British Journal of Pharmacology (1997) 122, 51–58

Inhibition by ATP of calcium oscillations in rat cultured hippocampal neurones

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1 The effect of adenosine 5′-triphosphate (ATP) on glutamatergic synaptic transmission in hippocampus was examined by an indicator of intracellular Ca^{2+} oscillations. These oscillations were postsynaptic responses by glutamate released from presynaptic sites. ATP completely inhibited the oscillations in a concentration-dependent manner.

2 The ATP-induced inhibition was mediated via P2-purinoceptors since ATP exhibited the inhibitory action even in the presence of P1-purinoceptor antagonists. Also non-hydrolysable ATP analogues and uridine 5′-triphosphate (UTP) inhibited the oscillation.

3 The rank order of agonist potency of ATP analogues for inhibition of the Ca^{2+} oscillation was as follows: 2-methyl-thio-adenosine 5′-triphosphate $\geqslant$ ATP > adenosine 5′-O-(3-thiotriphosphate) > UTP > α,β-methylene-adenosine 5′-triphosphate. These inhibitory effects were insensitive to suramin. Judging from this rank order of potency, the inhibitory P2-purinoceptor could be assigned to a subclass of GTP-binding protein coupled-type receptors.

4 The site of action of ATP was thought to be presynaptic since ATP did not affect the postsynaptic Ca^{2+} responses by glutamate. These results suggest the existence of a presynaptic inhibitory P2-receptor that inhibits glutamate release in the hippocampus.

Keywords: ATP; P2-purinoceptor; hippocampal neurone; Ca^{2+} oscillation; glutamate

Introduction

Adenosine 5′-triphosphate (ATP) has joined the growing list of neurotransmitters in the central nervous system. The cDNAs encoding P2-purinoceptors coupled with guanosine 5′-triphosphate (GTP)-binding proteins (G-proteins) (Lustig *et al*., 1993; Webb *et al*., 1993) and those forming cation channels (Brake *et al*., 1994; Valera *et al*., 1994; Chen *et al*., 1995; Lewis *et al*., 1995) have been cloned and characterized. Northern blot analysis shows that mRNAs coding for these purinoceptors are expressed in various brain regions including the hippocampus (Collo *et al*., 1996; Séguéla *et al*., 1996). It has been shown that ATP is released from hippocampal slices by electrical stimulation of Schaffer collateral-comissural afferents (Wieraszko *et al*., 1989), and that ATP mediates synaptic transmission in the rat cultured hippocampus to stimulate a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in postsynaptic neurones (Inoue *et al*., 1995), and induces long-lasting enhancement of the population spikes (Wieraszko & Seyfried, 1989; Nishimura *et al*., 1990). These findings suggest that ATP should have an excitatory role for the neuronal transmission in the hippocampus. Long term potentiation (LTP), a phenomenon which may underlie the process of memory and learning (see Bliss & Lynch, 1988; Malenka *et al*., 1989) has been extensively studied in relation to glutamate, the major excitatory neurotransmitter in the hippocampus. A rise in $[Ca^{2+}]_i$ mediated by glutamate receptors is an event considered to be essential for the induction of LTP, ATP, as well as other molecules, including arachidonic acid (Bliss *et al*., 1991), nitric oxide and carbon monoxide (Zhuo *et al*., 1993), are implicated in this event. ATP potentiates LTP in hippocampal preparations (Wieraszko & Ehrlich, 1994). We speculated that the mechanism of this potentiation was due to ATP-induced stimulation of glutamate release, because we had previously demonstrated by means of an electrophysiological technique, that ATP could evoke glutamate release in some synapses (Inoue *et al*., 1992). We next examined biochemically, the effect of ATP on the release of glutamate by use of a batch method. Unexpectedly, in these experiments ATP did not stimulate glutamate release but inhibited glutamate release from rat cultured hippocampal neurones (Inoue & Ohara-Imaizumi, unpublished data).

Long term depression (LTD), another form of synaptic plasticity, is also considered to be an important cellular mechanism for memory and learning in various brain regions including the hippocampus (Artola & Singer, 1993; Bear & Malenka, 1994). With regard to the mechanisms, several investigators have shown that LTD is due to a long term decrease in glutamate release from presynaptic terminals in the hippocampus (Bolshakov & Siegelbaum, 1994; Xiao *et al*., 1995). Therefore, it is very important to discover whether ATP inhibits or stimulates the release of glutamate. The discrepancies between the results on the effects of ATP may be due to variations in the neurones used. In an earlier study Ca^{2+} oscillations in cultured hippocampal neurones were examined as an indication of glutamate release (Ogura *et al*., 1987).

From the results obtained in the present study, we propose that ATP inhibits Ca^{2+} oscillations by attenuating release of glutamate via suramin-insensitive P2-purinoceptor-mediated mechanisms in cultured hippocampal neurones.

Methods

Cell preparations

Rat hippocampal neurones were isolated and cultured as described previously (Nakazawa *et al*., 1995). Hippocampal cortices were dissected from 17 day-foetal Wistar rats. The tissue was treated with papain (9 u ml^{-1}; Worthington Biochemical, Freehold, NJ, U.S.A.) dissolved in phosphate buffered saline solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin (Wako Pure Chemicals, Fraction V, Osaka, Japan). After en-

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zyme treatment at 37°C for 10 min, cells were dispersed by gentle agitation through a pipette and were plated on poly-L-lysine- and collagen-coated glass coverslips with a silicon rubber wall (Flexiperm, Heraeus Biotechnology, Hanau, Germany) at a density of 3×10^5 cells cm^2. The cells were maintained for up to 14 days in Dulbecco's modified Eagle's medium supplemented with 5% precolostrum newborn calf serum, 5% heat inactivated (56°C, 30 min) horse serum, 20 u ml^{-1} penicillin and 10 μg ml^{-1} streptomycin in a humidified atmosphere of 90% air and 10% CO_2 at 37°C.

Measurement of $[Ca^{2+}]_i$ in single cells

The changes in $[Ca^{2+}]_i$ in single cells was measured by the fura-2 method as described by Grynkiewicz *et al.* (1985) with minor modifications (Koizumi *et al.*, 1994; 1995). The cells were washed with balanced salt solution (BSS) of the following composition (mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.2, $MgCl_2$ 1.2, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) 25 and D-glucose 10, and incubated with 10 μM fura-2-acetoxymethylester (fura-2 AM) at 37°C in BSS. After 30 min incubation, the cells were washed with 0.2 ml of BSS. The coverslips were mounted on an inverted epifluorescence microscope (TMD-300, Nikon, Tokyo, Japan) equipped with a 75 W xenon-lamp and band-pass filters of 340 nm wavelength, for measurement of the Ca^{2+}-dependent signal (F340), and 360 nm wavelength, for measurement of the Ca^{2+}-independent signal (F360). Measurements were carried out at room temperature. Image data, recorded by a high-sensitivity silicon intensifer target camera (C-2741-08, Hamamatsu Photonics, Co., Hamamatsu, Japan) were regulated by a Ca^{2+} analysing system (Furusawa Laboratory Appliance Co., Kawagoe, Japan). The absolute $[Ca^{2+}]_i$ was estimated from the ratio of emitted fluorescence (F340/F360) according to a calibration curve obtained by using a standard Ca^{2+} buffer (Molecular Probes Inc., C-3712 with 1 mM $MgCl_2$). Drugs were dissolved in BSS and applied by superfusion. For the Ca^{2+}-depleted experiments, Ca^{2+} was omitted from BSS and 1 mM ethylene glycol-bis(β-aminoethyl ether) N,N,N′,N′-tetraacetic acid (EGTA) was added (Ca^{2+}-free BSS).

Chemicals

Drugs used were: adenosine 5′-triphosphate disodium salt (ATP; Yamasa Co., Choshi, Japan), adenosine 5′-monophosphate disodium salt (AMP; Oriental Yeast Co., Tokyo, Japan) and suramin monosodium salt (Bayer, Leverkusen, Germany). Fura-2 acetoxymethylester (fura-2 AM), HEPES, EGTA were purchased from Dojin (Kumamoto, Japan), α,β-Methylene-adenosine 5′-triphosphate ($\alpha\beta$-MeATP), 2-methylthio-adenosine 5′-triphosphate (2MeSATP), 8-cyclopentyl-1,3-dimethylxanthine (8-CPT), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and LY53857 (4-iso-propyl-7-methyl-9-(2-hydroxy-1-methylpropoxycarbonyl)-4,6,6A,7,8,9,10,10A-octahydroindolo-[4,3-fg]quinoline maleate) were from Research Biochemicals International (Natick, MA, U.S.A.), glutamate, adenosine 5′-O-(3-thiotriphosphate) (ATPγS), adenosine, D-amino-5-phosphonovaleric acid (D-APV), aminophylline and N_6-cyclohexyladenosine (CHA) were from Sigma (St. Louis, MO, U.S.A.). CP66713 (4-amino-8-chloro-1-phenyl-[1,2,4]-triazolo[4,3-a]-quinoxaline) was from Pfizer (CP, U.S.A.). CGS22492 (2-[(2-Cyclohexylethyl)amino]adenosine was a gift from Ciba-Geigy (Basle, Switzerland)) Tetrodotoxin (TTX) and other chemicals were from Wako Pure Chemical (Osaka, Japan). CNQX was dissolved in dimethyl sulphoxide at a concentration of 10 mM and then dissolved in BSS at the appropriate concentration. Other drugs were directly dissolved in BSS or Ca^{2+}-free BSS.

Results

Rat cultured hippocampal neurones exhibit synchronous Ca^{2+} oscillations via glutamate-mediated mechanisms

Figure 1 shows typical Ca^{2+} oscillations in 7-day-cultured hippocampal neurones. The Ca^{2+} oscillations were observed in cells cultured for 5–14 days. Although the amplitudes of each trace differ, traces (a)–(d) demonstrate synchronous Ca^{2+} oscillations whereas trace (e) shows non-synchronous oscillations, suggesting that cells (a)–(d) are coupled via synaptic connections. Removal of extracellular Ca^{2+} or the application

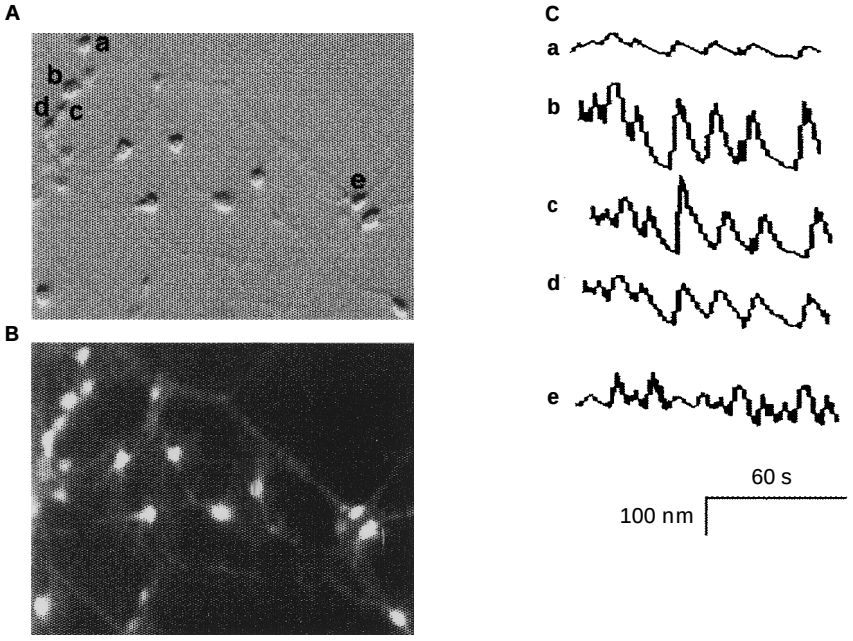

Figure 1 Typical periodical Ca^{2+} oscillations in the rat cultured hippocampal neurones. (A) Phase-contrast image of the hippocampal neurones (objective × 20). (B) Fluorescent image of the neurones (objective × 20). (C) Typical periodical Ca^{2+}-oscillation in the neurons. (a)–(e) Correspond to (a)–(e) shown in (A). Horizontal and vertical bars show 60 s and 100 nm, respectively.

of tetrodotoxin (TTX) abolished the Ca^{2+}-oscillations. (Figure 2a). In addition, CNQX (30 μM), a non-N-methyl-D-aspartate (NMDA) receptor antagonist, and D-APV (100 μM), a NMDA glutamate receptor antagonist also inhibited the Ca^{2+} oscillations (Figure 2b). Atropine (10 μM), a muscarinic cholinoceptor antagonist, inhibited the Ca^{2+} oscillations in some cells (12/46 cells tested), but hexamethonium (100 μM) had no effect on any of 62 cells tested. The Ca^{2+} oscillations observed in the atropine-sensitive neurones were also inhibited by CNQX. Noradrenaline (10 μM) (9 out of 64 cells) and 5-hydroxytryptamine (5-HT;10 μM) (6 out of 128 cells) but not dopamine (10 μM) (0 out of 128 cells) stimulated rise in $[Ca^{2+}]_i$ in some neurones and this effect was inhibited by phenoxybenzamine (10 μM), an adrenoceptor antagonist and LY53587 (10 μM), a 5-HT_2 receptor antagonist, respectively. Phenoxybenzamine ($n=63$) and LY53587 ($n=64$) had no effects on the Ca^{2+} oscillations by themselves (data not shown).

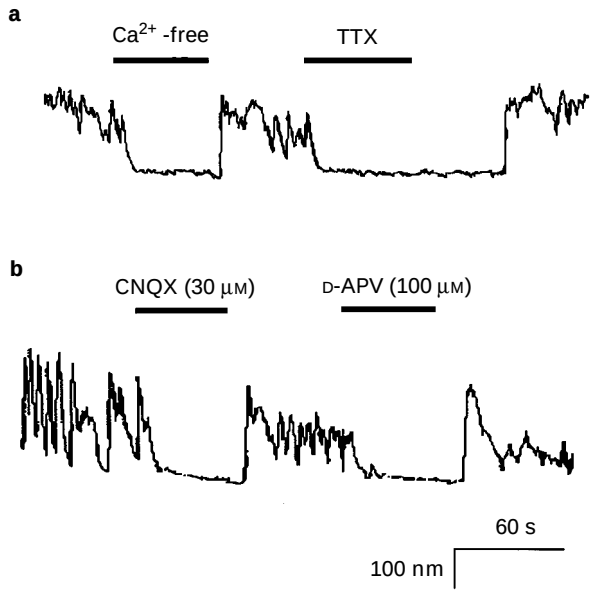

Figure 2 The effects of various reagents on the Ca^{2+} oscillations in the hippocampal neurones. (a) The effects of removal of extracellular Ca^{2+} or application of TTX on the Ca^{2+} oscillations. The perfusate was replaced by Ca^{2+}-free BSS (containing 1 mM EGTA) for 1 min or TTX (3 μM) was added to the cells for 1 min (horizontal bars). These treatments were separated by 1 min. (b) The effects of CNQX or D-APV on the Ca^{2+} oscillations. CNQX (30 μM) or D-APV (100 μM) was added to the cells for 1 min (horizontal bars) and each application was separated by 1 min. Horizontal and vertical scales show 60 s and 100 nm, respectively. At least three such experiments ($n=30-94$) were performed.

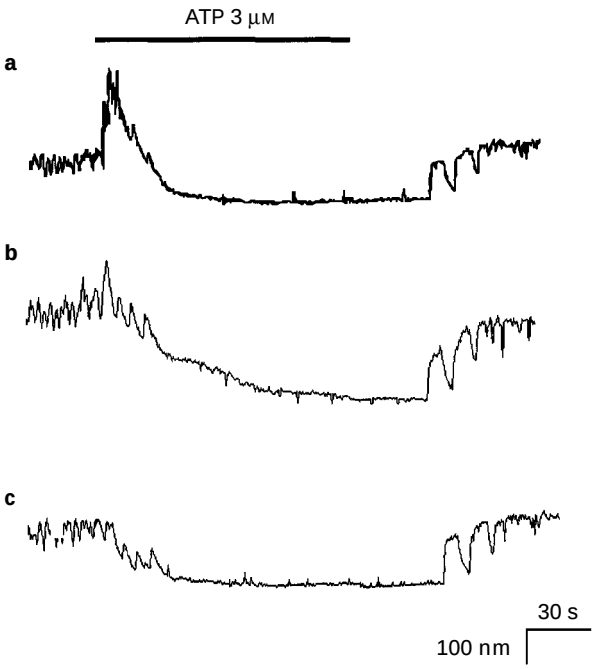

Figure 3 The effects of ATP on the Ca^{2+} oscillations in the hippocampal neurones. (a)–(c). Typical traces of Ca^{2+} oscillations evoked by 3 μM ATP. ATP was applied to the cells for 2 min (horizontal bar). (a) ATP produced a transient rise in $[Ca^{2+}]_i$ followed by a suppression of the oscillations. (b)–(c) ATP inhibited the Ca^{2+} oscillations without the transient $[Ca^{2+}]_i$ rise. Horizontal and vertical scales show 30 s and 100 nm, respectively. Four such separate experiments were performed ($n=128$).

Inhibition of Ca^{2+} oscillations by ATP

The effects of ATP on the Ca^{2+} oscillations were evaluated. As shown in Figure 3, at least two types of response were ob-

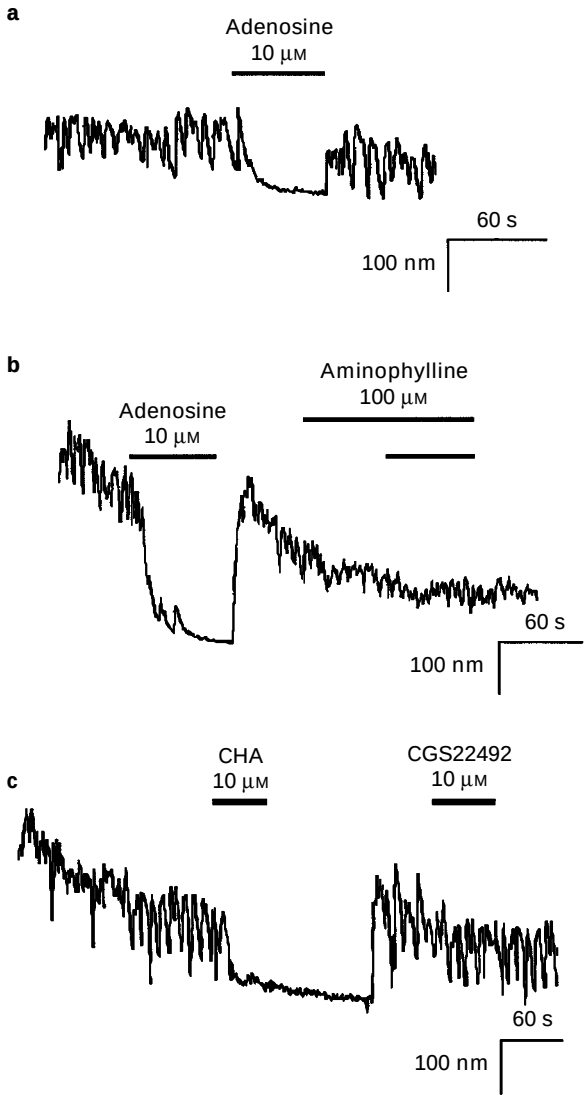

Figure 4 The effects of adenosine and its related compounds on the Ca^{2+} oscillations in the hippocampal neurones. Horizontal bars show the application of the chemicals shown. (a) When adenosine (10 μM) was applied to the cells for 1 min, it completely inhibited the Ca^{2+} oscillations. (b) The effects of aminophylline (100 μM) on the inhibition by adenosine (10 μM) of the Ca^{2+} oscillations. Aminophylline was applied to the cells 60 s before and during the application of adenosine. Adenosine was added to the cells twice for 60 s separated by 2 min. Aminophylline completely blocked the adenosine-induced inhibition. (c) The effects of CHA and CGS22492 on the Ca^{2+} oscillation. Each agent was applied to the cells for 60 s. Horizontal and vertical scales show 60 s and 100 nm, respectively. At least two such separate experiments were performed ($n=64-160$).

served. In some cells ATP induced a transient rise in $[Ca^{2+}]_i$ followed by inhibition of the Ca^{2+} oscillations (Figure 3a and b). In other cells, only inhibition of the Ca^{2+} oscillations was observed (Figure 3c). ATP-induced inhibition of Ca^{2+} oscillations was observed in almost all hippocampal neurones tested (344 out of 349 cells). ATP is metabolized to adenosine which is well known to inhibit several responses in the central nervous system (Phillis *et al*., 1975; Okada & Kuroda, 1980). Indeed, we demonstrated here that adenosine (10 μM) inhibited the Ca^{2+} oscillations in our cultured hippocampal preparation (Figure 4a, b), as shown previously (Kudo *et al*., 1991). Inhibition of the Ca^{2+} oscillations by adenosine was completely blocked by 100 μM aminophylline, a non-selective adenosine receptor antagonist (Figures 4b and 5a), and by 100 μM 8-CPT, an adenosine A_1 receptor antagonist, but not by CP66713, an antagonist of adenosine A_{2A} receptors (data not shown). The inhibition was mimicked by CHA (10 μM), an adenosine A_1 receptor agonist, but not by CGS22494 (10 μM), an adenosine A_{2A} receptor agonist (Figure 4c). These results support the hypothesis that adenosine-evoked inhibition of the Ca^{2+} oscillations is mediated by A_1 adenosine receptors.

Next, we tested the effects of aminophylline and 8-CPT on the ATP-induced inhibition of the Ca^{2+} oscillation (Figure 5). Although 100 μM aminophylline completely blocked the inhibitory action of adenosine (Figure 4b), it did not affect ATP-dependent inhibition (Figure 5b). Similarly, 8-CPT, at 100 μM, did not abolish the ATP-evoked inhibition although it did show partial attenuation of the ATP-dependent inhibitory action (Figure 5c). It is therefore unlikely that ATP inhibits the Ca^{2+} oscillation through adenosine receptors. This is supported by following experiments. Figure 6 shows the effects of various ATP analogues on the Ca^{2+} oscillations. ATP was inhibitory over a concentration range of 0.01 to 10 μM. ATPγS and α,β-MeATP, which are non-hydrolysable ATP analogues, also mimicked the inhibitory action. Moreover, UTP, which is not an adenosine derivative, mimicked the inhibition. We therefore concluded that P2-purinoceptor-mediated mechanisms are involved in the ATP-evoked inhibition on the Ca^{2+} oscillations.

Suramin, a non-selective P2-purinoceptor blocker, inhibited the rise in $[Ca^{2+}]_i$ evoked by ATP but did not affect the inhibition of the Ca^{2+} oscillation by ATP (Figure 5d). The rank order of agonist potency for the inhibition was: 2MeSATP (0.01–0.1) $\geqslant$ ATP (0.01–0.1) > ATPγS (1) > UTP (30) > α,β-MeATP (100). The values shown in parentheses are the appropriate minimum effective concentrations of the agonists (μM).

ATP did not affect the Ca^{2+} responses to glutamate

The effects of ATP on the Ca^{2+} responses to glutamate were investigated. Briefly glutamate (30 μM, 10 s) produced a transient increase in $[Ca^{2+}]_i$ in the neurones in the presence of TTX (Figure 7a). In these conditions, the $[Ca^{2+}]_i$ increase by exogenously applied glutamate is a response mediated through postsynaptic glutamate receptors. Glutamate was applied to the cells three times for 10 s, separated by 2 min and the first, second and third Ca^{2+} responses to glutamate were defined as S1, S2 and S3, respectively. ATP was applied to the cells 30 s

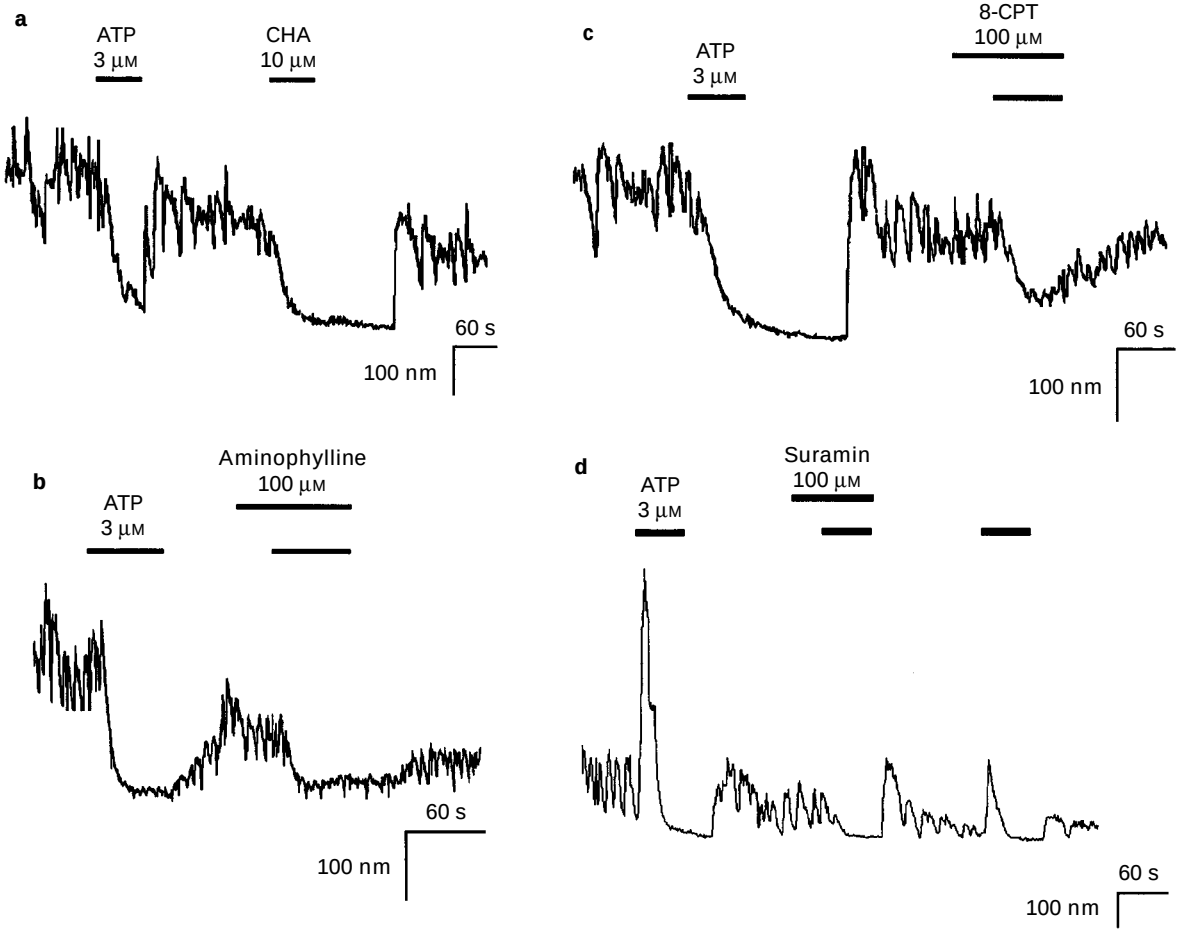

Figure 5 The effects of various reagents on the inhibition by ATP of the Ca^{2+} oscillations in the hippocampal neurones. Horizontal bars show the application of the various chemicals. (a) Inhibition by ATP or CHA of the Ca^{2+} oscillations in a cell. Both ATP (3 μM) and CHA (10 μM) inhibited the Ca^{2+} oscillations reversibly. (b,c) The effects of aminophylline (100 μM) (b) and 8-CPT (100 μM) (c) on the ATP-induced inhibition. Aminophylline and 8-CPT were applied to the cells 30 s before and during the ATP-application. (d) The effects of suramin (100 μM) of the ATP-evoked responses. Suramin was applied to the cells 30 s before and during the ATP-application. Horizontal and vertical scales show 60 s and 100 nm, respectively. At least five such separate experiments were performed ($n = 138-160$).

before and during the second glutamate application. The ratio of S2 over S1 was calculated and adopted as an index to compare differences between cells. In a control experiment, the $[Ca^{2+}]_i$ elevation evoked by the first glutamate stimulation was equivalent to that of the second and third glutamate application (Figure 7b). ATP (10 μM) did not affect the Ca^{2+} responses to glutamate ($n=25$). These data suggest that ATP (10 μM) does not inhibit the sensitivity of postsynaptic glutamate receptors.

Discussion

We demonstrated that ATP has an inhibitory role via P2-purinoceptors in the suppression of the release of glutamate, resulting in inhibition of the Ca^{2+} oscillations in the hippocampal neurones. There are several studies which support our findings. Exogenously applied ATP inhibits release of noradrenaline from sympathetic nerves in the rat vas deferens (Forsyth *et al*., 1991; von Kügelgen, 1994a), bovine adrenal

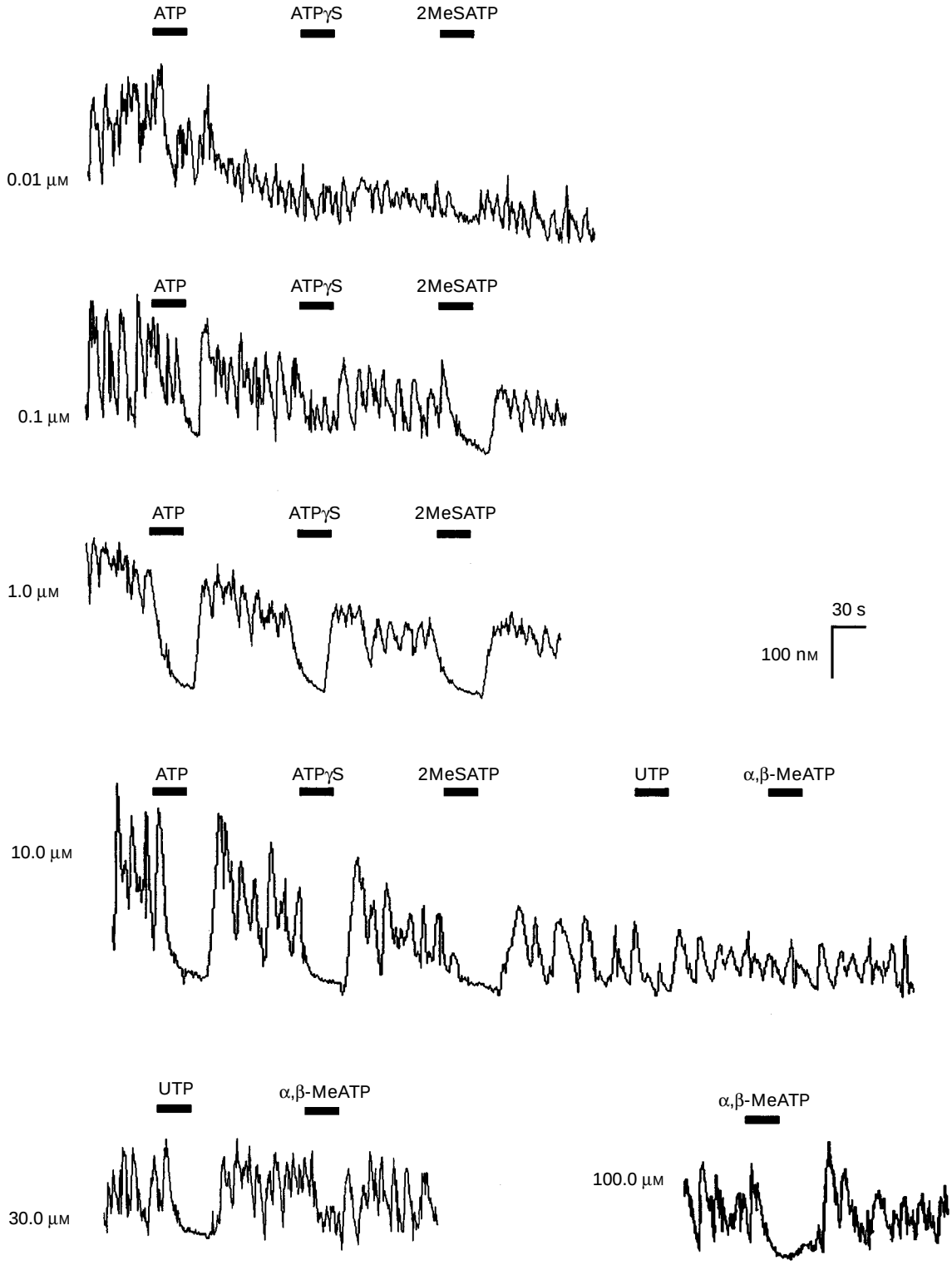

Figure 6 Comparison of the abilities of ATP and its analogues to inhibit the Ca^{2+} oscillations in the hippocampal neurones. Traces show representative results. Each compound was applied to the cells for 30 s (horizontal bars) at a concentration shown on the left-hand side of each trace. Horizontal and vertical scales show 30 s and 100 nm, respectively. At least three such separate experiments were performed ($n=96-128$).

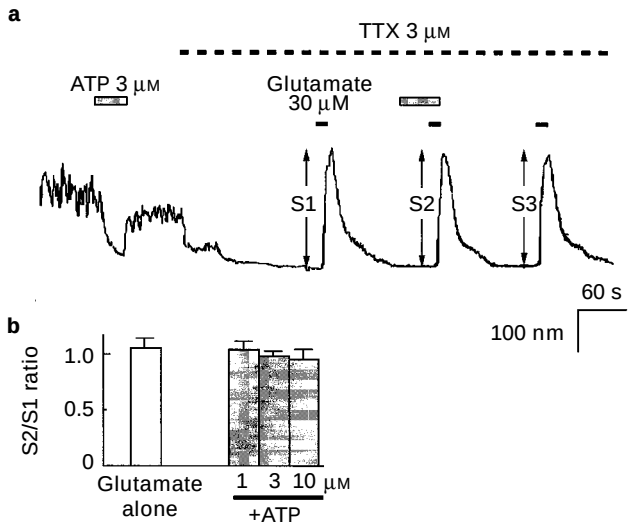

Figure 7 The effects of ATP on the Ca^{2+} responses to glutamate in the hippocampal neurones. (a) At first, ATP (3 μM) was applied to the cells for 60 s to confirm the existence of the ATP-induced inhibition. Next, TTX (3 μM) was applied to the cells in order to inhibit cell-to-cell communications and the Ca^{2+} oscillations (dotted horizontal bar). Then glutamate (30 μM) was applied to the cells three times for 15 s separated by 120 s. The first, the second and third Ca^{2+} responses to glutamate were defined as S1, S2 and S3, respectively. ATP (3 μM) was applied to the cells 30 s before and during the second ATP application. (b) The ratio of S2 to S1 in the presence of various concentrations of ATP was calculated and summarized. The data show mean $\pm$ s.e.mean of 28 (control) and 24–41 (+ATP) cells tested, obtained from three separate experiments.

chromaffin cells (Sasakawa *et al*., 1989; Currie & Fox, 1996) and rat brain cortex (von Kügelgen *et al*., 1994b).

Several investigators have shown the importance of the formation of the synapse for the appearance of Ca^{2+} oscillations in the cultured hippocampal (Ogura *et al*., 1987) and cortical neurones (Ichikawa *et al*., 1993; Muramoto *et al*., 1993). In our cultured hippocampal neurones the following observations support this view. (1) Ca^{2+} oscillations were observed only in the cells after at least 5 days of culture. (2) The Ca^{2+} oscillations were inhibited by removal of extracellular Ca^{2+}, a condition known to inhibit the release of neurotransmitters and by the application of TTX, which inhibits neuronal conduction. (3) In many cases, the Ca^{2+} oscillations appeared synchronized with other adjacent cells (Figure 1a–d), whereas more distant neurones displayed asynchronous Ca^{2+} oscillations (Figure 1e). (4) The Ca^{2+} oscillations were inhibited by CNQX or D-APV, glutamate receptor antagonists. Although atropine inhibited the Ca^{2+} oscillations in some cells, CNQX exhibited its inhibitory action even in such the atropine-sensitive cells (see Results). Therefore it is glutamatergic transmission that plays a critical role in the induction of Ca^{2+} oscillations. These data suggest that the oscillations are mediated via glutamatergic transmission through both NMDA- and non-NMDA glutamate receptors.

ATP inhibited the Ca^{2+} oscillations by a mechanism mediated by P2-purinoceptors. We previously found that ATP produces a rise in $[Ca^{2+}]_i$ in rat cultured hippocampal neurones (Inoue *et al*., 1995). Also we observed Ca^{2+} responses to ATP in this preparation in the presence of various blockers including TTX, CNQX, D-APV, atropine hexamethonium and cadmium ion, indicating that ATP directly stimulates excitatory P2-purinoceptors in the postsynaptic neurones to promote the increase in $[Ca^{2+}]_i$. The finding that ATP produced a transient rise in $[Ca^{2+}]_i$ in some cells (Figure 3a), and that this increase was inhibited by suramin (Figure 5d), suggests that the response is mediated by excitatory P2-purinoceptors in the postsynaptic neurones. In addition to the excitatory P2-purinoceptors, an inhibitory P2-purinoceptor also seems to be present in the hippocampal neurones. Adenosine, a metabolite of ATP, is well known to be an inhibitory neurotransmitter (Phillis *et al*., 1975; Okada & Kuroda, 1980), and has been suggested to be responsible for some inhibitions observed with ATP (Kudo *et al*., 1991). In agreement with these findings, both adenosine and CHA, an adenosine A_1 receptor agonist, potently inhibited the Ca^{2+} oscillations in the neurones (Figures 4 and 5). Our present results do not exclude the possibility that adenosine, quickly metabolized from ATP, may partly contribute to the ATP-evoked inhibition of the Ca^{2+} oscillation since 8-CPT, an adenosine A_1-receptor antagonist, slightly attenuated the ATP-evoked inhibition (Figure 5c). However, the majority of the ATP-evoked inhibition must be mediated by P2-purinoceptors because (1) 8-CPT could not abolish the ATP-induced inhibition, (2) aminophylline, a non-selective adenosine receptor antagonist, did not show any affect on the ATP-induced inhibition of the Ca^{2+} oscillations (Figure 5b) although aminophylline blocked the adenosine-evoked inhibition completely (Figure 4b), and (3) ATPγS and α,β-MeATP, non-hydrolyzable ATP analogues, and UTP, which is not an adenosine derivative, inhibited the Ca^{2+} oscillation (Figure 6). These results support the proposal that ATP acts directly on P2-purinoceptors to induce the observed inhibitory action, not via adenosine.

Another inhibitory neurotransmitter, γ-aminobutyric acid (GABA), may affect the Ca^{2+} oscillations. Ca^{2+} oscillations were inhibited by GABA and were amplified by bicuculline, an antagonist of $GABA_A$ receptors, in some neurones (data not shown). Inhibition by GABA of Ca^{2+} oscillations in various brain regions, including the hippocampus, has been observed only in mature neurones (Obrietan & Van den Pol, 1995), and a GABA-mediated inhibition was found to be developmentally-dependent in the hippocampus (Wagner & Alger, 1995). Thus, ATP may act as an inhibitor to compensate for the lack of an inhibitory action by GABA in this developmental stage.

Next we attempted to characterize the putative inhibitory P2-purinoceptors in the hippocampal neurones. Various concentrations of ATP, its analogues, and the pyrimidine nucleotide UTP were applied to the cells and their inhibitory potency was determined. The inhibitory potency rank order was: 2MeSATP $\geqslant$ ATP > ATPγS > UTP > α,β-MeATP. Judging from the rank order of potency of the ATP analogues, the inhibitory P2-purinoceptor may be assigned to a subclass of G-protein coupled-type receptors. As suramin was found to be a weak and ineffective antagonist of the ATP-induced inhibition of the Ca^{2+} oscillation, some type of suramin insensitive P2-purinoceptor (von Kügelgen *et al*., 1994b; Collo *et al*., 1996) may contribute to the inhibition. Moreover, a new type of inhibitory P2-purinoceptor, which has been suggested for the purinoceptors in vas deference (Forsyth *et al*., 1991; von Kügelgen *et al*., 1994a), caudal artery (Shinozuka *et al*., 1988) and rat cortex (von Kügelgen *et al*., 1994b), may be present in the hippocampus to suppress the Ca^{2+} oscillation. Further experiments are required to identify the subclass of P2-purinoceptors responsible for the inhibition of the Ca^{2+} oscillation.

We also examined the site of action of ATP. When glutamate is applied to the neurones in the presence of TTX, the neuron is independent of other neuronal networks due to the inhibition of neuronal conduction. We considered the elevation in $[Ca^{2+}]_i$ evoked by glutamate in the presence of TTX as a response mediated by postsynaptic glutamate receptors with little effects of other neuronal innervations. This glutamate-evoked rise in $[Ca^{2+}]_i$ was not affected by ATP (Figure 7). Thus, the inhibition by ATP appears not to be due to a decrease in sensitivity of postsynaptic glutamate responses. ATP has been shown to inhibit L-type voltage-gated Ca^{2+} channels (VGCCs) in PC12 cells (Nakazawa & Inoue, 1992) and N- and P/Q-type VGCCs in adrenal chromaffin cells (Currie & Fox, 1996). The release of glutamate in the hippocampal neurones is regulated by presynaptic both N- and Q-type VGCCs (Scholz & Miller, 1995). These findings raise the possibility that ATP

may exhibit its inhibitory action by suppressing presynaptic VGCCs to reduce the release of glutamate, which might result in inhibition of the Ca^{2+} oscillations.

Taken together, our results demonstrated that ATP, in addition to its excitatory action on the stimulation of a rise in $[Ca^{2+}]_i$ via postsynaptic P2-purinoceptors (inoue *et al*., 1995), has an inhibitory role in the suppression of the release of glutamate, probably via an effect on presynaptic P2-purinoceptors, leading to inhibition of the Ca^{2+} oscillations in the hippocampal neurones.

The authors appreciate Ms T. Obama for culturing the cells, Dr J. Kenimer for improving the manuscript and Dr Y. Ohno for continuous encouragement. This work was partly supported by the Japan Health Science Foundation.

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(Received March 14, 1997
Revised May 9, 1997
Accepted May 30, 1997)