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PURINERGIC P2 RECEPTORS TRIGGER ADENOSINE RELEASE LEADING TO ADENOSINE A_{2A} RECEPTOR ACTIVATION AND FACILITATION OF LONG-TERM POTENTIATION IN RAT HIPPOCAMPAL SLICES

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Abstract—Electrophysiological recordings were used to investigate the effects of ATP analogues on θ-burst-induced long-term potentiation (LTP) in rat hippocampal slices. α,β-Methylene ATP (α,β-MeATP; 20 μM) decreased LTP from 36±9% to 17±5%, an effect prevented by adenosine A_1 receptor blockade in accordance with the localised catabolism of ATP analogues into adenosine, leading to adenosine A_1 receptor activation. Thus, to probe the role of extracellular ATP, all experiments were performed with the A_1 receptor selective antagonist, 1,3-dipropyl-8-cyclopentylxanthine (50 nM). In these conditions, α,β-MeATP or 5′-adenylylimidodiphosphate (β,γ-ImATP; 20 μM) facilitated LTP by 120%, an effect prevented by the P2 receptor antagonists, pyridoxalphosphate-6-azophenyl-2′-4′-disulphonic acid (PPADS; 20 μM) or suramin (75 μM), as well as by the $P2X_{1/3}$-selective antagonist 8-(benzamido)naphthalene-1,3,5-trisulfonate (10 μM). The facilitations of LTP by either α,β-MeATP or β,γ-ImATP (20 μM) were also prevented by both 4-(2-[7-amino-2-(2-furyl(1,2,4)-triazolo(2,3a)-(1,3,5)triazin-5-yl-amino]ethyl)phenol (50 nM) or 7–2(-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]-1,2,4-triazolo[1,5-c] pyrimidine (50 nM), antagonists of facilitatory adenosine A_{2A} receptors, were occluded by the A_{2A} receptor agonist, CGS 21680 (10 nM) and were prevented by the protein kinase C inhibitor, chelerythrine (6 μM) and unaffected by the protein kinase A inhibitor, H89 (1 μM). Furthermore, β,γ-ImATP (20 μM) enhanced [^{3}H]adenosine outflow from rat hippocampal slices by nearly 150%, an effect prevented by PPADS (20 μM) or suramin (75 μM). The adenosine transport inhibitors, nitrobenzylthioinosine (5 μM) and dipyridamole (10 μM) also prevented β,γ-ImATP (20 μM)-induced [^{3}H]adenosine outflow and facilitation of LTP. These results suggest that ATP analogues facilitate LTP through P2 receptor activation that mainly triggers adenosine release leading to the activation of adenosine A_{2A} receptors.

Key words: ATP, neuromodulation, LTP, hippocampus.

Both types of ATP receptors, ionotropic (P2X) and metabotropic (P2Y), are widely distributed in the brain (Ralevic and Burnstock, 1998). The P2X receptors comprise a family of ATP-gated nonselective cation channel-receptors that mediate fast excitatory transmission in some peripheral nervous system and CNS pathways (Ralevic and Burnstock, 1998) and also have a neuromodulatory role, particularly at the presynaptic level (reviewed in Cunha and Ribeiro, 2000a). Seven subtypes of P2X receptors have been cloned ($P2X_{1-7}$) (North and Surprenant, 2000), of which $P2X_1$, $P2X_2$, $P2X_4$ and $P2X_6$ have overlapping expressions in the brain (Valera et al., 1994; Collo et al., 1996; Séguéla et al., 1996; Soto et al., 1996; Tanaka et al., 1996; Lê et al., 1998a). In fact, localisation of ATP receptors by radioligand binding techniques (Bo and Burnstock, 1994; Balcar et al., 1995), *in situ* hybridisation (Valera et al., 1994; Kidd et al., 1995; Collo et al., 1996; Soto et al., 1996; Tanaka et al., 1996; Lê et al., 1998a) and immunocytochemistry (Lê et al., 1998b; Kanjhan et al., 1999; Worthington et al., 1999; Rubio and Soto, 2001) show a broad distribution of P2X receptors in the brain, with the hippocampus being one of the areas expressing the highest levels.

However, in the hippocampus and in other brain structures where P2X receptors are abundantly expressed, the neuroactive role of ATP is still ill-defined. In fact, ATPergic transmission has been demonstrated in the medial habenula (Edwards et al., 1992), in the spinal cord (Bardoni et al., 1997), in the locus coeruleus (Nieber et al., 1997) and in the nucleus tractus solitarius (Thomas and Spyer, 2000) whereas in the hippocampus it only contributes to less than 10% of excitatory transmission in glutamatergic synapses (Pankratov et al., 1998; Mori et al., 2001). When probing the effect of extracellular ATP, it is mostly concluded that its main role is to act as a substrate for ecto-nucleotidases yielding adenosine (Dunwiddie et al., 1997; Cunha et al., 1998), a neuromodulator on its own (reviewed by Cunha, 2001). In fact, the inhibitory effect of ATP on basal hip-

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Abbreviations: aCSF, artificial cerebrospinal fluid; CGS 21680, 2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine; DMSO, dimethylsulphoxide; 1,3-dipropyl-8-cyclopentylxanthine, DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; fEPSP, field excitatory post-synaptic potential; β,γ-Imido ATP, 5-adenylylimido-diphosphate; LTP, long-term potentiation; α,β-MeATP, α,β-methylene ATP; NBTI, *S*-(*p*-nitrobenzyl)-6-thioinosine; NF023, 8-(benzamido)naphthalene-1,3,5-trisulfonate; PPADS, pyridoxal phosphate-6-azophenyl-2,4-disulphonic acid; SCH 58261, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; XAC, 8-{4-[(2-aminoethyl) amino]carbonylmethyl-oxyphenyl}xanthine; ZM 241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5ylamino]ethyl)phenol.

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pocampal synaptic transmission is abolished in adenosine A_1 receptor knockout animals (Masino et al., 2002).

But, interestingly, ATP is released in greater amounts upon conditions of high frequency stimulation (Wieraszko et al., 1989; Cunha et al., 1996a) associated with phenomena of synaptic plasticity. Use-dependent long-term changes in synaptic efficiency, like long-term potentiation (LTP), are thought to form the basis for learning and memory (Bliss and Collingridge, 1993). In hippocampal CA1 neurons, extracellularly applied ATP causes synaptic potentiation similar to LTP (ATP-induced LTP; Wieraszko and Seyfried, 1989; Wieraszko and Ehrlich, 1994; Fujii et al., 1995; O'Kane and Stone, 2000). This may occur either because extracellular ATP can play a role in ATP-induced LTP as a orthophosphate donor for the extracellular phosphorylation of membrane proteins (Fujii et al., 1995; Chen et al., 1996) or because ATP may directly control neuronal function by activating hippocampal P_2 receptors (Inoue et al., 1996; Pankratov et al., 1998, 2002; Mori et al., 2001).

We re-evaluated the modulatory role of extracellular ATP on synaptic plasticity in hippocampal slices now 1) controlling for the possibility that some effects may be due to the formation of adenosine, 2) using ATP analogues not prone to act as phosphate donors, and 3) choosing a θ-burst pattern to induce LTP, since modulatory effects may not be observed with robust high-frequency stimulation protocols (de Mendonça and Ribeiro, 2001).

EXPERIMENTAL PROCEDURES

Drugs

Adenosine deaminase (type VI; 1803 U/ml; EC 3.5.4.4), *S*-(*p*-nitrobenzyl)-6-thioinosine (NBTI), α,β-methylene ATP (α,β-MeATP) and 5-adenylylimido-diphosphate (β,γ-Imido ATP) were from Sigma (Reagente 5, Porto, Portugal), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 8-{4-[(2-aminoethyl)amino]carbonylmethyl-oxyphenyl}xanthine (XAC), suramin, 2-[4-(2-*p*-carboxyethyl)-phenylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), pyridoxal phosphate-6-azophenyl-2,4-disulphonic acid (PPADS), and 8-(benzamido)naphthalene-1,3,5-trisulfonate (NF023) were from RBI (Reagente 5, Porto, Portugal), dipyridamole was from Boehringer Ingelheim, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5ylamino]ethyl)phenol (ZM 241385) was from Tocris, 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 58261) was a kind gift of E. Ongini (Shering-Plough, Milan), [2,3,8′-^{3}H]adenosine (specific activity: 55.8 Ci/mmol) and [^{3}H]CGS 21680 (specific activity 38.4 Ci/mmol) were from DuPont NEN (Anagene, Lisbon, Portugal) and chelerythrine and H89 were from Calbiochem (Darmstadt, Germany).

ZM241385, CGS 21680, XAC, dipyridamole, NBTI, H89 and chelerythrine were made up as 5 mM stock solutions in dimethylsulphoxide (DMSO). SCH 58261 was made up as a 10 mM stock solution in DMSO. DPCPX was made up into a 5 mM stock solution in 99% DMSO and 1% NaOH (1M). All drug stock solutions were diluted directly into the superfusion solution to the appropriate final concentration. DMSO, in the maximal concentration applied to the preparations, was devoid of effects on synaptic plasticity experiments and [^{3}H]adenosine release from rat hippocampal slices.

Preparation of hippocampal slices

The experiments were performed on hippocampal slices taken from male young adult Wistar rats (5–6 weeks old, from Harlan Iberica, Barcelona, Spain) that were handled and killed according to EU guidelines, the number of animals used being kept to an absolute minimum for the results to reach statistical significance. To minimise suffering, the animals were anaesthetized with halothane before being decapitated, and the right hippocampus was dissected free within an ice-cold artificial cerebrospinal fluid (aCSF) of the following composition (in mM): NaCl 124, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 26, $MgSO_4$ 1, $CaCl_2$ 2, glucose 10, and gassed with a 95% O_2 and 5% CO_2 mixture. Slices were cut 400 μm thick perpendicularly to the long axis of the hippocampus with a McIlwain tissue chopper, and kept in a resting chamber within the same gassed aCSF at room temperature (22 °C–25 °C) for at least 1 h to allow their energetic and functional recovery.

Electrophysiology studies

A slice was transferred to the submerged recording chamber (1 ml capacity) where it was continuously superfused at a rate of 3 ml/min with the same gassed solution at 30.5 °C. Stimulation (rectangular pulses of 0.1 ms) was delivered through a bipolar concentric wire electrode placed on the Schaffer collateral/commissural fibers in the stratum radiatum. Two separate sets of the Schaffer pathway (S1 and S2) were stimulated, as previously described (Costenla et al., 1999). Responses were evoked alternately on the two pathways, each pathway being stimulated once every 20 s (basal frequency). The initial intensity of the stimulus was that eliciting a field excitatory post-synaptic potential (fEPSP) of about 1 mV amplitude, whilst minimising contamination by the population spike, and of similar magnitude in both pathways. Evoked fEPSP were recorded extracellularly from CA1 stratum radiatum using micropipettes filled with 4 M NaCl and 2–6 MΩ resistance, and displayed on a Tektronix digitising oscilloscope. The averages of eight consecutive responses from each pathway were obtained, plotted graphically and recorded for further analysis with a personal computer using locally developed software. The fEPSP were quantified as the slope of the initial phase of the potential. The independence of the two pathways was tested by using paired-pulse facilitation across both pathways, less than 10% facilitation being usually observed.

LTP was induced by a θ-burst stimulation pattern (three trains of 100 Hz, each with four stimuli, separated by 200 ms). In all the experiments, the stimulation protocol to induce LTP was applied after having a stable baseline for at least 30 min. LTP was quantified as the percentage change in the average slope of the potentials taken from 50 to 60 min after the induction protocol, in relation to the average slope of the fEPSP measured during the 10 min that preceded the induction protocol. LTP was always induced in one pathway in the control conditions, and afterward induced in the other pathway, in the presence of the drug to be tested. It is important to note that the use of two stimulation pathways to compare the effects of drugs on θ-burst-induced LTP is particularly important since there is a large variation in the amplitude of LTP between slices, whereas we confirmed that LTP amplitudes obtained on stimulation of two independent sets of Schaffer pathways are similar.

[^{3}H]adenosine release studies

The release of [^{3}H]adenosine from rat hippocampal slices was performed as previously described (Cunha et al., 1996a). Briefly, the slices were resuspended in 2 ml of oxygenated aCSF, equilibrated at 37 °C for 10 min and loaded with [2,3,8′-^{3}H]adenosine (30 μCi/ml, 0.125 μM) for 20 min. The excess of radioactivity was removed by washing the preparations twice with 2 ml of oxygenated aCSF at 37 °C. Groups of three slices were then placed in 100 μl chambers and superfused with oxygenated aCSF at 37 °C with a flow rate of 0.6 ml/min. After 30 min superfusion, the effluent was collected every 2 min to quantify released tritium. When the effect of any drug was tested, this drug was added 6 min after the

sample collection was started, only into two chambers out of four chambers, and remained in the bath until the end of the release period. At the end of the experiments (22 min after starting the sample collection), the slices were sonicated in 0.5 ml of 3 M perchloric acid with 2% Triton X-100, and a 100 μl aliquot of the homogenised slices was used to determine the amount of tritium retained by the preparations (Cunha et al., 1996a).

The release of [^{3}H]adenosine from rat hippocampal synaptosomes followed a protocol similar to that used for slices, as previously described (Cunha et al., 2000), using 0.4–0.6 mg of protein in each assay.

The release of [^{3}H]adenosine from primary cultures of rat hippocampal neurons or astrocytes was also evaluated. Hippocampal neurons were dissociated from hippocampi of E18–E19 rat embryos, as previously described (Ambrósio et al., 1999) and cultured in serum free Neurobasal medium (GIBCO, Life Technologies, UK) with B27 supplement (GIBCO), 25 μM glutamine, 0.5 mM glutamate and 0.12 mg/ml gentamicin. Cultures were plated on poly-D-lysine-coated (0.1 mg/ml) glass coverslips at a density of 0.2×10^6 cells/cm^2 and kept at 37 °C in a humidified incubator in 5% CO_2/95% air for 7–8 days, the time required for maturation of hippocampal neurons. Cultured astrocytes were obtained from P5 rat pups and cultured in a humidified incubator in 5% CO_2/95% air for 10–14 days at 37 °C on poly-D-lysine-coated (0.1 mg/ml) 75 cm^2 Costar containers at a density of 1.14×10^5 cells/cm^2 in Dulbecco's Modified Eagle medium (GIBCO) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% foetal calf serum (Seromed; e.g. Saneto and de Vellis, 1987). These cultures of neurons or astrocytes were incubated for 4 h at room temperature with aCSF solution buffered with 25 mM HEPES (aCSF-HEPES solution) and then incubated for a further 30 min period with [2,3,8′-^{3}H]adenosine (30 μCi/ml, 0.125 μM). They were then superfused for 90 min at a flow rate of 0.5 ml/min with aCSF-HEPES solution to remove non-incorporated radioactivity, the excess of medium being removed by vacuum suction to ensure a constant volume of 2 ml (neurons) or 5 ml (astrocytes). Drugs were added through this superfusion solution and tritium release was quantified in the collected vacuum aspirate after exposure of the cultures for 15 min to the tested drugs. At the end of the experiment, the cultured neurons or astrocytes were incubated with 0.5 or 2 ml of 3 M perchloric acid with 2% Triton X-100, and a 100 μl aliquot of the supernatant was used to determine the amount of tritium retained by the preparations.

Radioactivity was expressed in terms of disintegrations per second per milligram of protein (Bq/mg) in each chamber, protein being quantified by the method of Peterson (1977). In some experiments, a 500 μl aliquot of the collected sample was separated by HPLC and the peak corresponding to adenosine was collected to quantify [^{3}H]adenosine outflow, as previously described (Cunha et al., 1996a).

Binding to adenosine A_{2A} receptors

The competition by ATP analogues (1–1000 μM) of [^{3}H]CGS 21680 binding was performed as previously described (Cunha et al., 1996b) using membranes from the rat hippocampus. Briefly, [^{3}H]CGS 21680 (30 nM) binding was carried out for 4 h at room temperature (23–25 °C), with 242–310 μg of membrane protein in a final volume of 300 μl in an incubation solution containing 50 mM Tris–HCl and 10 mM $MgCl_2$, pH 7.4, with 5 U/ml adenosine deaminase. Specific binding was determined by subtraction of the non-specific binding which was measured in the presence of 2 μM XAC. The binding reactions were stopped by vacuum filtration through Whatman GF/C glass fibre filters, followed by washing of the filters and reaction tubes with 10 ml of the incubation solution, kept at 4 °C. The filters were then placed in scintillation vials, and 5 ml of scintillation liquid (Scintran Cocktail T; Wallac, Turku, Finland) were added. Radioactivity bound to the filters was determined after 12 h with an efficiency of 55–60% for 2 min. Membrane protein was determined according to Peterson (1977).

Statistical analysis

Values are presented as the mean±S.E.M. The significance of the differences between the means was calculated with the two-tailed paired Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

ATP analogues facilitate LTP when adenosine A_1 receptors are blocked

The application of θ-burst stimulation (three trains of 100 Hz, four stimuli, separated by 200 ms) in one pathway caused LTP with an amplitude of 36.4±8.7% ($n=4$). The application of a similar θ-burst stimulation in the other pathway in the presence of αβ-MeATP (20 μM) elicited a smaller LTP (16.6±5.0%, $n=4$, $P<0.05$). This inhibitory effect of the ATP analog is similar to the inhibition of LTP by adenosine A_1 receptor activation (de Mendonça and Ribeiro, 1990) and it is known that, under basal conditions of stimulation, ATP analogues are locally catabolised into adenosine and channelled to A_1 receptor activation (Cunha et al., 1998). In fact, the P2 receptor antagonists, PPADS (10 μM) or suramin (75 μM), did not modify the inhibitory effect of the ATP analog on LTP (the values of LTP were 29.0±5.0% in control solution and 14.0±5.3% in the presence of αβ-MeATP (20 μM) and PPADS (10 μM); and 21.5±5.2% in control solution and 12.2±5.1% in the presence of αβ-MeATP (20 μM) and suramin (75 μM; $n=4$, $P<0.05$). In contrast, in the presence of the selective adenosine A_1 receptor antagonist, DPCPX (50 nM), we no longer observed the inhibitory effect of the ATP analog. In fact, αβ-MeATP (20 μM) facilitated LTP from 21.2±7.5% to 47.4±5.6% ($n=5$; $P<0.05$; Fig. 1A, B). We also tested another ATP analog, βγ-Imido ATP (20 μM), which did also facilitate LTP from 16.1±8% to 41.5±5.1%, in the presence of 50 nM DPCPX ($n=4$; $P<0.05$; Fig. 1C). Thus, the inhibitory effects of the ATP analogues on LTP may be due to adenosine A_1 receptor activation rather than to a genuine P2 receptor-mediated effect. When blocking adenosine A_1 receptors, an excitatory effect of the ATP analogues on LTP became apparent. Therefore, to further investigate the role of P2 receptors in the modulation of LTP, all the subsequent experiments were performed under blockade of adenosine A_1 receptors with DPCPX (50 nM). To test the modification by different drugs of the facilitation by ATP analogues of θ-burst LTP, we always compared in two different pathways of the same slice the effect in the first pathway of DPCPX together with the tested drug (control) with the effect in the second pathway of the ATP analog in the presence of DPCPX and the tested drug. This enabled us to determine if the effect of the ATP analogues was modified by the tested drug in the same slice, irrespective of the effect of the tested drugs on LTP and on basal synaptic transmission. Again, it is important to stress that comparisons need to be carried out in the same slice since we noted a considerable variation of the amplitude of θ-burst LTP between slices.

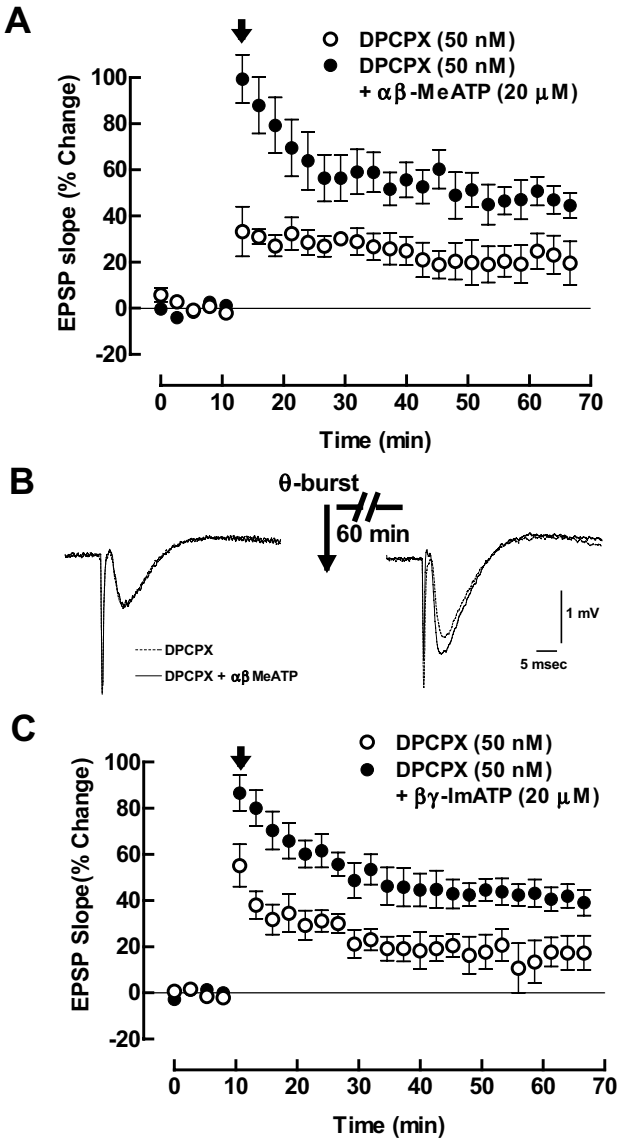

Fig. 1. Effect of the ATP analogues, αβ-MeATP (20 μM) and βγ-Imido ATP (20 μM), on LTP induced by θ-burst stimulation pattern in rat hippocampal slices in the presence of the adenosine A_1 receptor antagonist, DPCPX (50 nM). Panels A and B show the time course of the changes (%) in fEPSP slope. The arrow represents the time of application of the θ-burst stimulation pattern. Panel A shows the facilitatory effect of αβ-MeATP (20 μM) on θ-burst-induced LTP (●) compared with DPCPX (50 nM) alone (○). Each point represents the mean±S.E.M. of four experiments. Panel B presents averages of eight consecutive fEPSP obtained before (potentials on the left) and 60 min after induction of LTP with the θ-burst stimulation pattern (potentials on the right) in the presence of DPCPX (50 nM) alone (dashed lines) and in the simultaneous presence of DPCPX (50 nM) and αβ-MeATP (20 μM). The experiment was carried out in the same hippocampal slice on recording extracellularly in CA1 dendrites after stimulation of two converging but independent afferent pathways. Panel C shows the facilitatory effect of βγ-Imido ATP (20 μM) on θ-burst-induced LTP (●) compared with DPCPX (50 nM) alone (○). Each point represents the mean±S.E.M. of four experiments. Note that DPCPX (50 nM) caused a 11±2% facilitation of basal fEPSP slope and αβ-MeATP (20 μM) or βγ-Imido ATP (20 μM) were virtually devoid of effects on basal fEPSP slope when added in the presence of DPCPX.

ATP antagonists prevent the facilitatory effect of ATP analogues on LTP

We then tested the ability of antagonists of P2 receptors to modify the facilitatory effect of ATP analogues on LTP-induced by θ-burst stimulation pattern, observed in the presence of DPCPX (50 nM; Fig. 1). The facilitatory effect of αβ-MeATP (20 μM) on LTP was prevented by the P2 receptor antagonist PPADS (20 μM), a value of 14.6±1.6% being obtained in the presence of PPADS and αβ-MeATP compared with a value of 16.9±3.7% when αβ-MeATP was not present ($n=3$; $P>0.05$; Fig. 2A). Likewise, the facilitatory effect of βγ-Imido ATP (20 μM) on LTP was also prevented by the P2 receptor antagonist PPADS (20 μM), a value of 15.1±2.4% being obtained in the presence of PPADS and βγ-Imido ATP compared with a value of 15.9±2.9% when βγ-Imido ATP was not present ($n=3$, $P>0.05$). The facilitatory effect of βγ-Imido ATP (20 μM) on LTP was also prevented by another P2 receptor antagonist, suramin (75 μM), a value of 15.1±1.7% being obtained in the presence of suramin and βγ-Imido ATP compared with a value of 17.6±2.0% when βγ-Imido ATP was not present ($n=3$; $P>0.05$; Fig. 2B).

The pharmacological characterisation of the P2 receptors likely to be involved in the facilitation by ATP analogues of θ-burst induced LTP is problematic due to the poor selectivity of the P2 receptor ligands currently available (Ralevic and Burnstock, 1998). But, since βγ-Imido ATP was equi-effective with αβ-MeATP, the later being considered a $P2X_{1/3}$ receptor preferring agonist (Ralevic and Burnstock, 1998), we decided to test the ability of a purported $P2X_1/P2X_3$ receptor antagonist, NF023 (Soto et al., 1999; Lambrecht, 2000) to prevent the facilitatory effect of ATP analogues. NF023 (10 μM) blocked the facilitatory effect of βγ-Imido ATP (20 μM) on LTP, a value of 25.6±10.6% being obtained in the presence of NF023 and βγ-Imido ATP and a value of 25.3±10.5% when βγ-Imido ATP was not present, ($n=3$; Fig. 2C).

Adenosine A_{2A} antagonists prevent the facilitatory effect of the ATP analogues on LTP

Since it has previously been shown that endogenously released adenine nucleotides lead to preferential activation of adenosine A_{2A} receptors (Cunha et al., 1996c) and activation of these A_{2A} receptors is known to facilitate LTP (de Mendonça and Ribeiro, 1994), we thought important to probe the participation of facilitatory adenosine A_{2A} receptors in this enhancement of LTP by ATP analogues.

Indeed, the A_{2A} receptor antagonists, ZM 241385 (50 nM) and SCH 58261 (50 nM) prevented the facilitatory effects of αβ-MeATP (20 μM) and of βγ-Imido ATP (20 μM) on LTP (see Fig. 3). Thus, a value for LTP of 18.2±0.7% was obtained in the presence of ZM 241385 and αβ-MeATP compared with a value of 17.9±0.3% when αβ-MeATP was not present ($n=4$; $P>0.05$; Fig. 3A). The facilitatory effect of βγ-Imido ATP (20 μM) on LTP was also prevented by ZM 241385 (50 nM), a value of 21.2±1.4% being obtained in the presence of ZM 241385 and βγ-Imido ATP compared with a value of 22.7±1.4%

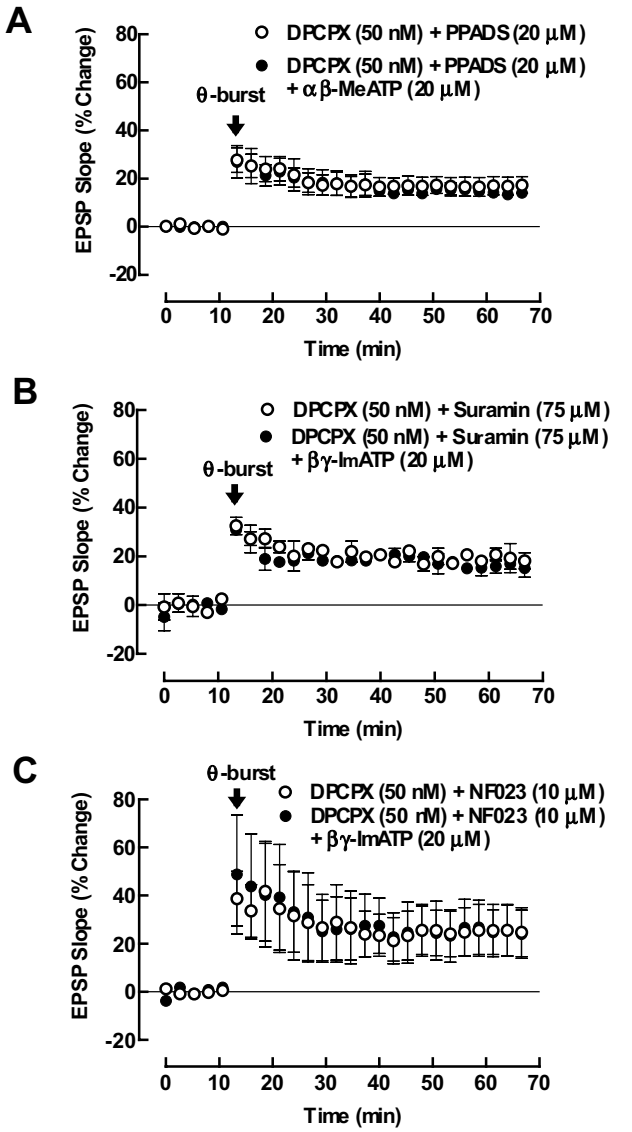

Fig. 2. Effect of the P2 receptor antagonists, PPADS (20 μM, panel A), suramin (75 μM, panel B) and NF023 (10 μM, panel C), on the facilitation by ATP analogues of LTP induced by θ-burst stimulation pattern in rat hippocampal slices. All panels show the time course of the changes (%) in fEPSP slope. The arrow represents the time of application of the θ-burst stimulation pattern. All the experiments were performed in the presence of the adenosine A_1 receptor antagonist, DPCPX (50 nM). Panel A shows that the facilitatory effect of αβ-MeATP (20 μM) on θ-burst-induced LTP is prevented in the presence PPADS (20 μM). Panel B shows that the facilitatory effect of βγ-Imido ATP (20 μM) on θ-burst-induced LTP is prevented in the presence of suramin (75 μM). Panel C shows that the facilitatory effect of βγ-Imido ATP (20 μM) on θ-burst-induced LTP is prevented in the presence of NF023 (10 μM). In all panels, each point represents the mean±S.E.M. of three experiments.

when βγ-Imido ATP was not present ($n=3$; $P>0.05$). Finally, the facilitatory effect of βγ-Imido ATP (20 μM) on LTP was also prevented by SCH 58261 (50 nM), a value of 24.2±3.6% being obtained in the presence of SCH 58261 and βγ-Imido ATP compared with a value of 24.7±3.7% when βγ-Imido ATP was not present ($n=3$; $P>0.05$; Fig. 3B).

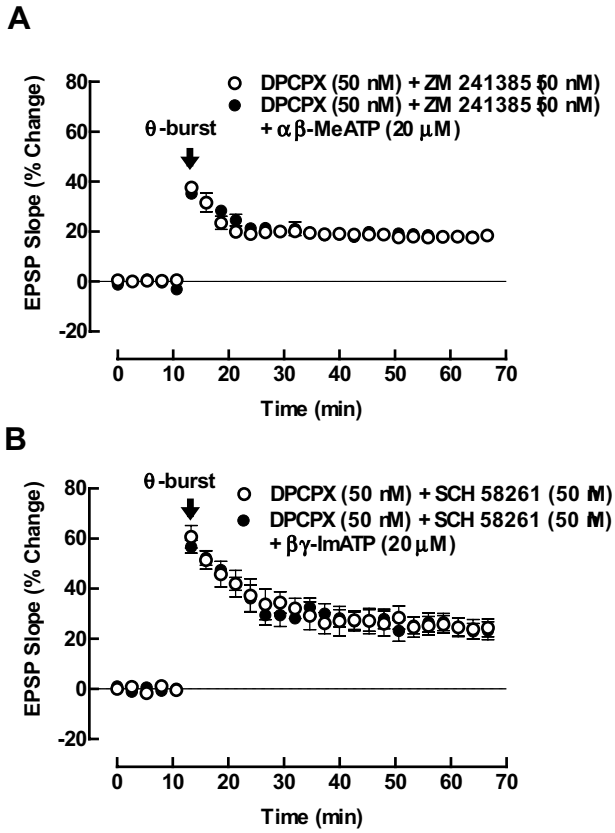

Fig. 3. Effect of A_{2A} receptor antagonists on the facilitation by ATP analogues of LTP induced by θ-burst stimulation pattern in rat hippocampal slices. The panels show the time course of the changes (%) in fEPSP slope. The arrow represents the time of application of the θ-burst stimulation pattern. All the experiments were performed in the presence of the adenosine A_1 receptor antagonist, DPCPX (50 nM). Panel A shows that the facilitatory effect of αβ-MeATP (20 μM) on θ-burst-induced LTP is prevented in the presence of A_{2A} receptor antagonist, ZM 241385 (50 nM). Each point represents the mean±S.E.M. of four experiments. Panel B shows that the facilitatory effect of βγ-Imido ATP (20 μM) on θ-burst-induced LTP is also prevented in the presence of another A_{2A} receptor antagonist, SCH 58261 (50). Each point represents the mean±S.E.M. of three experiments.

ATP analogues do not bind to hippocampal adenosine A_{2A} receptors

Since adenosine A_{2A} receptors antagonists prevented the facilitatory effect of ATP analogues on LTP, we sought to exclude the possibility that ATP analogues might directly interact with adenosine A_{2A} receptors. For that purpose, we carried out displacement binding curves to investigate the ability of ATP analogues to displace the binding of the selective A_{2A} receptor agonist [^{3}H]CGS 21680 to rat hippocampal membranes. As shown in Fig. 4, both αβ-MeATP and βγ-Imido ATP failed to displace the binding of [^{3}H]CGS 21680 (30 nM) to rat hippocampal membranes in concentrations up to 100 μM. This indicates that the tested ATP analogues are unable to interact directly with adenosine A_{2A} receptors in the rat hippocampus, similarly to what was previously observed in the striatum (Pirotton and Boeynaems, 1993). We also found that PPADS also failed

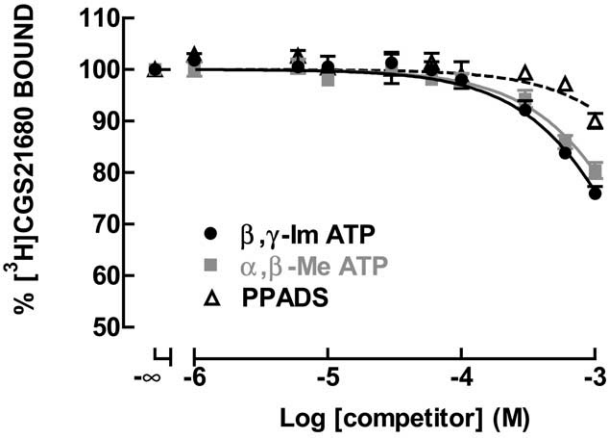

Fig. 4. ATP analogues and a P2 receptor antagonist are not able to displace the binding of the selective A_{2A} receptor agonist, [^{3}H]CGS 21680, to rat hippocampal membranes. The ordinates represent the specific binding of [^{3}H]CGS 21680 (30 nM) on subtraction of non-specific binding determined in the presence of 2 μM XAC from the total binding, in the presence of αβ-MeATP (filled squares, filled grey line), of βγ-Imido ATP (filled circles, filled black line) or of PPADS (filled triangles, dashed line). The results are means±S.E.M from four experiments performed in triplicate.

to displace the binding of [^{3}H]CGS 21680 (30 nM) to rat hippocampal membranes in concentrations up to 100 μM (Fig. 4).

ATP analogues trigger [^{3}H]adenosine outflow from rat hippocampal slices

Another hypothesis that would reconcile the simultaneous involvement of P2 and A_{2A} receptors in the facilitation of LTP by ATP analogues would be a P2 receptor-induced release of adenosine that would then activate adenosine A_{2A} receptors. Thus, we investigated if ATP analogues could trigger adenosine outflow from rat hippocampal slices. Fig. 5a shows that βγ-Imido ATP (20 μM) induced a release of tritium from hippocampal slices previously labelled with [^{3}H]adenosine to a value 147.7±2.3% greater than baseline (n=4; P<0.05). The P2 receptor antagonists, PPADS (10 μM; n=4) and suramin (75 μM; n=3; data not shown), were devoid of effects on basal tritium outflow but prevented this facilitatory effect of βγ-Imido ATP (20 μM) on tritium outflow (Fig. 5A).

We then tried to explore if there was any particular component in the slices that might contribute more intensely for this ATP-induced adenosine release. As illustrated in Fig. 5B, βγ-Imido ATP (20 μM) triggered an outflow of tritium that was greater in nerve terminals (n=4) than in slices (n=3) and had similar amplitude in slices and in cultured astrocytes (n=3). The βγ-Imido ATP (20 μM)-induced outflow of tritium from cultured neurons (n=4) was consistently lower than in all other tested hippocampal preparations (Fig. 5B). This suggests that the P2 receptor-evoked release of adenosine may mainly be derived from nerve terminals and astrocytes although it should be kept in mind that the expression and function of P2 receptors and nucleoside transporters may be modified in cultured neurons or astrocytes. We also confirmed that βγ-Imido

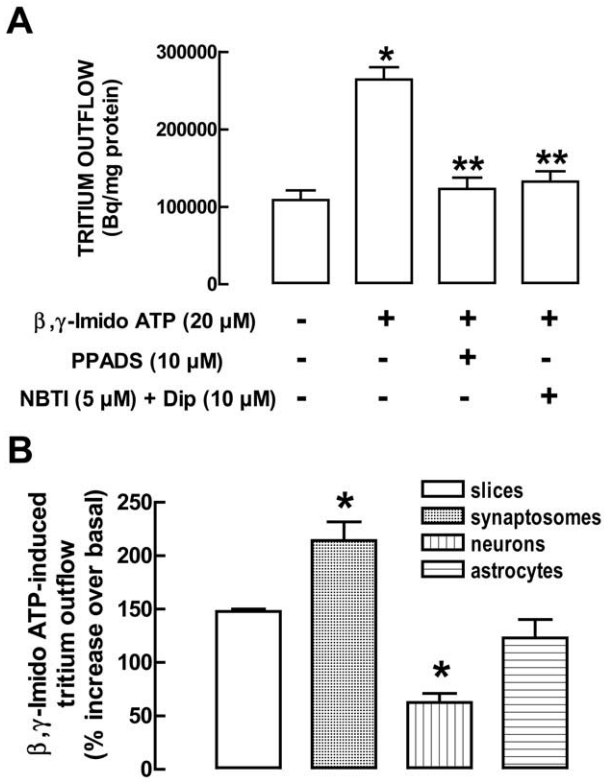

Fig. 5. ATP analogues trigger the release of tritium from rat hippocampal preparations previously loaded with [^{3}H]adenosine. All the experiments were performed in the presence of the adenosine A_1 receptor antagonist, DPCPX (50 nM). Panel A shows that βγ-Imido ATP (20 μM) triggers an outflow of tritium from rat hippocampal slices that is prevented by the P2 receptor antagonist, PPADS (10 μM), and by the adenosine transport inhibitors, NBTI (5 μM) and dipyridamole (Dip; 10 μM), which by themselves do not modify significantly the basal outflow of tritium. Each bar is the mean±S.E.M. of four experiments. Panel B compares the ability of βγ-Imido ATP (20 μM) to trigger tritium release from hippocampal slices (first bar from the left), hippocampal nerve terminals (second bar from the left), cultured hippocampal neurons (third bar from the left) and astrocytes (fourth bar from the left), previously loaded with [^{3}H]adenosine. Each bar is the mean±S.E.M. of three to four experiments. * P<0.05 when compared with the βγ-Imido ATP-induced tritium release from hippocampal slices (first bar).

ATP (20 μM) indeed enhanced [^{3}H]adenosine release. In fact, βγ-Imido ATP (20 μM) enhanced by 216±12% the outflow of adenosine from hippocampal nerve terminals (n=3). This indicates that tritium release might be a good measure of adenosine release and show that nerve terminals might be major contributors for this P2 receptor-induced release of adenosine.

Adenosine transport blockers prevent the ATP-induced [^{3}H]adenosine outflow

The extracellular accumulation of adenosine may result from two different sources: either from a release of adenosine as such through the bi-directional non-concentrative nucleoside transporters or from the formation of adenosine by the extracellular catabolism of released adenine nucleotides (Cunha, 2001). We tested the ability of inhibitors of

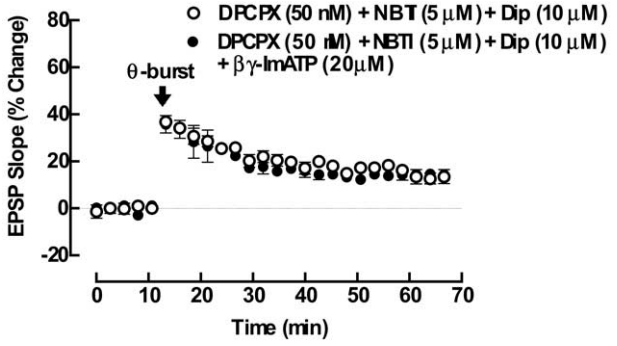

Fig. 6. Effect of the two inhibitors of the bi-directional non-concentrative adenosine transporters, NBTI and dipyridamole (Dip), on the facilitation by ATP analogues of LTP induced by θ-burst stimulation pattern in rat hippocampal slices. The figure shows the time course of the changes (%) in fEPSP slope. The arrow represents the time of application of the θ-burst stimulation pattern. All the experiments were performed in the presence of the adenosine A_1 receptor antagonist, DPCPX (50 nM). The figure shows that the facilitatory effect of βγ-Imido ATP (20 μM) on θ-burst-induced LTP is prevented in the presence of NBTI (5 μM) and dipyridamole (Dip, 10 μM). Each point represents the mean±S.E.M. of three experiments.

nucleoside transporters, dipyridamole and NBTI, to modify the P2 receptor-induced outflow of adenosine from rat hippocampal slices. We observed that the simultaneous presence of NBTI (5 μM) and dipyridamole (10 μM) virtually abolished the ability of βγ-Imido ATP (20 μM) to trigger tritium outflow from rat hippocampal slices previously labelled with [^{3}H]adenosine (Fig. 5A). This strongly suggests that the ATP-induced outflow of adenosine might essentially result from a release of adenosine mediated by the bi-directional non-concentrative adenosine transporters.

Adenosine transport blockers prevent the facilitatory effect of ATP analogues on LTP

To further reinforce our hypothesis that the facilitation by ATP analogues of hippocampal LTP involves a P2 receptor-mediated release of adenosine followed by activation of adenosine A_{2A} receptors, we tested the effect of NBTI and dipyridamole on the facilitatory effect of βγ-Imido ATP (20 μM) on LTP. The facilitatory effect of βγ-Imido ATP (20 μM) on LTP was prevented in the presence of NBTI (5 μM) and dipyridamole (10 μM), a value of 12.4±2.9% being obtained in the presence of NBTI, dipyridamole and βγ-Imido ATP, compared with a value of 15.5±1.2% when βγ-Imido ATP was not present (n=3; Fig. 6).

Activation of adenosine A_{2A} receptors facilitates LTP and occludes the facilitation of LTP by ATP analogues

If indeed adenosine A_{2A} receptor activation occurs downstream of P2 receptor activation, then an exogenously added high affinity agonist of A_{2A} receptors should by itself facilitate θ-burst LTP. In fact, the prototypical A_{2A} receptor agonist, CGS 21680 (10 nM), enhanced θ-burst LTP, a value of 47.7±2.7% being obtained in the presence of CGS 21680 (10 nM), compared with a value of 32.6±5.6% in the absence of CGS 21680 (n=3; P<0.05; Fig. 7A).

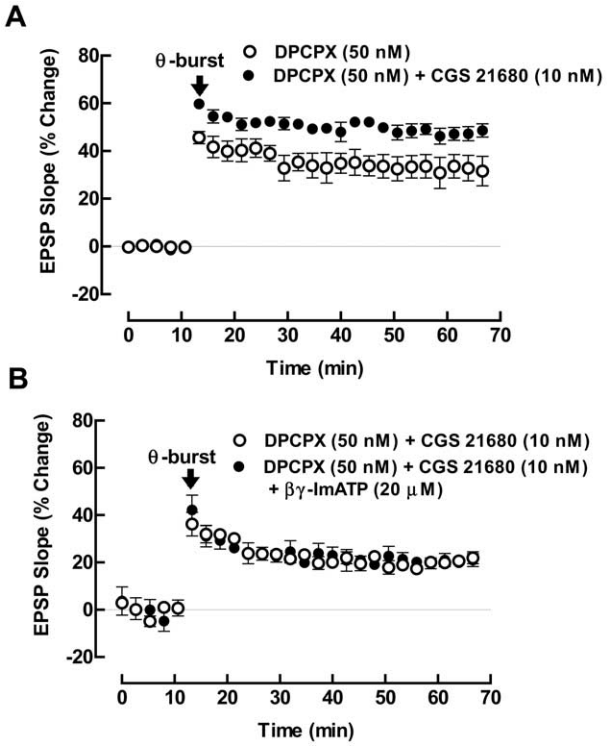

Fig. 7. The adenosine A_{2A} receptor agonist, CGS 21680, enhances LTP and occludes the facilitatory effect of ATP analogues on LTP induced by θ-burst stimulation pattern in rat hippocampal slices. Both panels show the time course of the changes (%) in fEPSP slope. The arrow represents the time of application of the θ-burst stimulation pattern. All the experiments were performed in the presence of the adenosine A_1 receptor antagonist, DPCPX (50 nM). Panel A shows that CGS 21680 (10 nM; ●) facilitates LTP compared with its absence (○). Panel B shows that the facilitatory effect of βγ-Imido ATP (20 μM) on θ-burst-induced LTP is prevented in the presence of CGS 21680 (10 nM). Each point is the mean±S.E.M. of three experiments.

Also, if the P2 receptor-mediated facilitation of LTP indeed depends on adenosine release followed by A_{2A} receptor activation then one would expect that the direct activation of A_{2A} receptors with CGS 21680 should occlude the facilitation of LTP by ATP analogues. As predicted, CGS 21680 (10 nM) prevented the facilitation of LTP by βγ-Imido ATP (20 μM), a value of 32.1±6.6% being obtained in the presence of CGS 21680 and βγ-Imido ATP, compared with 30.4±5.3% in the absence of βγ-Imido ATP (n=3; P>0.05; Fig. 7B).

Protein kinase C, but not protein kinase A, prevents the facilitatory effect of ATP analogues on LTP

The results so far obtained show that the effect of ATP analogues on LTP requires the recruitment of adenosine A_{2A} receptors after P2 receptor-induced adenosine release. Since adenosine A_{2A} receptor facilitation of hippocampal synaptic transmission requires the activation of protein kinase C but not protein kinase A (Cunha and Ribeiro, 2000b), we tested the effect of the inhibitor of the protein kinase C, chelerythrine, on the facilitatory effect of βγ-Imido ATP on LTP. The facilitatory effect of βγ-Imido

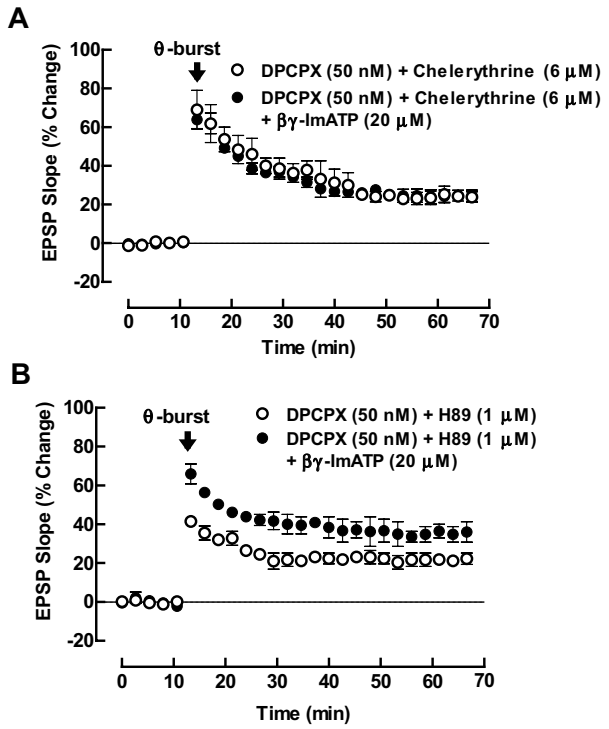

Fig. 8. Effect of a protein kinase C inhibitor (chelerythrine) and of a protein kinase A inhibitor (H89) on the facilitation by ATP analogues of LTP induced by θ-burst stimulation pattern in rat hippocampal slices. The panels show the time course of the changes (%) in fEPSP slope. The arrow represents the time of application of the θ-burst stimulation pattern. All the experiments were performed in the presence of the adenosine A_1 receptor antagonist, DPCPX (50 nM). Panel A shows that the facilitatory effect of βγ-Imido ATP (20 μM) on θ-burst-induced LTP is prevented in the presence of chelerythrine (6 μM; ●) compared with the its absence (○). Panel B shows that the facilitatory effect of βγ-Imido ATP (20 μM) on θ-burst-induced LTP was not modified in the presence of H89 (1 μM; ●) compared with its absence (○). Each point represents the mean±S.E. of two experiments.

ATP (20 μM) on LTP was prevented by chelerythrine (6 μM), a value of 24.5±3.2% being obtained in the presence of chelerythrine and βγ-Imido ATP compared with a value of 24.1±3.2% when βγ-Imido ATP was not present (n=2, Fig. 8A). We also tested the effect of an inhibitor of the protein kinase A, H89, on the facilitatory effect of βγ-Imido ATP on LTP. The facilitatory effect of βγ-Imido ATP (20 μM) on LTP was not prevented by H89 (1 μM), a value of 35.9±4.1% being obtained in the presence of H89 and βγ-Imido ATP compared with a value of 21.8±2.7% when βγ-Imido ATP was not present (n=2; Fig. 8B).

DISCUSSION

The present results show that the modulation by extracellular ATP analogues of θ-burst-induced LTP in rat hippocampal slices requires the activation of P2 receptors, but ultimately depends on the induced release of adenosine and activation of adenosine receptors. Upon blockade of inhibitory adenosine A_1 receptors, ATP activates P2 receptors that trigger an enhanced adenosine outflow through nucleoside transporters, particularly from nerve terminals and astrocytes, and this released adenosine then activates facilitatory A_{2A} receptors causing an enhancement of LTP via a protein kinase C-dependent pathway.

Under basal frequencies of stimulation in rat hippocampal slices (one stimulus every 20 s), the effects of exogenously added ATP analogues are due to A_1 receptor activation upon extracellular catabolism of ATP into adenosine through the ecto-nucleotidase pathway (Dunwiddie et al., 1997; Cunha et al., 1998). Although in Schaffer fibre synapses it is possible to reveal a minor component of the excitatory postsynaptic current mediated by P2X receptors (Pankratov et al., 1998, 2002), it is observed that both the neuromodulatory effects and the direct effects of exogenously added ATP on the post-synaptic component are abolished by selective blockade of adenosine A_1 receptors (Dunwiddie et al., 1997) and are not observed in A_1 receptor knockout animals (Masino et al., 2002). In fact, the kinetic efficiency of the ecto-nucleotidase pathway determines a fast and localised catabolism of adenine nucleotides into adenosine and the channelling of the minor amounts of adenosine formed to the highly abundant A_1 receptors (Dunwiddie et al., 1997; Cunha et al., 1998), and this occurs both presynaptically (Cunha et al., 1998) and postsynaptically (Dunwiddie et al., 1997). We now found that the same occurs during θ-burst LTP, i.e. that exogenously added ATP analogues inhibited LTP in a way reminiscent of the known inhibitory effects of adenosine on LTP (de Mendonça and Ribeiro, 1990; Arai et al., 1990). Indeed, this inhibitory effect of ATP analogues on LTP was prevented by DPCPX, but was not modified by the P_2 receptor antagonists, PPADS or suramin, indicating that the inhibitory effects were due to the formation of adenosine, and did not involve a P2 receptor-mediated action, as recently suggested in clamped pyramidal neurons (Pankratov et al., 2002). ATP analogues can thus exert inhibitory effects on LTP, which are apparently not mediated by purinergic P2 receptors and involve formation of adenosine acting through adenosine A_1 receptors. This likely results from the broad distribution of ecto-nucleotidases in different cell types in the CNS (reviewed by Zimmermann and Braun, 1999) and to the abundance and broad distribution of A_1 receptors in the hippocampus (e.g. Fastbom et al., 1987) that biases the effect of exogenously added ATP analogues. In fact, we have previously shown that exogenously added and endogenously released adenine nucleotides produce opposite effects in the modulation of hippocampal synaptic transmission (Cunha et al., 1996c). Indeed, we now observed that, when blocking the adenosine A_1 receptors, excitatory effects for the ATP analogues could be revealed, which were genuinely mediated by P2 receptors, since they were blocked by the P2 receptor antagonists, PPADS or suramin.

At this stage, it is not possible to reliably study the role of endogenously released ATP on LTP since the available antagonists of P2 receptors also interfere with different ionotropic receptors, namely with NMDA receptor function (Nakazawa et al., 1995; Ong et al., 1997; Peoples and Li, 1998) that is of critical importance for LTP induction (Col-

lingridge et al., 1983). Thus, we studied the mechanisms involved in these excitatory effects of the ATP analogues on LTP, which might be of particular relevance since ATP is released from stimulated hippocampal nerve terminals (Cunha et al., 2000) and the release of ATP is disproportionally larger upon conditions of stimulation known to induce LTP (Wieraszko et al., 1989; Cunha et al., 1996). As previously justified, it is only possible and reasonable to study the role of P2 receptors upon blockade of A_1 receptors. There are, at present, few compounds selective for either P2X or P2Y receptor subtypes. The observation that NF023, an antagonist moderately selective for the $P2X_{1/3}$ subunit containing receptors (Soto et al., 1999; Lambrecht, 2000), prevented the facilitatory effects of ATP analogues on LTP suggests that these receptor subtypes might be involved, a contention that is reinforced by the immunological confirmation of the localisation of P2X1, P2X2 and P2X3 receptor subunits in hippocampal nerve terminals (Rodrigues et al., manuscript in preparation). However, a detailed pharmacological characterisation must await the availability of more selective compounds and, at this stage, it is not possible to exclude the eventual involvement of P2Y receptors.

The facilitatory effects of ATP analogues on LTP were prevented by two selective antagonists of adenosine A_{2A} receptors. This result was not unexpected, since activation of P2 receptors was proposed to induce the release of adenosine (Nikbakht and Stone, 2000a,b) and we had previously reported that endogenously released adenine nucleotides trigger A_{2A} receptor activation in the hippocampus at low frequencies of stimulation (Cunha et al., 1996c). By directly measuring the amount of tritiated adenosine released from hippocampal slices, we confirmed that ATP analogues markedly enhanced the outflow of adenosine (Sperlagh et al., 2003). It is important to emphasise that this enhanced adenosine outflow is actually due to de novo purine release rather than from the extracellular catabolism of the added ATP analogues since the former is tritiated whereas the latter are not. The observation that the inhibitors of adenosine transporters nearly blocked this P2 receptor-induced adenosine outflow strongly suggests that the enhanced levels of extracellular adenosine may essentially result from a release of adenosine as such (see Cunha, 2001). We also found that the facilitatory effects of the ATP analogues on LTP were prevented in the presence of the blockers of bi-directional non-concentrative nucleoside transporters, NBTI plus dipyridamole. The mechanism whereby activation of P2 receptors modifies the function of nucleoside transporters is at present unknown. But the increase in extracellular adenosine levels activating adenosine A_{2A} receptors seems to be mandatory for the facilitatory effects of the ATP analogues on LTP, since these effects were blocked by two adenosine A_{2A} receptor selective antagonists, ZM 241385 and SCH 58261. Activation of the adenosine A_{2A} receptors is known to facilitate LTP irrespective of A_1 receptor blockade (de Mendonça and Ribeiro, 1994). We now replicated and extended this observation, showing that the adenosine A_{2A} receptor selective agonist, CGS 21680, facilitated θ burst-induced LTP and it has recently been reported that NMDA receptor activation bursts A_{2A} receptor-mediated modulation of hippocampal synaptic transmission (Nikbakht and Stone, 2001). In accordance with the previously observed coupling of adenosine A_{2A} receptors with protein kinase C rather than with protein kinase A (see Cunha and Ribeiro, 2000b), we found that the facilitatory effect of the ATP analogues on LTP were prevented by a protein kinase C inhibitor, chelerythrine, but not by a protein kinase A inhibitor, H89.

Thus, the present results indicate that ATP, which is released upon stimulation conditions that induce LTP, activates P2 receptors that lead to facilitation of LTP by a adenosine A_{2A} receptor-mediated and protein kinase C-dependent pathway. This concatenated effect of P2 and P1 (A_{2A}) receptors illustrates the tight interrelation between ATP and adenosine neuromodulatory systems and reinforces our previous contention (Cunha and Ribeiro, 2000) that blockade of ATP responses by P2 receptor antagonists does not exclude the involvement of P1 receptors in physiological effects triggered by ATP. Certainly, the present findings do not exclude that ATP released upon stimulation might exert other effects relevant for LTP not involving P2 receptors. In particular, ATP was reported to extracellularly phosphorylate membrane proteins (Fujii et al., 1995; Chen et al., 1996), although the presently tested ATP analogues were selected because they are poor phosphate donors (Wieraszko and Ehrlich, 1994; Kimiura et al., 1985). Interestingly, a brief decrease in the activities of the enzymes that metabolise ATP to adenosine, ecto-ATP diphosphohydrolase and ecto-5′-nucleotidase, was observed in the hippocampus when learning an inhibitory avoidance task (Bonan et al., 1998, 2000). We could speculate that this decreased catabolism would lead to higher levels of ATP acting upon P2 receptors to transiently facilitate the phenomena of synaptic plasticity important for memory acquisition.

In conclusion, the present work demonstrates a functional role for P2 receptors in the modulation of LTP that operate indirectly through induction of adenosine release.

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