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Extracellular ATP reduces optically monitored electrical signals in hippocampal slices through metabolism to adenosine

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Abstract

Electrical signals in rat hippocampal slices were optically monitored using a voltage-sensitive dye to determine whether extracellular ATP exhibits direct effects through its own receptors or indirect effects after its hydrolysis to adenosine. The dentate gyrus was stimulated and electrical signals in the CA1 and the CA3 region were analyzed. The signals were divided into two components: a transient component peaking within 10 ms (fast component) and a subsequent sustained component (slow component). ATP (10 to 100 μM) inhibited both the fast and the slow components in the CA1 region by about 30% and 70%, respectively. ADP, AMP and adenosine also inhibited the fast and the slow components. The inhibition by ATP was antagonized by aminophylline and other adenosine receptor antagonists, and by α,β-methylene ADP, an inhibitor of 5′-nucleotidases. These results suggest that extracellular ATP inhibits neuronal electrical signals in hippocampal slices after its metabolism to adenosine.

Keywords: ATP; Hippocampal slice; Electrical signal; Optical recording; Adenosine receptor

1. Introduction

Extracellular ATP has been shown to be a neurotransmitter in the central nervous system (Edwards et al., 1992; Inoue et al., 1995; Shen and North, 1993; Robertson and Edwards, 1998). Adenosine, a metabolite of ATP, is also known as a neuromodulator in the central nervous system (Latini et al., 1996; De Mendonça and Ribeiro, 1997). Although exogenously applied ATP is able to induce cellular responses directly through P2 receptors (e.g., Shen and North, 1993; Nakazawa et al., 1994; Koizumi and Inoue, 1997), several reports have also shown that ATP exhibits pharmacological effects after its hydrolysis to adenosine. Adenosine metabolized from ATP elicited a rise in intracellular Ca^{2+} concentration in rat hippocampal neurons (Dunwiddie et al., 1997) and inhibited neurotransmission in rat hippocampal CA1 pyramid synapses (Cunha et al., 1998).

Optical recording with voltage-sensitive dyes is recognized as a good strategy to analyze synaptic connections, neuronal circuits and spatial–temporal patterns of neuronal activity (Grinvald et al., 1982; Bonheffer et al., 1989; Salzberg, 1989; Ikeda et al., 1998; Murase et al., 1998). Nakagami et al. (1996, 1997a,b) applied this method to rat organotypic hippocampal cultures, where neuronal morphology and architecture have been retained, and have demonstrated that this system is suitable for the clarification of the basic properties of physiological propagation of neuronal excitability and its modulation by neurotrophic factors. The optical recording could also be used in pharmacological experiments aiming at the clarification of the effects of compounds in the presence of interneuronal communications, though such attempts have not been made. In the present study, we used this quasi-physiological tissue preparation to examine whether or not extracellular ATP exhibits direct effects through its own receptors or indirect effects through adenosine receptors after its metabolism to adenosine in rat hippocampal neurons.

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2. Materials and methods

2.1. Organotypic culture of hippocampus

Organotypic cultures of the hippocampus were made by the interface method (Stoppini et al., 1991). Brains were isolated from 9-day-old Wistar rats, and 200-μm-thick horizontal entorhino-hippocampal slices were prepared using a microslicer (DKT 1500; Dosaka E.M., Japan). Slices were maintained in ice-cold Gey's balanced salt solution gassed with 95% O_2 and 5% CO_2. The slices were then placed on a transparent membrane (Millicell-CM; Millipore, Bedford, MA, USA) and transferred into six-well plates with culture medium. The culture medium consisted of 50% minimal essential medium (Gibco, Grand Island, NY, USA), 25% Hank's balanced salt solution (HBSS) and 25% donor horse serum, and was supplemented with 6.5 mg/ml glucose (final concentration), 50 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate. The cultures were grown at 37°C with 5% CO_2 under a humidified atmosphere. The culture medium was changed every second day.

2.2. Optical recording

Optical recordings were performed according to Nakagami et al. (1996, 1997a,b) with some modifications. Preparations cultured for 10 to 14 days were used for optical recordings. The cultures were stained for 5 min in 0.2 mg/ml RH482 (Kankoh-Shikiso Kenkyusho, Okayama, Japan) dissolved in artificial cerebrospinal fluid (ACSF) containing 127 mM NaCl, 1.6 mM KCl, 1.24 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$ and 10 mM glucose. The cultures dissected with the transparent membrane were then transferred into an experimental chamber (about 2.5 ml in volume) placed on an inverted microscope, and superfused with ACSF for at least 15 min at 30°C. ACSF was continuously gassed with 95% O_2 and 5% CO_2 throughout the experiment. A bipolar tungsten electrode whose tips were coated for electrical isolation was inserted into the mossy fiber area of the dentate gyrus, and a rectangular pulse (0.5 to 1.0 mA) of 100 μs duration was delivered from a stimulator (SEN-3301; Nihonkohden, Japan) every 15 s. A conventional glass microelectrode filled with 0.9% NaCl was inserted into the CA3 region to record evoked population spikes extracellularly. While the population spikes were monitored on an oscilloscope (VC-6023; Hitachi, Japan) via an amplifier (MEG-1200; Nihonkohden, Japan), the intensity of the electrical stimulation to the dentate gyrus was adjusted such that the population spike amplitude reached its maximum.

The optical recording system (HR Deltaron 1700; Fuji Photo Film, Tokyo, Japan) consisted of a 128 × 128 photodiode array and a processing unit (Matsumoto and Ichikawa, 1990). Transmitted light with wavelength of 700 ± 20 nm was projected. The duration of light exposure was shortened to 1 s by a mechanical shutter to avoid photodynamic damage and dye bleaching. Before stimulation, an image of background light signals obtained by averaging 128 frames was stored in a reference memory. The stored reference data were continuously subtracted from real-time images and transferred to the processing unit sequentially at a frame rate of 0.6 ms. Every trial consisted of 512 real-time images, and 16 trial images were averaged to improve the signal-to-noise ratio. Off-line analysis of the averaged images was done with software attached to the recording system (ver. 1.22 and 1.30).

The application of ATP and other compounds was made by superfusion at a flow rate of about 1.5 ml/min. In previous experiments, a superfusion period of about 10 min was required until the block by NMDA receptor antagonists of glutamatergic neurotransmission was

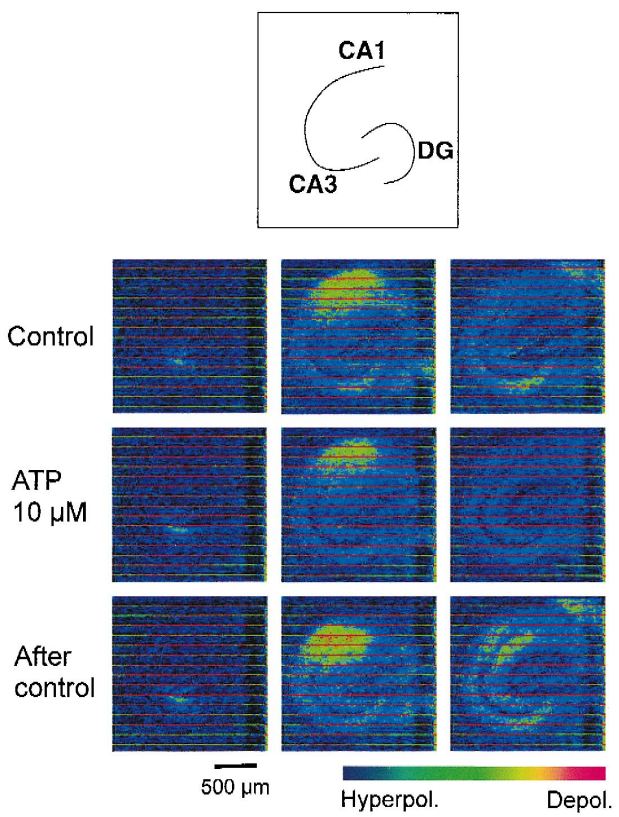

Fig. 1. Effects of ATP on optically monitored electrical excitation propagated from the dentate gyrus to the CA1 region via the CA3 region in a rat cultured hippocampal slice. Fractional absorbance changes were coded by pseudo-color scale shown below. Three frames are arranged for the responses before the application of ATP (Control; upper panels), during the application of ATP (ATP 10 μM; middle panels) or after the washout of ATP (After control; lower panels). The frames were made from optical signals measured 0, 9 and 15 ms (Control and ATP 10 μM; left to right) or 0, 6 and 12 ms after electrical stimulation (after control).

achieved in rat cultured hippocampal slices (Nakagami, unpublished observation), and so ATP and other substances were superfused for 20 min before optical recordings in most experiments in this report. The superfusion was stopped during optical recordings to avoid vibration of the water surface. The effects of ATP or other compounds were determined by analyzing optical signals in pyramidal cell layers of the CA1 and the CA3 region. The heights of peak signals were measured as indexes of the fast and the slow components (see Fig. 2A). In a part of the experiments, the areas under optical signal curves were calculated for 20 frames, beginning from the 10th frame after the fast peak excitation, where the peak of the slow component was involved, and these areas under the curves were adopted as indexes of the slow component.

2.3. Drugs

Drugs used were ATP (adenosine 5′-triphosphate disodium salt; Sigma, St. Louis, MO, USA), ADP (adenosine 5′-diphosphate sodium salt, Sigma), AMP (adenosine 5′-monophosphate, Sigma), adenosine (Sigma), UTP (uridine 5′-triphosphate trisodium salt; Wako, Osaka, Japan), UDP (uridine 5′-diphosphate sodium salt, Sigma), aminophylline (Sigma), α,β-methylene ADP sodium salt (Sigma), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; Sigma), and 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM241385; Tocris Crookson, UK).

3. Results

3.1. Inhibition by exogenous ATP of hippocampal electrical excitation

Fig. 1 illustrates optical monitoring of excitation propagating in a pathway from the dentate gyrus to the CA1 region via the CA3 region. Major peak excitation was found within 10 ms after the stimulation of the dentate gyrus in the CA3 or the CA1 region. In addition to this transient excitation (‘‘fast component’’), a sustained excitation (‘‘slow component’’), which gradually decreased over a period of 100 ms or longer, was also found (Fig. 2A). These biphasic properties of the excitation were similar to those reported for this preparation previously (Nakagami et al., 1996, 1997a,b). The effects of ATP on the two components were examined. When treated with 10 μM ATP for 20 min before and during the electrical stimulation, the fast component was significantly inhibited in the CA1 region, but not in the CA3 region (Fig. 2B). The slow component was also significantly inhibited in the CA1 region (Fig. 2C). The slow component in the CA3

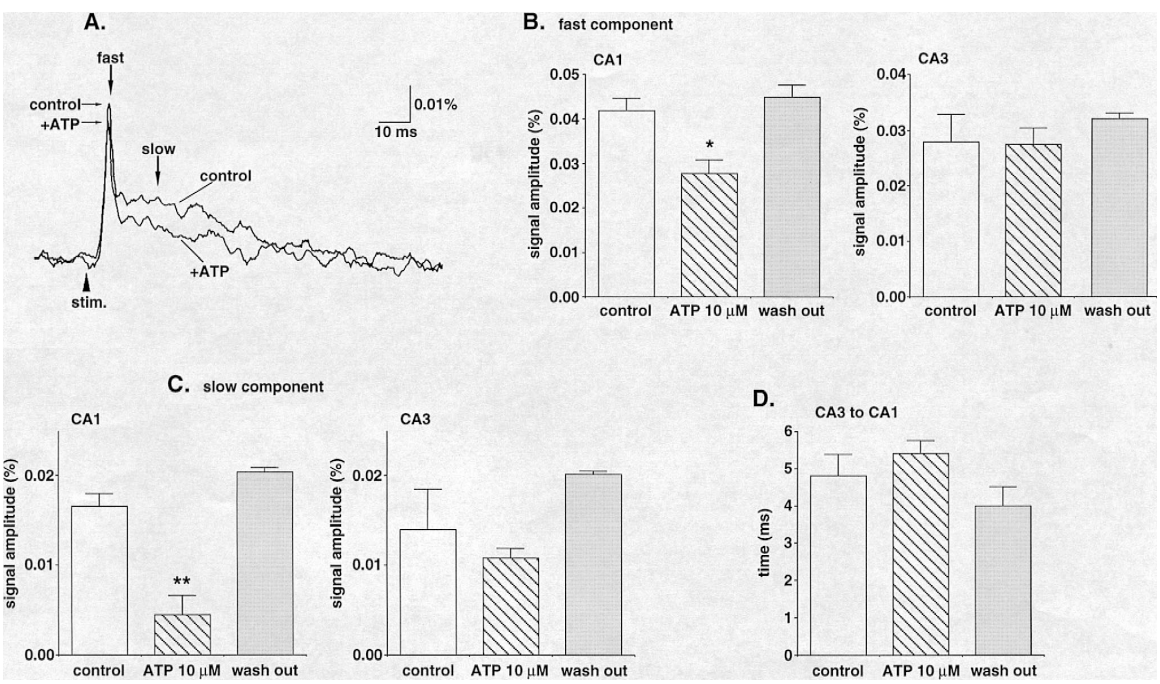

Fig. 2. (A) Effects of ATP (10 μM) on the time course of optically monitored electrical signals in the CA1 region. The signals consist of a fast and a slow component as indicated by arrows. ATP reduced both the fast and the slow components. (B, C) Effects of ATP (10 μM) on the fast (A) and the slow components (B) of electrical signals in the CA1 (left panels) and the CA3 region (right panels). Peak percent changes in absorbance in response to electrical stimulation were measured from three preparations before (control) and during the application of ATP (ATP 10 μM) and after the washout of ATP (wash out). Bars are S.E. Asterisks indicate significant difference from control signals (Tukey's test; $^{*}P < 0.05$, $^{**}P < 0.01$). (D) Propagation time from the CA3 region to the CA1 region. The data were obtained from the same preparations as in (B) and (C).

region was also decreased by ATP, though the decrease was not statistically significant. The inhibition by ATP was reversible both for the fast and the slow component, and the amplitude was restored to the same or somewhat higher levels compared with that before ATP application. The propagation velocity, determined as the time lag between the fast component in the CA3 region and that in the CA1 region, was not significantly affected by 10 μM ATP (Fig. 2D).

The inhibition by ATP of electrical signals in the CA1 region was further characterized. Fig. 3 shows the concentration–response relationship for the inhibition by ATP of electrical signals in the CA1 region. Because absolute signals were variable among batches, which may have depended on the culture conditions, the signals in the presence of ATP were normalized to control signals recorded before the ATP application in individual preparations. When determined in this manner, the inhibition was significant at 30 μM or higher for the fast component, and it was significant at 10 μM or higher for the slow component. The magnitude of the inhibition by 30 μM ATP was not significantly affected by shortening the pretreatment period of 20 min (see Materials and methods) to 10 min or prolonging it to 30 min (not shown).

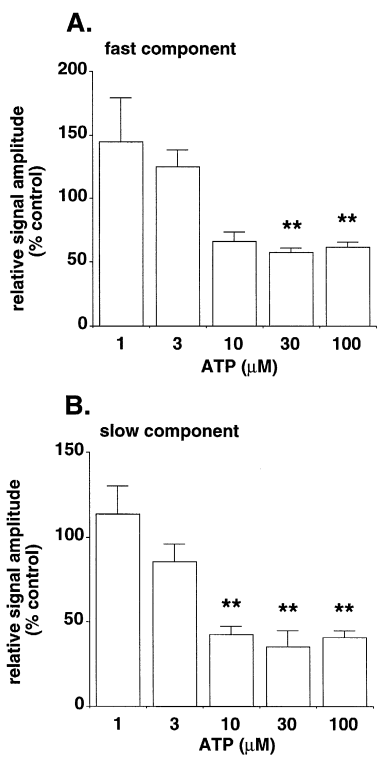

Fig. 3. (A, B) Dose–response relationship for the inhibition by ATP of the fast (A) and the slow components (B) of electrical signals in the CA1 region. Peak percent changes in absorbance in response to the electrical stimulation in the presence of ATP were normalized to those before the ATP application in individual preparations. Bars are S.E. Asterisks indicate significant differences from control signals when the data before normalization were compared by the paired *t*-test ($^{**}P < 0.01$; $n = 4$).

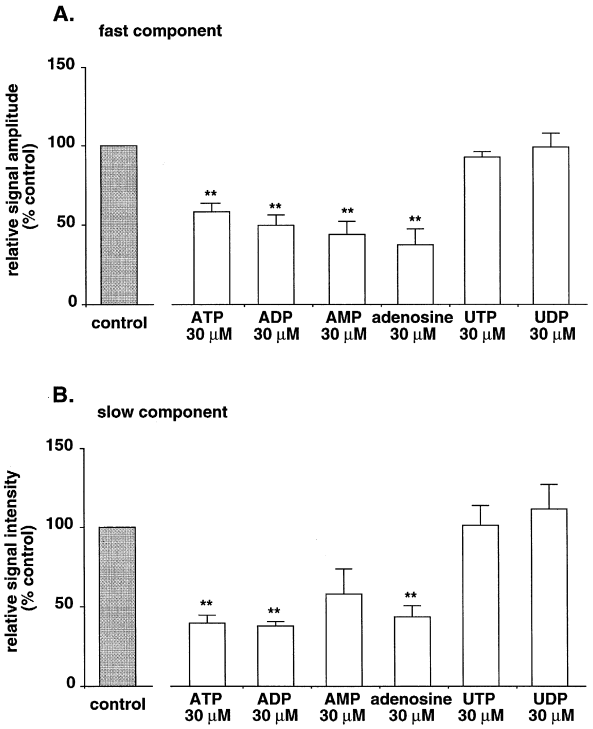

Fig. 4. (A, B) Effects of various nucleotides and adenosine (30 μM) on the fast (A) and the slow (B) components of electrical signals in the CA1 region. The data were normalized to those obtained before the application of the compounds. For the slow component shown in (B), the area under signal curve was measured as an index (see Materials and methods). Each column and bar indicates mean and S.E. obtained from four preparations. Asterisks indicate significant differences from control signals when the data before normalization were compared by the paired *t*-test ($^{**}P < 0.01$).

3.2. Effects of nucleotides and adenosine on electrical excitation

Fig. 4 shows the effects of various nucleotides and adenosine on the fast and the slow components in the CA1 region. ADP, AMP or adenosine (30 μM) reversibly inhibited the fast component to an extent similar to that obtained with 30 μM ATP (Fig. 4A). These compounds also reduced the slow component, though the reduction induced by 30 μM AMP was not statistically significant (Fig. 4B). UTP or UDP (30 μM) did not affect the fast or the slow component. Like ATP, the inhibition produced by adenine nucleotides and adenosine was reversible.

3.3. Breakdown of ATP to adenosine mediates its inhibitory effects

Because both adenine nucleotides including ATP and adenosine exhibited inhibitory actions in the CA1 regions, it was expected that the inhibition by ATP might have been mediated through the metabolism of ATP to adenosine. Thus, the influence of aminophylline, a classical

inhibitor of adenosine receptors, on the inhibition by ATP was examined. Aminophylline (100 μM) alone did not affect the fast or the slow component in the CA1 region. The inhibition by adenosine of the fast or the slow component was antagonized by aminophylline (Fig. 5A and B; left). Similarly, the inhibition by ATP of the fast or the slow component was antagonized by aminophylline (Fig. 5A,B; right).

Fig. 6 shows the influence of α,β-methylene ADP, a compound known to inhibit the breakdown of ATP by blocking 5′-nucleotidases (Dunwiddie et al., 1997). α,β-Methylene ADP (100 and 300 μM) attenuated the reduction by ATP of the fast component in a dose-dependent manner (Fig. 6A). This compound also attenuated the inhibition of the slow component though the effect was not statistically significant (Fig. 6B).

The effects of compounds known as antagonists selective for subclasses of adenosine receptors on the ATP- and the adenosine-induced inhibition of electrical excitability were examined. In the presence of DPCPX (1 μM), an antagonist selective for the adenosine A_1 receptor, or ZM241385 (100 nM), an antagonist selective for the adenosine A_{2A} receptor (Ralevic and Burnstock, 1998), ATP failed to inhibit the fast component of the electrical excitation (Fig. 7A). Similarly, adenosine failed to inhibit the fast component in the presence of these compounds (Fig. 7C). DPCPX also attenuated the inhibition by ATP or adenosine of the slow component (Fig. 7B and D). ZM241385 also attenuated the inhibition by ATP or adenosine of the slow component, though the attenuation was less remarkable than that exerted by DPCPX (Fig. 7B and D).

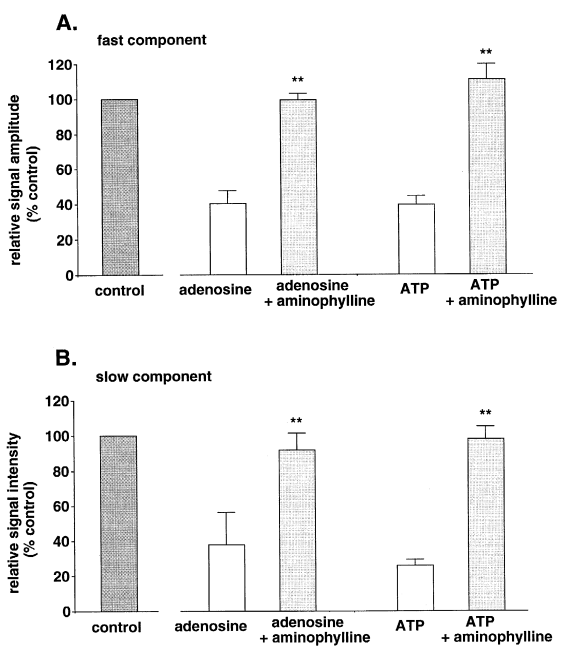

Fig. 5. (A, B) Antagonism by aminophylline (100 μM) of the inhibition by adenosine or ATP (30 μM) of the fast (A) or the slow component (B) in the CA1 region. The signals were normalized to those before the application of ATP or adenosine in individual preparations. Each column and bar indicates mean and S.E. from four preparations. Asterisks indicate significant differences from the signals with ATP or adenosine alone determined by the paired *t*-test ($^{**}P < 0.01$).

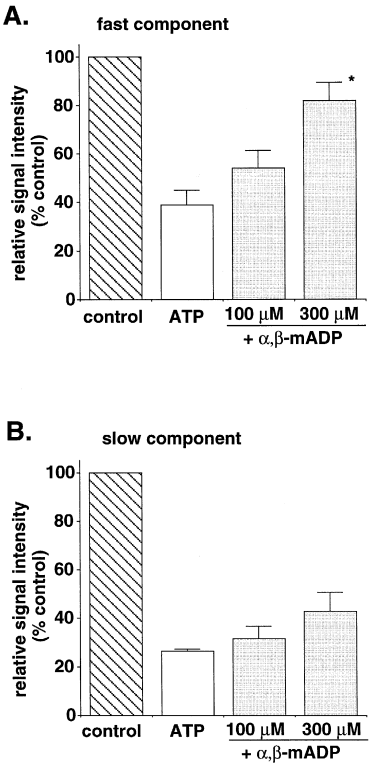

Fig. 6. Effects of α,β-methylene ADP (α,β-mADP) on the inhibition by 30 μM ATP of the fast (A) or the slow component (B). The slow component was measured as the area under the signal curve. The signals were normalized to those before the application of ATP. Each column and bar indicates mean and S.E. from four preparations. An asterisk indicates significant differences from the signals with ATP alone determined by the paired *t*-test ($^{*}P < 0.05$).

4. Discussion

Extracellular ATP exhibited inhibitory actions on optically monitored electrical signals in rat cultured hippocampal slices. The inhibitory actions appear to be mediated through the breakdown of extracellular ATP because (1) the inhibition was mimicked by ADP or adenosine (Fig. 4), and (2) the inhibition by ATP was abolished or attenuated by aminophylline or other adenosine receptor antagonists (Figs. 5 and 7), or α,β-methylene ADP (Fig. 6). A similar conversion of exogenously applied ATP to adenosine has recently been reported in rat cultured hippocampal neurons (Dunwiddie et al., 1997) and rat hippocampal CA1 pyramid synapses (Cunha et al., 1998). Our results, obtained by optical measurement of electrical excitability, also support the idea that ATP modulates neuronal activity after its metabolism to adenosine in the hippocampus.

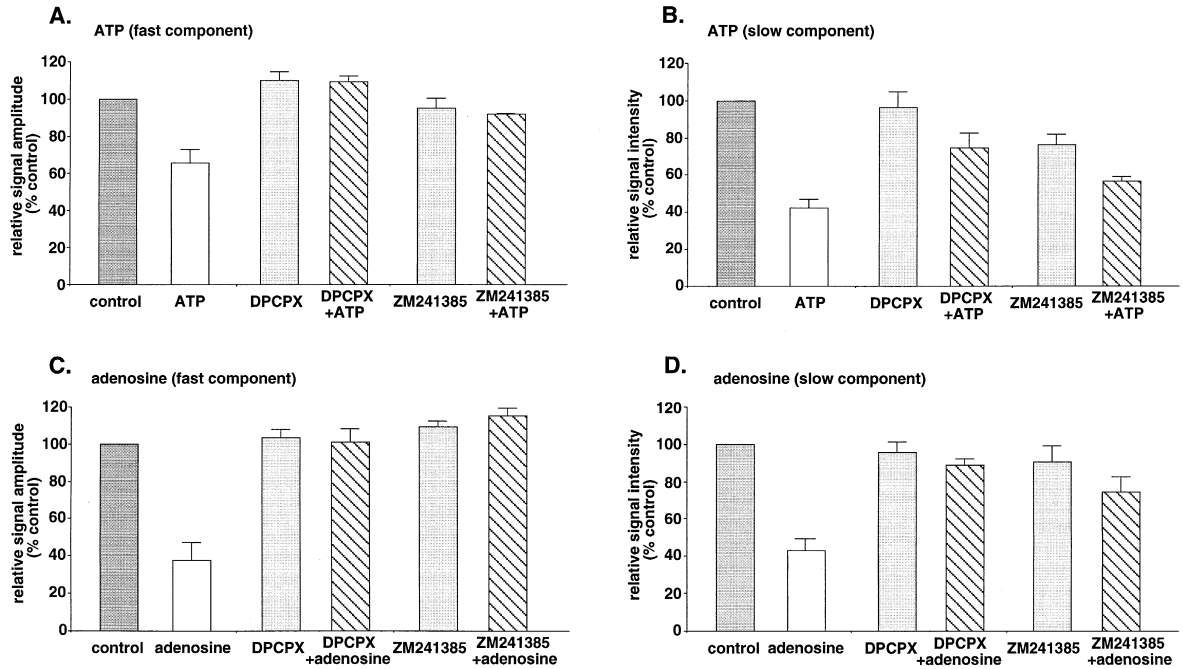

Fig. 7. (A–D) Inhibition by 30 μM ATP (A, B) or adenosine (C, D) of the fast (A, C) or the slow component (B, D) in the absence or presence of an adenosine A_1 receptor antagonist (1,3-dipropyl-8-cyclopentylxanthine; DPCPX; 1 μM) and an adenosine A_{2A} receptor antagonist (ZM241385; 100 nM). The slow component was measured as the area under the signal curve. The signals were normalized to those before the application of ATP. Each column and bar indicates mean and S.E. from four preparations.

The optical signals corresponding to the fast component are believed to reflect Na^+-dependent action potentials in neurons (Grinvald et al., 1982; Nakagami et al., 1996). It has been reported that the slow component reflects post-synaptic potentials (Grinvald et al., 1982), though other mechanisms such as local associated connections (Nakagami et al., 1997a) or glial depolarization (Konnerth et al., 1987) have also been proposed for this component. The inhibition by exogenously applied adenosine or adenosine metabolized from exogenous ATP of neurotransmitter release has been reported in the rat hippocampus (Cunha et al., 1998) and the rat medial habenula nucleus (Robertson and Edwards, 1998). The inhibition of neurotransmitter release may underlie the inhibition of the fast component in the present study because it reduces the generation of action potentials. The inhibition of neurotransmitter release may also contribute to the inhibition of the slow component if this component arises from post-synaptic potentials.

Excitatory actions of extracellular ATP on hippocampal neurons or other neurons in the brain have been reported. For example, exogenously applied ATP (Inoue et al., 1992) causes glutamate release in rat cultured hippocampal neurons (Inoue et al., 1992) and enhances fast excitatory neurotransmitter release from rat trigeminal mesencephalic nucleus (Khakh and Henderson, 1998). Such excitatory actions were not observed in the present study. We adopted a relatively long application period of ATP (20 min) because a rapid exchange of solutions was not possible with our optical recording system (see Materials and methods). This may be a limitation of the optical recording method when applied to pharmacological studies. The development of a special method for the application of ATP for a short period such as seconds is necessary to clarify whether or not excitatory actions are also observed with this method.

The inhibition by ATP of the electrical signals appeared to be mediated exclusively through adenosine receptors stimulated by adenosine metabolized from ATP, judging by the abolishment by aminophylline of the inhibition (Fig. 5). The lack of a direct contribution of ATP indicates that functional ATP receptors are not involved in the observed inhibition of the electrical signals. Unlike aminophylline, α,β-methylene ADP largely attenuated the inhibition by ATP of the fast component, but only weakly attenuated the inhibition of the slow component (Fig. 6). These results can be explained, for example, by assuming that the amount of adenosine required for the inhibition of the slow component is smaller than that for inhibition of the fast component, and that the reduced level of adenosine in the presence of α,β-methylene ADP is still sufficient to inhibit a large part of the slow component.

The inhibition by ATP or adenosine of the fast component was abolished in the presence of DPCPX or ZM241385 (Fig. 7A and C). These results suggest that both adenosine A_1 and adenosine A_{2A} receptors contribute to the inhibition. The contribution of these two subclasses of receptors may be cooperative, and, if one of these subclasses is blocked, the inhibition of the fast component may be abolished, as seen in the present study. The inhibition of the slow component was also attenuated by

these antagonists (Fig. 7B and D), indicating that the two subclasses are also involved in this inhibition. It is also noted that ZM241385 was reported to inhibit responses mediated through the adenosine A_1 receptor in the rat hippocampus (Lopes et al., 1999). Thus, it is possible that the antagonism by this compound of adenosine in the present study is attributable to its antagonism of adenosine A_1 receptors. Further pharmacological characterization is necessary to determine the exact adenosine receptor subclasses contributing to the inhibition. Cunha et al. (1998) reported that the inhibition by adenosine metabolized from ATP of rat hippocampal CA1 neurotransmission was largely (by 80%) prevented by DPCPX, though they did not test the effects of A_2 antagonists.

In conclusion, we have demonstrated that extracellular ATP inhibits optically monitored electrical signals in cultured rat hippocampal slices, and that this inhibition is mediated by the conversion of ATP to adenosine. This finding in the slices may provide evidence for functional roles of extracellular ATP and its metabolite adenosine in the brain: ATP released from nerve terminals may be converted to adenosine to serve as an inhibitory modifier of the propagation of neuronal excitation under physiological conditions, or, alternatively, adenosine metabolized from ATP leaking from damaged cells under pathological conditions may serve as a factor that protects surviving cells from cell death by preventing hyper-excitation.

Acknowledgements

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