to suggest that, under normal conditions, when the transporter is loaded from the extracellular space with physiological amount of nucleosides, it is taken up and is rapidly interconverted to adenine nucleotides and inosine by

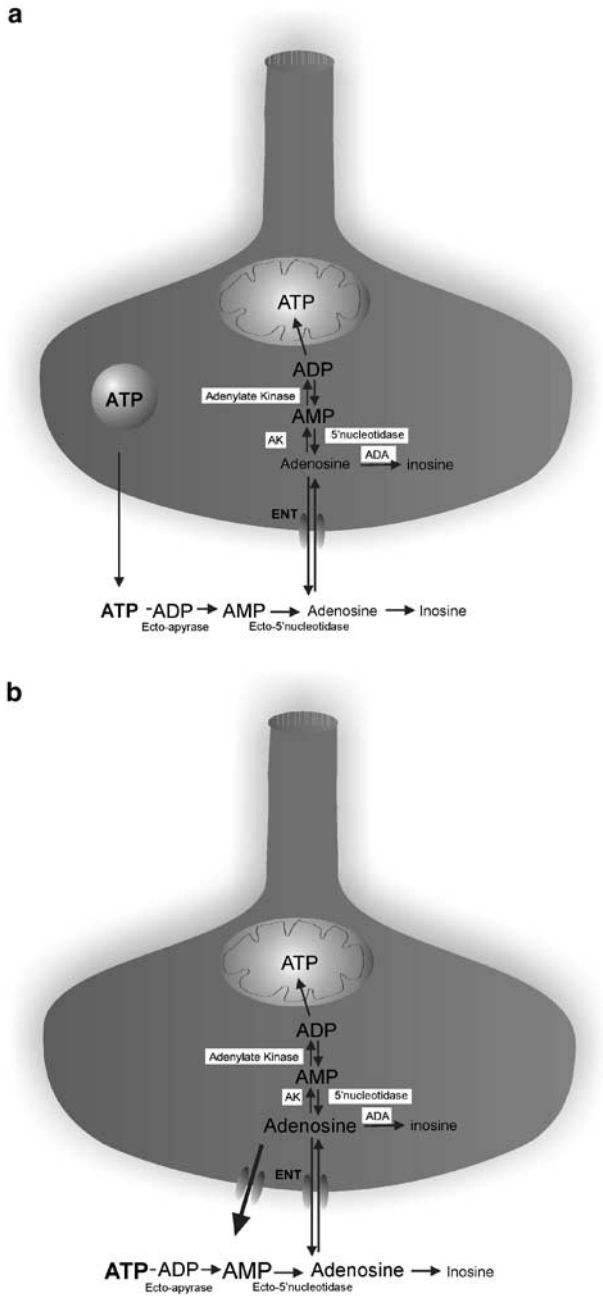

Figure 7 Schematic representation of the presumed subcellular mechanism involved in the effect of nucleotides and nucleosides to release tritiated and endogenous purines in the hippocampus. (a) Under physiological condition, ATP is released by neuronal activity from axon terminals, and is rapidly metabolized to adenosine, which is taken up by the ENT transporter and reincorporated into nucleotide pools, clearing up the extracellular space from biologically active purines and maintaining nanomolar cytoplasmic concentration of adenosine. (b) When the extracellular compartment is loaded pharmacologically with high concentration of purines, they are also taken up by the ENT transporter, but intracellular elimination pathways might not keep up with the uptake causing intracellular adenosine accumulation. This could reverse the direction of the transporter and elicit purine release.

the cytosolic adenosine kinase and ADA enzymes, respectively, maintaining intracellular adenosine concentration at the nanomolar level and the inward driving force of the transporter (Figure 7a). However, when the transporter is loaded from the extracellular space by a higher, 'pharmacological' amount of purines, intracellular elimination by adenosine kinase and ADA might not keep up with the inward transport (Figure 7b). This process could lead to cytoplasmic adenosine accumulation and the subsequent reversal of the transporter. This assumption is consistent with the idea that the v_{max} and K_m values of the nucleoside transporters are higher than that of the adenosine kinase and ADA enzymes (Phillips & Newsholme, 1979; Yao *et al*., 1997), and explains why the effect of ATP and ADP was manifest only at high concentrations and appeared nonsaturating in this concentration range. It remains, however, unclear whether the same or different transporters are involved in the uptake and efflux of purines demonstrated by the present experiments. Since we could not inhibit the response by other known transport inhibitors, such as ABC transporter inhibitors, it seems likely that the ENT transporters are involved in both processes. Yet another possibility is that ATP is secreted by CD39 protein, recently recognized as an ATP-transporting protein (Bodas *et al*., 2000).

As the ENT1 nucleoside transporters are distributed on nerve terminals and also on astrocytes and endothelial cells (Anderson *et al*., 1999), the cellular source of ATP-evoked purine efflux could be either nerve terminals, glial cells or endothelial cells. Since no similar ATP- or ADP-induced purine accumulation was observed in isolated hippocampal nerve terminals (Cunha, 2001b), it seems likely that non-neuronal elements expressing the ENT1 transporter provide a major contribution to this effect. In addition, further investigation is required on the exact contribution of this kind of purine release to extracellular purine levels under different patterns of neuronal activity or pathological situations. Since EFS-evoked [^{3}H]purine efflux was not sensitive to dipyridamole, at least under our experimental conditions, it appears that this mechanism primarily acts when exogenous purines are added to tissue in relatively high concentrations. Under these conditions, exogenous purines, which generate or in themselves are substrates of nucleoside transporter, could elicit a change in the composition of their own extracellular level, influencing also the active ligand concentrations at the vicinity of pre- and postsynaptic adenosine and P2 receptors, and might cause unexpected pharmacological effects. Alternatively, under pathological conditions, extracellular ATP accumulation due to cellular loss might result in a sufficiently high extracellular purine concentration able to trigger this mechanism.

In conclusion, here we suggest a new mechanism whereby extracellular ATP and other purines could enter the extracellular space. Purine outflow by homo- or heteroexchange might change the composition and contribute to extracellular purine levels during physiological and pathological activation of the purinergic signalling system, resulting in a suitably high extraneuronal adenosine load to cause the reversal of the ENT1 nucleoside transporter.

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