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Effect of P_2-purinoceptor antagonists on glutamatergic transmission in the rat hippocampus

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1 A study has been made of the effects of P_2-purinoceptor antagonists on the evoked excitatory postsynaptic currents (e.p.s.cs) generated in CA1 pyramidal cells on stimulation of Schaffer collaterals and in CA3 pyramidal cells on stimulation of mossy fibres. The effects of these antagonists on currents generated in the cells on application of glutamate has also been determined.

2 Suramin blocked the evoked e.p.s.cs with an 50% inhibition (ID_{50}) of 62 ± 8 μM (mean ± s.e.mean, $n = 17$), spontaneous miniature e.p.s.cs and the currents induced by application of 100 μM glutamate with an $ID_{50} = 121 \pm 36$ μM ($n = 15$) in all the cells studied.

3 Reactive Blue 2 (RB-2) in a concentration of 200 μM decreased the e.p.s.cs by $80 \pm 10\%$ ($n = 6$) and the glutamate-activated currents by $83 \pm 3\%$ ($n = 6$).

4 Pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) in the concentration-range of 40–500 μM decreased the amplitude of the e.p.s.cs in 12 out of 13 cells studied. PPADS at 200 μM reduced the amplitude of the e.p.s.cs by $60 \pm 10\%$ ($n = 3$). PPADS did not affect the glutamate-induced currents in 4 cells and produced potentiation of the current amplitude by $60 \pm 10\%$ in 4 other cells.

5 These results suggest that both presynaptic and postsynaptic P_2-purinoceptors in the hippocampus can modulate the release and action of endogenous glutamate.

Keywords: P_2-purinoceptors; patch-clamp; hippocampal cells; suramin; Reactive Blue 2; PPADS

Introduction

It is recognised that fast excitatory synaptic transmission in hippocampus is mediated by endogenous glutamate which acts on AMPA/KA ionotropic receptors in the postsynaptic membrane (see Monaghan *et al.*, 1989; Headly & Grillner, 1990). Adenosine triphosphate (ATP), which is a transmitter at autonomic neuromuscular junctions (Burnstock, 1990) and ganglia (Silinsky *et al.*, 1992; Evans *et al.*, 1992; Silinsky & Gerzanich, 1993) acts through P_2-purinoceptors. ATP may also be a transmitter in the central nervous system (see Hoyle, 1992; Edwards *et al.*, 1992). Stimulation of nerves in the brain releases ATP (Wu & Phillis, 1978) and the RNA for the P_2-purinoceptor has recently been isolated and found in the brain (Brake *et al.*, 1994). Michel & Humphrey (1993), in radioligand binding studies, found high and low affinity binding sites for [^{3}H]-α,β-methylene-ATP which indicated the presence of P_{2X}-purinoceptors in hippocampus. Bo & Burnstock (1994) as well as Balkar *et al.* (1995) found a relatively high density of [^{3}H]-α,β-methylene-ATP binding sites in Ammon's horn of the hippocampus. Stimulation of Schaffer collaterals releases ATP; this release is dependent on the extracellular calcium concentration, and is not evoked by glutamate itself, leading to the suggestion that ATP may be stored with glutamate in synaptic vesicles so that they are released together (Wieraszko *et al.*, 1989). Furthermore, ATP at low concentrations (0.5 μM) increases the amplitude of the population spike in the CA1 region of the hippocampus due to stimulation of the Schaffer collaterals (Wieraszko & Seyfried, 1989).

Stone & Cusack (1989), however, could find no effects of non-hydrolysable ATP analogues for P_2-purinoceptors (Burnstock & Kennedy, 1985) in a concentration of 10 μM on the population spike or synaptic potential in CA1 due to stimulation of Schaffer collaterals; nor did these agonists at 10 μM change the neuronal responses to N-methyl-D-aspartate (NMDA). The possibility arises then that ATP exerts an excitatory effect at low concentrations indirectly because of its hydrolysis to adenosine, which is known to produce an enhancement of the postsynaptic field potentials in the hippocampus at low concentrations (Nishimura *et al.*, 1990; Okada *et al.*, 1990). Yet ATP (100 μM) induces rapid depolarization leading to action potential firing in some dissociated hippocampal neurones, perhaps because of the absence of ectoenzymes, and this is blocked by the P_2-purinoceptor antagonist, suramin (300 μM; Inoue *et al.*, 1992; Balachandran & Bennett 1995).

The present work describes modulation of glutamatergic transmission in rat hippocampus by P_2-purinoceptor antagonists.

Methods

Tissue preparation

Hippocampal slices were obtained from 10–14 days old Wistar rats. Animals were decapitated under deep halothane anaesthesia. Transverse slices were cut with a manual Vibroslice (Campden Instr., England). The thickness of the slices varied between 200 μM for experiments examining glutamate activated currents and 400 μM for e.p.s.c. generation. Thickness of the slices varied because in one set of experiments a small thickness enhanced visualization of cells on a surface of the slice, but in the second set of experiments it was more critical to keep the neuronal pathways intact. Slices were maintained on nylon net at 37°C in artificial cerebrospinal fluid (ACSF) contained (in mM): NaCl 126, KCl 2.5, $MgCl_2$ 1.2, $CaCl_2$ 2.4, NaH_2PO_4 1.2, $NaHCO_3$ 26, glucose 10. The solution was bubbled with carbogen (95% O_2 and 5% CO_2) to maintain oxygenation and pH = 7.4. Slices were used within 1–8 h after dissection.

Patch-clamp recordings

Patch-clamp recording was similar to the methods described by Edwards *et al.* (1989). Hippocampal slices were transferred

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into a low volume recording chamber and superfused with ACSF at room temperature of 23–25°C. In experiments involving L-glutamate-induced current measurements, 200–500 nM of TTX (from RBI, U.S.A.) were added to the perfusing solution. All P_2-purinoceptor antagonists were bath-applied. In most cases it was possible to identify cells in the pyramidal cell layer using an upright microscope with interfering contrast optics (Diaplan Letz, Germany) equipped with 40 × water immersion objective (Nikon, Japan).

Pipettes were pulled from borosilicate glass capillaries 1.5 mm o.d. (Clark Electromedical Instr., England) with a vertical patch-clamp pipette puller PP-83 (Narishige Instr., Japan). The resistance of the pipettes varied between 4–7 MΩ. The pipette solution (intracellular solution) contained (in mM): K-gluconate 110, KCl 10, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, K-ATP 2 (pH = 7.3 by KOH).

The series resistance was typically 10–60 MΩ and was monitored throughout the experiment. It was not compensated, but if it changed more than 15% during the experiment, the results were rejected.

In experiments designed to measure e.p.s.cs, mossy fibres and the Schaffer collateral fibres were stimulated with a glass bipolar electrode filled with ACSF. The electrodes were pulled from theta style glass capillaries (Clark Electromedical Instr., England). The stimulus intensity varied between 10–70 V and the duration was 200 μs. During the experiment the duration and stimulus strength were kept constant. The stimulating electrode was placed 10–20 μm into the slice close to the dentate granule cell layer when stimulating mossy fibres or on stratum lacunosum moleculare near CA3 region while stimulating Schaffer collateral fibres.

In experiments examining glutamate-activated current, 100 μM L-glutamate was applied by pressure ejection from a pipette with a resistance of 1–2 MΩ. The pipette was positioned perpendicularly to the perfusing solution flow at the distance from the soma which gave maximal response to the agonist with minimal time of onset of the current. Pressure of 3–6 Psi was applied to the end of the pipette with a pressure application device (Picospritzer, General Valve Corp., U.S.A.) controlled by a IBM-PC compatible computer.

Whole-cell membrane currents were monitored with an Axopatch1C patch-clamp amplifier (Axon Instr., U.S.A.), filtered at 1 kHz. The currents were sampled at 3.9 kHz in e.p.s.cs recording or at 200 Hz while measuring glutamate-evoked currents. Digitized data were stored on the hard drive of IBM-compatible computer for further analysis.

Drugs

Pyridoxal - phosphate - 6 - azophenyl - 2',4' - disulphonic acid (PPADS), Reactive Blue 2 (RB-2), tetrodotoxin (TTX) were obtained from Research Biochemicals Inc. (U.S.A.) and suramin from Bayer (Germany). All P_2-purinoceptor antagonists were diluted immediately before the experiment to make up a 10 mM stock solution and were then added to the perfusion solution. 6,7Dinitroquinoxaline-2,3-dione (DNQX, Tocris Cookson, England) was first diluted in dimethylsulphoxide (DMSO) to make up 100 mM stock which was stored frozen at −20°C. The perfusion solution contained less than 0.01% DMSO.

Results

Excitatory post-synaptic currents (e.p.s.cs) were recorded from CA1 or CA3 pyramidal neurones voltage clamped at −80 to −60 mV during stimulation of Schaffer collaterals or the mossy fibre axons at 0.5 Hz. E.p.s.cs were blocked completely and reversibly by 200 nM TTX or 10 μM DNQX. The latter indicates that synaptic transmission was due to operation of AMPA/KA channel-receptor complexes. The P_2-purinoceptor antagonist suramin was bath-applied (20 μM to 1 mM) to the hippocampal slices and in all cases ($n = 11$ for CA1 neurones

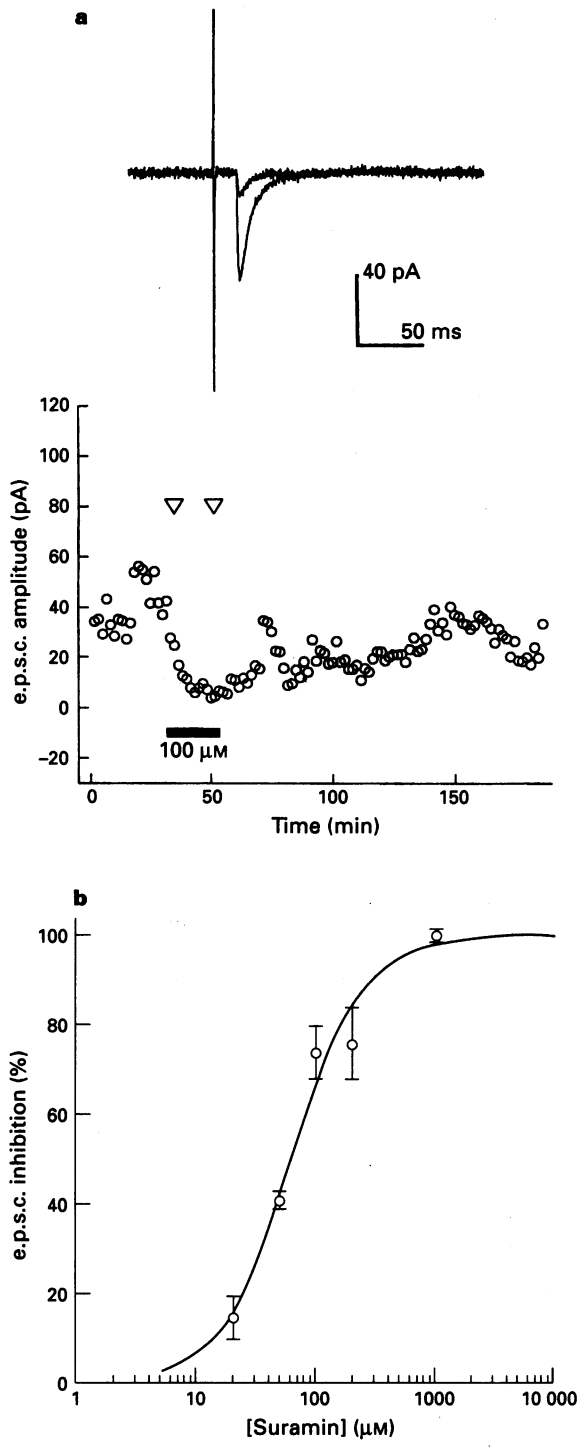

Figure 1 The effect of suramin on excitatory post-synaptic currents (e.p.s.cs) at Schaffer collateral synapses on CA1 pyramidal cells. (a) The effect of suramin at concentration of 100 μM (lower panel) on the e.p.s.c. amplitude. E.p.s.cs were evoked by stimulation of the mossy fibres pathway at 0.5 Hz. Each circle gives the average of 20 e.p.s.cs. The period of application of suramin is given by the horizontal line. In the upper panel, individual e.p.s.cs are presented taken at the times shown by triangles on the lower panel. Holding potential was −70 mV. (b) Dose-response curve for the effects of suramin on the e.p.s.cs; each point gives the average results (± s.e.mean) for an $n = 3$ to 7. The curve is drawn according to the logistic equation:

$$1 - \frac{I}{I_C} = \left(1 + \left(\frac{62\mu M}{[\text{Suramin}]}\right)\right)^{-1.4}$$

in which I and I_C are e.p.s.c. amplitudes in the presence and the absence of suramin respectively.

and $n=6$ for CA3 neurones) decreased the size of the e.p.s.cs within ~300 s of application. A representative example of the chart giving the time course of e.p.s.c. amplitude change is given in Figure 1a. A similar antagonism took place at a holding potential of +10 mV indicating blockade of the NMDA component of synaptic transmission ($n=2$). The dose-response curve for inhibition of e.p.s.cs by suramin (Figure 1b) shows that 50% inhibition was achieved by 62±8 μM suramin with a cooperativity of c=1.4±0.3. Suramin at a concentration of 100–200 μM blocked spontaneous synaptic activity in CA1 neurones ($n=5$, not shown).

The effect of suramin on the currents generated by application of L-glutamate (100 μM) to the hippocampal neurones voltage clamped at −80 to −60 mV was next determined. The agonist was applied to the cells from the pyramidal cell layer in CA1 region at intervals of 20 s. Suramin was bath applied in the concentration-range 20 μM to 1000 μM. In all 15 neurones tested the current was decreased by suramin. An example of the effect is given in Figure 2a. The dose-response curve shown in Figure 2b indicated that the ID_{50} for the effect of suramin was 121±36 μM with a cooperativity of c=0.5±0.1.

In order to determine if the effect of suramin might be attributed to its blocking a P_{2x}-purinoceptor, the effects of the P_{2x}-purinoceptor antagonist PPADs on synaptic transmission to CA1 pyramidal neurones was ascertained. PPADs, in the concentration-range of 80 μM to 200 μM, reduced the size of the e.p.s.cs at synapses in 12 out of 13 CA1 neurones tested.

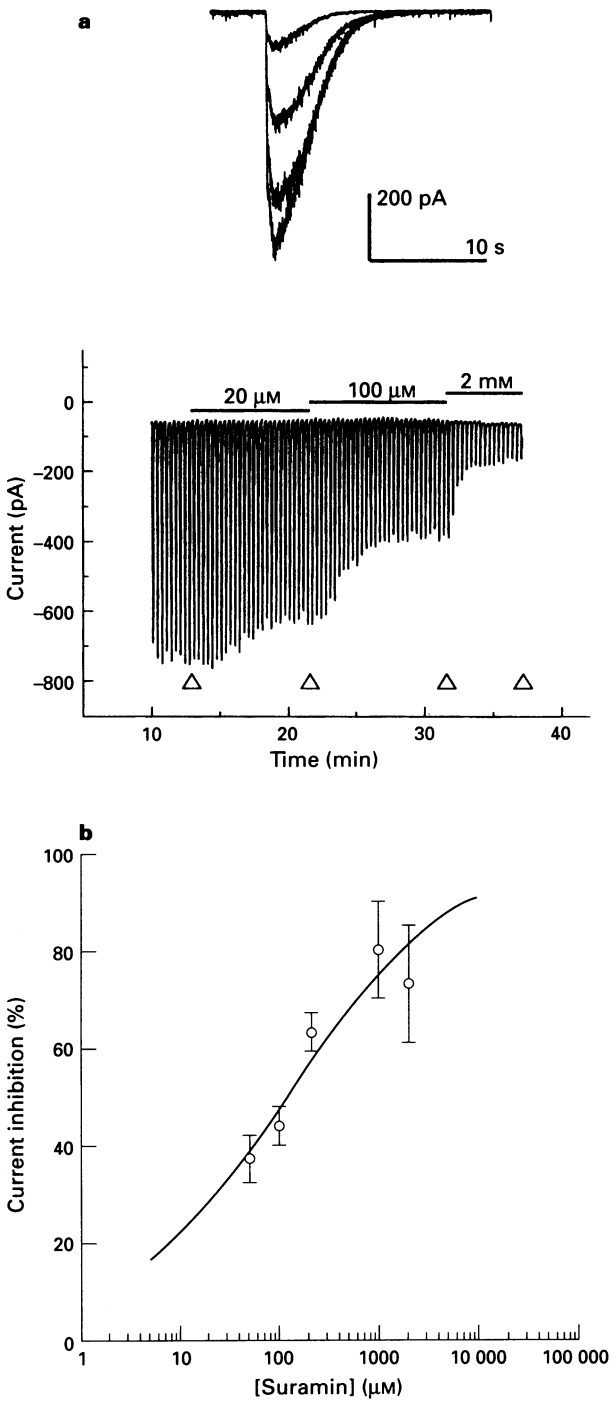

Figure 2 The effect of suramin on currents elicited by applications of 100 μM L-glutamate to hippocampal CA1 pyramidal neurones voltage clamped at −60 mV. (a) Lower panel, results are shown for one cell in which the concentration of suramin was increased successively from 20 μM to 2 mM, as indicated by the horizontal lines. Each peak corresponds to 500 ms application of 100 μM L-glutamate. Traces on the upper panel are taken at times marked by (△) on the lower panel. (b) Dose-response curve for the effects of suramin on the amplitude of the glutamate-evoked current; each point gives the average results (±s.e.mean) for n of 3 to 8. The curve is drawn according to the logistic equation:

$$1-\frac{I}{I_C}=\left(1+\left(\frac{121\mu M}{[\text{Suramin}]}\right)\right)^{-0.52}$$

in which I and I_C are amplitudes of the glutamate-activated currents in the presence and the absence of suramin respectively.

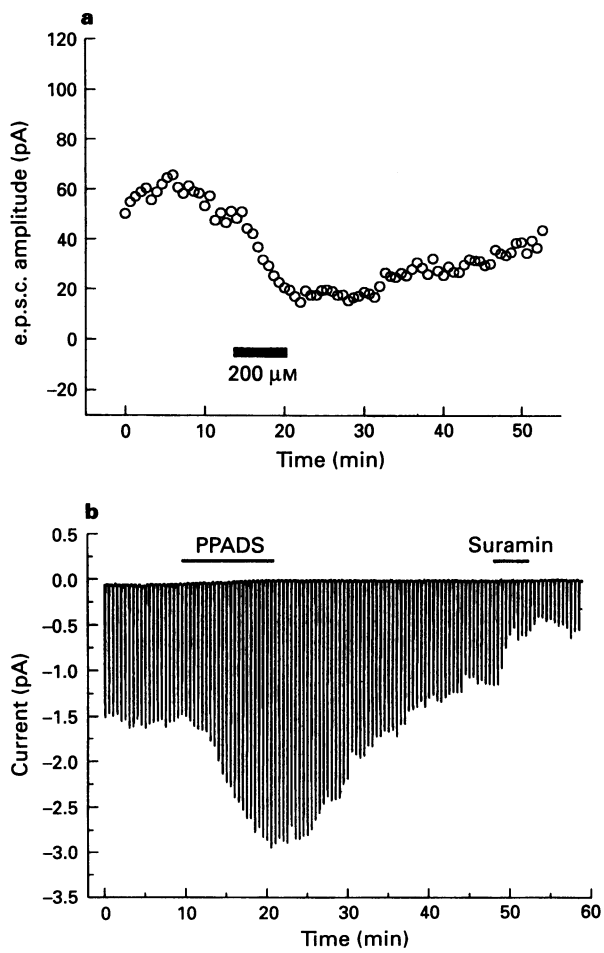

Figure 3 The effects of PPADS on CA1 pyramidal cells. (a) Reduction of the e.p.s.cs generated by stimulating the Schaffer collateral at 0.5 Hz. Each circle gives the average of 20 e.p.s.cs. The period of application of PPADs is given by the horizontal line. Holding potential was −70 mV. (b) The comparative effect of 200 μM PPADS and 500 μM suramin (the period of application is shown by horizontal lines). Currents were elicited in CA1 pyramidal cells by 1 s applications of 200 μM L-glutamate at intervals of 30 s. The holding potential was −60 mV.

PPADS at a concentration of 200 μM, decreased the size of the e.p.s.cs by 60±20% (Figure 3a). However, PPADS (20–500 μM) did not consistently affect the amplitude of the currents generated by application of glutamate. In 8 CA1 neurones the glutamate currents were either unaffected ($n=4$) or there was an increase in the amplitude of the current (Figure 3b). The enhancement of the current by 200 μM PPADS was 60±10% ($n=4$).

Reactive Blue 2 is supposed to be an antagonist of P_{2y}-purinoceptors in a particular concentration-range, so this was next tested to see if these purinoceptors might be implicated in synaptic transmission to hippocampal pyramidal neurones. Reactive Blue 2 (200 μM) reduced the e.p.s.c. amplitude in 6 CA1 cells by 80±10% (Figure 4a). It also decreased the amplitude of the glutamate-induced currents by 83±3% in 6 other CA1 neurones (Figure 4b). It was difficult to observe a recovery from the effect of Reactive Blue 2, perhaps because of staining of the slice with this antagonist which persisted in the presence of continual washing.

Discussion

In the present work, e.p.s.cs in both CA1 and CA3 pyramidal neurones were blocked by suramin (ID_{50} of 62 μM) and by the P_{2X}-purinoceptor antagonist, PPADS, as well as Reactive Blue 2. The question arises as to whether the action of the P_2 antagonists in blocking glutamatergic transmission is presynaptic or postsynaptic. It seems most likely that one site of action is postsynaptic as both suramin and Reactive Blue 2 blocked the effects of applied glutamate on the pyramidal neurones although PPADS did not. The failure of PPADs to antagonize the currents generated by applied glutamate points to a presynaptic action of this classical P_{2x}-purinoceptor blocker. It may be that one action of ATP is to modulate the release of glutamate, as the nucleotide is known to modulate the release of noradrenline from sympathetic neurones in culture (Allgaier *et al.*, 1994) and of acetylcholine from neurones in the brain (Cunha *et al.*, 1994). If this is the case then it would have to be claimed that the P_{2x}-purinoceptor antagonist blocked glutamatergic transmission as a consequence of blocking a presynaptic action of ATP necessary for glutamate release. Sun & Stanley (1994) have recently shown that ATP activates a fast inward current in a cholinergic nerve terminal which is insensitive to both suramin or Reactive Blue 2 and has a reversal potential of 0 mV. This presynaptic receptor is similar to the presynaptic ATP purinoceptor previously reported for adrenergic nerve terminals, sometimes referred to as the P_3-purinoceptor (Shinozuka *et al.*, 1988). If such an inward current, carried by calcium ions, is activated by ATP at glutamatergic terminals it would facilitate transmitter release.

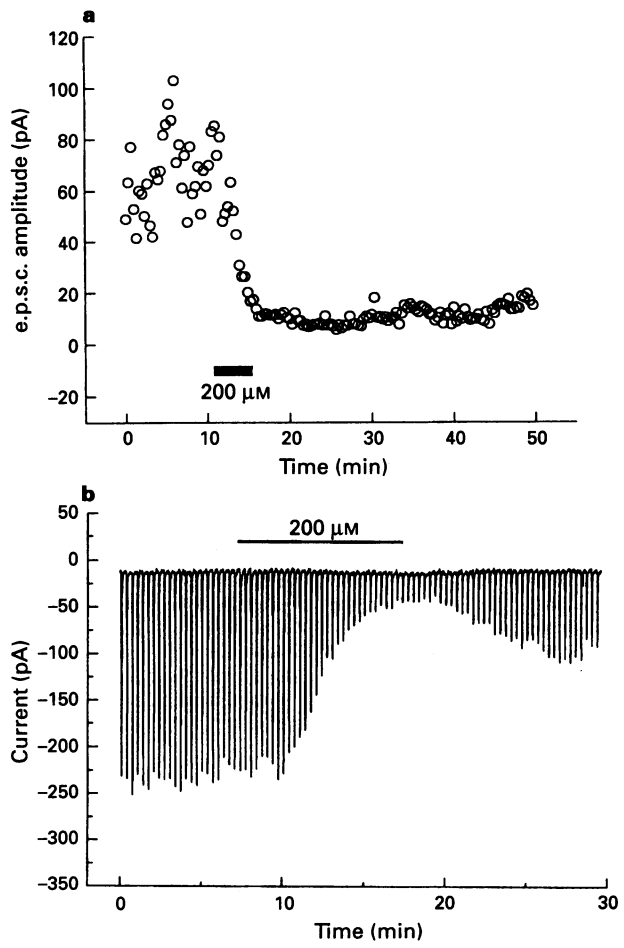

Figure 4 The effect of Reactive Blue-2 (RB-2) on CA1 pyramidal cells. (a) Inhibition of the e.p.s.cs generated by stimulating the Schaffer collateral at 0.5 Hz. Each circle gives the average of 10 e.p.s.cs. The period of application of RB-2 is given by the horizontal line. Holding potential was −65 mV. (b) The effect of 200 μM RB-2 on the currents elicited by 500 ms applications of 100 μM L-glutamate at intervals of 20 s. The holding potential was −62 mV.

Suramin and Reactive Blue 2 blocked both excitatory synaptic transmission to the pyramidal cells as well as the action of applied glutamate. A classical P_{2y}-purinoceptor in the postsynaptic membrane may therefore modulate the glutamate receptors perhaps in a way similar to the modulation of these receptors by κ_2-opioid receptors (Caudle *et al.*, 1994). Such a modulation may occur through several different mechanisms. ATP may be a coagonist on the glutamate receptor. The fact that glutamate alone produces an inward current indicates that ATP is unlikely to play a role analogous to that of the obligatory co-agonist glycine on the NMDA receptor, although it is possible that the applied glutamate released ATP in the slices. Another possibility is that there may be intermembrane coupling between P_{2y}-purinoceptors and the glutamate receptors. Exogenous ATP can induce long-term potentiation in pyramidal neurones, an effect that is antagonized by non-hydrolysable analogues of ATP (Wieraszko & Ehrlich, 1994). One possibility is that this is due to ATP acting on a P_{2y}-like purinoceptor that is coupled to the glutamate receptors. P_{2y}-purinoceptors have been identified that mediate a metabotropic effect due to ATP which increases the calcium level in 30% of dissociated hippocampal neurones (Mironov, 1994); such an increase in intracellular calcium could lead to modulation of glutamate receptors. Another possibility is that ATP acts on ecto-protein kinases that in turn phosphorylate a duplex of proteins which are coupled in the membrane to glutamate receptors (Chen *et al.*, 1994). The possibility that the action of ATP is mediated by the latter mechanism is supported by the observation that specific antagonists of ecto-protein kinases block the maintenance phase of long-term potentiation (Fujii *et al.*, 1994).

Another explanation for the action of both suramin and Reactive Blue 2 in blocking both synaptic currents and glutamate induced currents is that the glutamate receptors possess binding sites for both ATP and glutamate. It is interesing in this regard that the nicotinic receptor in *Torpedo* possesses ATP binding sites on the beta and delta subunits distinct from that of the ACh binding sites on the two alpha subunits (Carlson & Raftery, 1993). ATP increases the open-time of the α-bungarotoxin sensitive channel in skeletal muscles (Igusa, 1988; Lu & Smith, 1991); furthermore ATP activates both junctional and extrajunctional ACh receptors, giving rise to single channel currents that have the same slope conductance that characterize these receptors (Mozrzymas & Ruzzier, 1992). In both sympathetic neurones and phaeochromocytoma cells, the currents produced by ATP and ACh are not additive, desensitization to one agonist increases the current that can be generated by the other; the currents are selectively blocked by

suramin and hexamethonium respectively (Nakazawa *et al.*, 1991; Nakazawa, 1994). However, given that P_2-purinoceptor antagonists block glutamatergic transmission, it cannot be argued that the binding sites for ATP and glutamate are different, as it has been for the binding sites for ATP and ACh on nicotinic receptors. It is possible that both ATP and glutamate have the same binding site. In this case the currents due to ATP and glutamate would not be additive, but unlike the case of the nicotinic receptor they would both be blocked by the others antagonists. Perhaps one is left with the analogy provided by the action of 5-hydroxytryptamine in blocking peripheral nicotinic receptors, so that ligand-gated channels activated by their specific neurotransmitter may be regulated by a different transmitter through a direct action on the receptor molecule (Grassi *et al.*, 1993). In this case the 5-hydroxytryptamine receptor in neurones is blocked by the nicotinic receptor antagonist, curare (Bobker & Williams, 1990). It will be very interesting to determine experimentally which of these various possibilities for the action of P_2-purinoceptor antagonists on glutamatergic transmission in the hippocampus is correct.

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