Regulation of muscarinic acetylcholine receptor-mediated synaptic responses by adenosine receptors in the rat hippocampus

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1. Intracellular current clamp recordings were made from CA1 pyramidal neurones in rat hippocampal slices. Experiments were performed in the presence of ionotropic glutamate receptor antagonists and γ-aminobutyric acid (GABA) receptor antagonists to block all fast excitatory and inhibitory synaptic transmission. A single stimulus, delivered extracellularly in the stratum oriens, caused a reduction in spike frequency adaptation in response to a depolarizing current step delivered 2 s after the stimulus. A 2- to 10-fold increase in stimulus intensity evoked a slow excitatory postsynaptic potential (EPSP) which was associated with a small increase in input resistance. The peak amplitude of the EPSP occurred approximately 2·5 s after the stimulus and its magnitude (up to 30 mV) and duration (10–50 s) increased with increasing stimulus intensity.

2. The slow EPSP was unaffected by the metabotropic glutamate receptor antagonist (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG; 1000 μM) but was greatly enhanced by the acetylcholinesterase inhibitor physostigmine (1–5 μM). Both the slow EPSP and the stimulus-evoked reduction in spike frequency adaptation were inhibited by the muscarinic acetylcholine receptor (mAChR) antagonist atropine (1–5 μM). These results are consistent with these effects being mediated by mAChRs.

3. Both the mAChR-mediated EPSP ($EPSP_m$) and the associated reduction in spike frequency adaptation were reversibly depressed (up to 97%) by either adenosine (100 μM) or its non-hydrolysable analogue 2-chloroadenosine (CADO; 0·1–5·0 μM). These effects were often accompanied by postsynaptic hyperpolarization (up to 8 mV) and a reduction in input resistance (up to 11%). The selective adenosine A_1 receptor agonists 2-chloro-N^6-cyclopentyladenosine (CCPA; 0·1–0·4 μM) and $R(-)N^6$-(2-phenylisopropyl)-adenosine (R-PIA; 1 μM) both depressed the $EPSP_m$. In contrast, the adenosine A_{2A} receptor agonist 2-p-(2-carboxyethyl)-phenethylamino-5′-N-ethylcarboxamidoadenosine (CGS 21680; 0·5–1·0 μM) did not significantly affect the $EPSP_m$.

4. The selective adenosine A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 0·2 μM) fully reversed the depressant effects of both adenosine (100 μM) and CADO (1 μM) on the $EPSP_m$ and the stimulus-evoked reductions in spike frequency adaptation.

5. DPCPX (0·2 μM) alone caused a small but variable mean increase in the $EPSP_m$ of 22 ± 19% and enabled activation of an $EPSP_m$ by a previously subthreshold stimulus. In contrast, the selective adenosine kinase inhibitor 5-iodotubercidin (5-IT; 10 μM) inhibited the $EPSP_m$ by 74 ± 10%, an effect that was reversed by DPCPX.

6. The concentration–response relationship for the depressant action of CADO on the $EPSP_m$ more closely paralleled that for its presynaptic depressant action on glutamate-mediated EPSPs than that for postsynaptic hyperpolarization. The respective mean IC_{50} and EC_{50} concentrations for these effects were 0·3, 0·8 and 3·0 μM.

7. CADO (1–5 μM) did not have a significant effect on the postsynaptic depolarization, increase in input resistance and reduction in spike frequency adaptation evoked by carbachol (0·5–3·0 μM). All these effects were abolished by atropine (1 μM).

8. These data provide good evidence for an adenosine A_1 receptor-mediated inhibition of mAChR-mediated synaptic responses in hippocampal CA1 pyramidal neurones. This inhibition is mediated predominantly presynaptically, is active tonically and can be enhanced when extracellular levels of endogenous adenosine are raised.

CA1 pyramidal neurones in the hippocampus receive multiple intrinsic synaptic inputs as well as a number of extrinsic synaptic inputs from diverse areas of the brain. One of the most characterized extrinsic inputs is the septohippocampal input (Dutar, Bassant, Senut & Lamour, 1995), which comprises a heterogeneous population of afferents that mediate their effects through the release of numerous neurotransmitters including γ-aminobutyric acid (GABA), acetylcholine (ACh) and a variety of neuropeptides (Decker & McGaugh, 1991; Dutar *et al.* 1995). Of these heterogeneous inputs the cholinergic aspect of the septohippocampal input to the hippocampus has probably received most attention due to its involvement in mnemonic processing (Cole & Nicoll, 1983; Decker & McGaugh, 1991; Dutar *et al.* 1995). This input has been shown to activate a slow excitatory postsynaptic potential (EPSP) and to reduce spike frequency adaptation (sometimes referred to as action potential accommodation) via muscarinic acetylcholine receptor (mAChR)-mediated inhibition of K^+ conductances (Cole & Nicoll, 1983, 1984; Madison, Lancaster & Nicoll, 1987; Segal, 1988; Pitler & Alger, 1990). This prolonged excitation of CA1 pyramidal neurones, if left unregulated, can be detrimental to these cells and may result in epileptogenesis (Lothman, Bertram & Stringer, 1991; Wasterlain, Fujikawa, Penix & Sankar, 1993). As such, mechanisms that control the magnitude of the mAChR-mediated postsynaptic responses are likely to be of major importance in maintaining the normal functioning of the CNS.

A common mechanism of control of synaptic inputs within the brain is via activation of different receptors from those that mediate the postsynaptic response, e.g. activation of autoreceptors, or presynaptic heteroreceptors (Thompson, Capogna & Scanziani, 1993). The aim of this study, therefore, was to investigate the possibility that a neurotransmitter/neuromodulator other than ACh may restrict the activation of the mAChR-mediated postsynaptic responses. We chose to study adenosine since this neuromodulator plays an important role in controlling the excitability of neuronal networks by inhibiting other excitatory synaptic inputs, e.g. glutamate (Thompson, Haas & Gähwiler, 1992; Thompson *et al.* 1993). To do this we isolated stimulus-evoked mAChR-mediated synaptic responses (i.e. the mAChR-mediated EPSP ($EPSP_m$) and the stimulus-evoked reduction in spike frequency adaptation in CA1 pyramidal neurones) using a cocktail of ionotropic glutamate and GABA receptor antagonists to block all 'fast' excitatory and inhibitory synaptic transmission. Using this approach we have been able to investigate how drugs that affect the function of adenosine at the levels of the receptor and its metabolism influence both the $EPSP_m$ and the mAChR-mediated reduction in spike frequency adaptation. Preliminary accounts of part of this work have appeared in abstract form (Morton & Davies, 1996*a*, *b*).

METHODS

Female Wistar rats (2–4 weeks old) were cervically dislocated and subsequently decapitated in accordance with UK Home Office guidelines. The brain was removed rapidly and transverse hippocampal slices were prepared by hemisecting the whole brain minus the cerebellum and cutting 400 μm thick transverse coronal slices containing hippocampal slices, using a vibroslicer (Campden Instruments, Loughborough, UK). The CA3 region of each slice was then cut away to eliminate changes in network function that can occur due to epileptiform bursting in area CA3 when picrotoxin is applied to the slice. The resultant CA3-ectomized slices were placed on a nylon mesh at the interface of a warmed (32–34 °C), perfusing (1–2 ml min^{-1}) artificial cerebrospinal fluid and an oxygen-enriched (95% O_2–5% CO_2) humidified atmosphere. The standard perfusion medium comprised (mM): NaCl, 124; KCl, 3; $NaHCO_3$, 26; NaH_2PO_4, 1·25; $CaCl_2$, 2; $MgSO_4$, 1; D-glucose, 10; and was bubbled with 95% O_2–5% CO_2.

Following a 1 h equilibration period, intracellular recordings were obtained from the CA1 pyramidal cell body region using 2 M potassium methylsulphate-filled microelectrodes (60–110 MΩ). An Axoclamp-2B amplifier (Axon Instruments) was used in discontinuous (3–5 kHz switching frequency) current clamp mode. All impalements were made in control medium. Once stable recordings had been made for at least 10 min, all fast ionotropic glutamate receptor-mediated synaptic transmission was blocked using a combination of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 20 μM; Tocris Cookson Ltd, Bristol, UK) or 6-nitro-7-sulphamoylbenzo(*f*)quinoxaline-2,3-dione (NBQX; 2–4 μM; Tocris Cookson Ltd), and the *N*-methyl-D-aspartate (NMDA) receptor antagonists D-(*E*)-2-amino-4-methyl-5-phosphono-3-pentanoic acid (CGP 40116; 50 μM; Ciba-Geigy Ltd) or D-2-amino-5-phosphonopentanoate (AP5; 50 μM; Tocris Cookson Ltd), and all GABA-mediated synaptic transmission was abolished using the $GABA_A$ receptor antagonist picrotoxin (50 μM; Sigma) and the $GABA_B$ receptor antagonist 3-*N*-[1-(*S*)-(3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*p*-benzyl-phosphonic acid (CGP 55845A; 1 μM; Ciba-Geigy Ltd). Bipolar stimulating electrodes, made from 55 μm diameter insulated nickel–chromium wire, were positioned in the stratum oriens close to the recording electrode in the stratum pyramidale (Fig. 1*A*) to provide extracellular orthodromic activation of CA1 neurones. In each series of experiments, stimuli comprised square-wave pulses (20–200 μs; 5–30 V) delivered homosynaptically at a fixed intensity every 5–10 min. Data were captured using pCLAMP 6 software (Axon Instruments) and digitized records were stored on the hard disk of a PC and on DAT tape (DTR-1404; Biologic Scientific Instruments, Claix, France) for off-line analysis using Clampfit software (Axon Instruments). During the period between stimuli the input resistance and the extent of spike frequency adaptation of each neurone were measured routinely every 2 min using 300–600 ms long hyperpolarizing and depolarizing current steps (±0·15–0·40 nA), respectively. In all experiments in which $EPSP_m$s or stimulation-induced reductions in spike frequency adaptation were evoked, baseline recordings comprised either three successive $EPSP_m$s which had peak amplitudes that differed by no more than 15%, or reductions in spike frequency adaptation that were consistent over 20–30 min, respectively. All drugs were applied by addition to the perfusion medium. To compare the $EPSP_m$s evoked in the presence and absence of a drug at the same membrane potential, DC was injected through the

electrode to compensate for any drug-induced changes in membrane potential. 2-*p*-(2-Carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine (CGS 21680), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 5-iodotubercidin (5-IT), (+)-α-methyl-4-carboxyphenylglycine ((+)-MCPG) and nitrendipine were obtained from Research Biochemicals International. Adenosine, atropine, carbachol, 2-chloroadenosine (CADO), 2-chloro-N^6-cyclopentyladenosine (CCPA), $R(-)N^6$-(2-phenylisopropyl)-adenosine (R-PIA), physostigmine and picrotoxin were purchased from Sigma.

Data are presented as means ± standard error of the mean (S.E.M.) and statistical significance was assessed using Student's paired or unpaired *t* tests performed on raw data with $P < 0.05$ being taken as indicating statistical significance. *n* values refer to the number of times a particular experiment was performed, each in a different slice taken from a different rat.

RESULTS

Data were obtained from 168 stable intracellular recordings (2–6 h) from CA1 pyramidal neurones with overshooting action potentials, resting membrane potentials that were more negative than −55 mV and input resistance values of 30 MΩ or greater.

Characterization of a slow EPSP

Single shock stimulation in the stratum oriens evoked an EPSP which was followed by a biphasic inhibitory postsynaptic potential (IPSP). All components of this response were abolished by the combined application of NBQX (1–3 μM) or CNQX (20 μM), AP5 (50 μM) or CGP 40116 (50 μM), picrotoxin (50 μM) and CGP 55845A (1 μM) (Fig. 1*B*). A 2- to 10-fold increase in the stimulus intensity evoked a much slower EPSP in 126 out of 129 neurones in which this was attempted (Fig. 1*C*). This EPSP could be evoked reproducibly every 5–10 min. In a random group of cells the slow EPSP had a mean amplitude of 9·6 ± 2·1 mV, a time to peak of 2·6 ± 0·4 s and an overall duration of 32·6 ± 6·9 s ($n = 6$). The peak amplitude and duration of the EPSP were highly dependent upon the stimulus strength used, both increasing with increasing stimulus intensity (Fig. 1*C*). The EPSP often resulted in intense discharges of action potentials and in some neurones the EPSP depolarized the membrane beyond the action potential firing threshold and into a region where action potential generation was inactivated. The slow EPSP was accompanied by an increase in cell input resistance as measured by comparing responses to hyperpolarizing current steps applied to the cell before and during the EPSP (Fig. 1*D*).

As illustrated in Fig. 2*A*, the slow EPSP was not affected significantly by the metabotropic glutamate receptor antagonist (+)-MCPG (1000 μM; $n = 3$). Addition of the acetylcholinesterase inhibitor physostigmine (1–5 μM), however, caused a large enhancement of both the peak amplitude and duration of the slow EPSP ($n = 5$; Fig. 2*B*). Physostigmine also caused a reduction in spike frequency adaptation as measured by the number of action potentials fired in response to a 300 ms long +0·2 or +0·3 nA current step ($n = 6$; not illustrated). This effect presumably occurred as a result of raising extracellular levels of spontaneously released ACh due to inhibition of acetylcholinesterase activity by physostigmine (Cole & Nicoll, 1984; Azouz, Jensen & Yaari, 1994). In contrast, atropine (2–5 μM) reduced or abolished the slow EPSP ($n = 7$; Fig. 2*C*). These results are entirely consistent with the slow EPSP being mediated by activation of mAChRs and, as such, this EPSP will be referred to as a mAChR-mediated EPSP ($EPSP_m$).

Effects of adenosine receptor activation on the $EPSP_m$

We next addressed whether activation of adenosine receptors might affect the $EPSP_m$. The non-hydrolysable adenosine receptor agonist CADO (0·2–1 μM) caused a depression of the $EPSP_m$ that was maintained for the period of the agonist application and was fully reversed on washout ($n = 4$; Fig. 3*A*). This effect was concentration dependent and, at a concentration of 0·2 μM, CADO sometimes caused a substantial depression of the $EPSP_m$ without any effect on the postsynaptic membrane potential, input resistance (Fig. 3*B*) or spike frequency adaptation in response to a depolarizing current step. In contrast, at concentrations of 0·5 μM and above the depressant action of CADO on the $EPSP_m$ was invariably accompanied by postsynaptic hyperpolarization (up to 8 mV) and a reduction in input resistance (up to 11%). In one neurone the $EPSP_m$ was unaffected by CADO. The maximal effect of CADO on the $EPSP_m$ was 97% inhibition. Similar results were obtained using adenosine itself. Thus, at a concentration of 100 μM, adenosine inhibited the $EPSP_m$ by 78 ± 10% ($n = 3$).

Pharmacology of the adenosine receptor mediating the depression of the $EPSP_m$

To elucidate which adenosine receptor subtype mediated the CADO-induced depression of the $EPSP_m$, we investigated the effects of a number of adenosine receptor subtype-specific agonists. The adenosine A_1 receptor agonists CCPA (0·1–0·4 μM; $n = 7$; Fig. 4*A*) and R-PIA (1 μM; $n = 2$) depressed the $EPSP_m$ and caused postsynaptic hyperpolarizations associated with reductions in input resistance. Thus, at a concentration of 0·4 μM, CCPA depressed the peak amplitude of the $EPSP_m$ to 34 ± 6% of that in control medium and produced an accompanying hyperpolarization of 4–5 mV and a reduction in input resistance of 5–10% ($n = 4$). In contrast, the adenosine A_{2A} receptor agonist CGS 21680, at concentrations up to 1 μM, neither affected the $EPSP_m$ nor the passive membrane properties of the neurones in which it was tested ($n = 3$; Fig. 4*B*).

The effects of the selective adenosine A_1 receptor antagonist DPCPX (0·2 μM) were also tested. DPCPX fully reversed, or prevented, the inhibition of the $EPSP_m$ (Fig. 5*A*) as well as the postsynaptic hyperpolarizations and decreases in input resistance (see Fig. 8*A*) evoked by CADO (1–2 μM; $n = 8$) and adenosine (100 μM; $n = 3$; not illustrated).

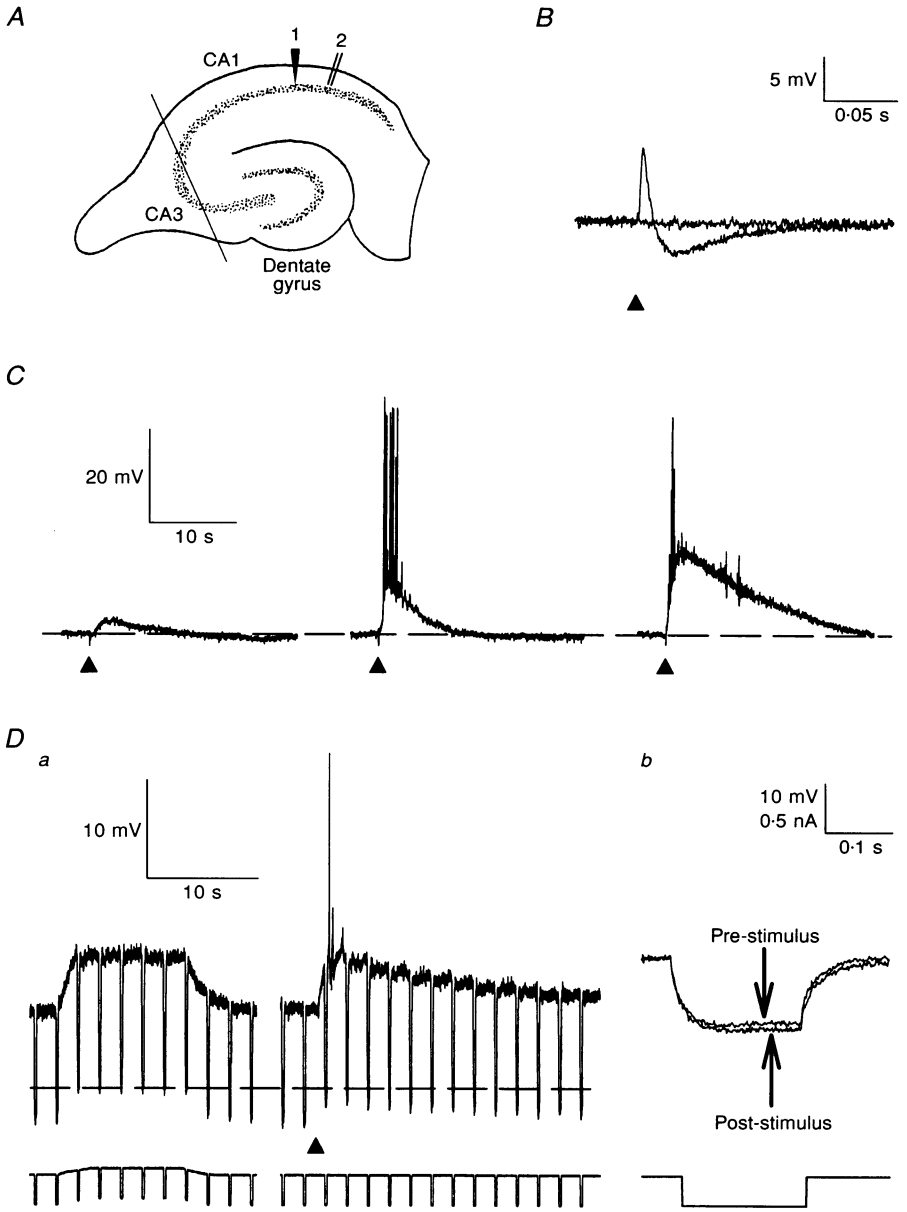

Figure 1. Characteristics of the pharmacologically isolated slow EPSP evoked in response to a single high intensity stimulus in the stratum oriens

A, schematic diagram of a rat hippocampal slice illustrating the positioning of recording (1) and stimulating (2) electrodes required to evoke the synaptic responses in *B–D*. *B*, intracellular recordings from a CA1 neurone in response to single shock stimulation in the stratum oriens. Note that in control medium an EPSP is followed by an IPSP and that the combination of CNQX (20 μM), CGP 40116 (50 μM), picrotoxin (50 μM) and CGP 55845A (1 μM) abolished this response (superimposed horizontal line). Traces are averages of four successive records taken at the same membrane potential. In *C*, the traces are single sweeps which illustrate the stimulus-dependent activation of a slow EPSP in the presence of the four amino acid receptor antagonists listed above. The stimulus intensities used to evoke the responses from left to right were 1·8, 2·3 and 4 mA, respectively. (The intensity that was required to evoke an AMPA/kainate receptor-mediated EPSP that was just suprathreshold for evoking an action potential was 0·04 mA.) The action potentials evoked by the slow EPSP are truncated due to the low sampling rate used to capture the entire response. The membrane potential of this neurone was −64 mV. *D*, the increase in input resistance associated with the slow EPSP. The input resistance was measured from the change in membrane potential in response to hyperpolarizing current steps of −0·3 nA applied for 0·2 s every 2·0 s. In *Da*, the cell membrane potential was manually clamped at a depolarized level before the stimulus so that the voltage response to the hyperpolarizing current steps could be compared with that evoked near the peak of the slow EPSP. *Db*, comparison of the responses in *a* on an expanded time scale. The current injected into the cell is shown below the voltage traces. The initial membrane potential of this cell was −64 mV. In this and all subsequent figures, the arrowheads below each synaptic trace indicate the time of afferent stimulation.

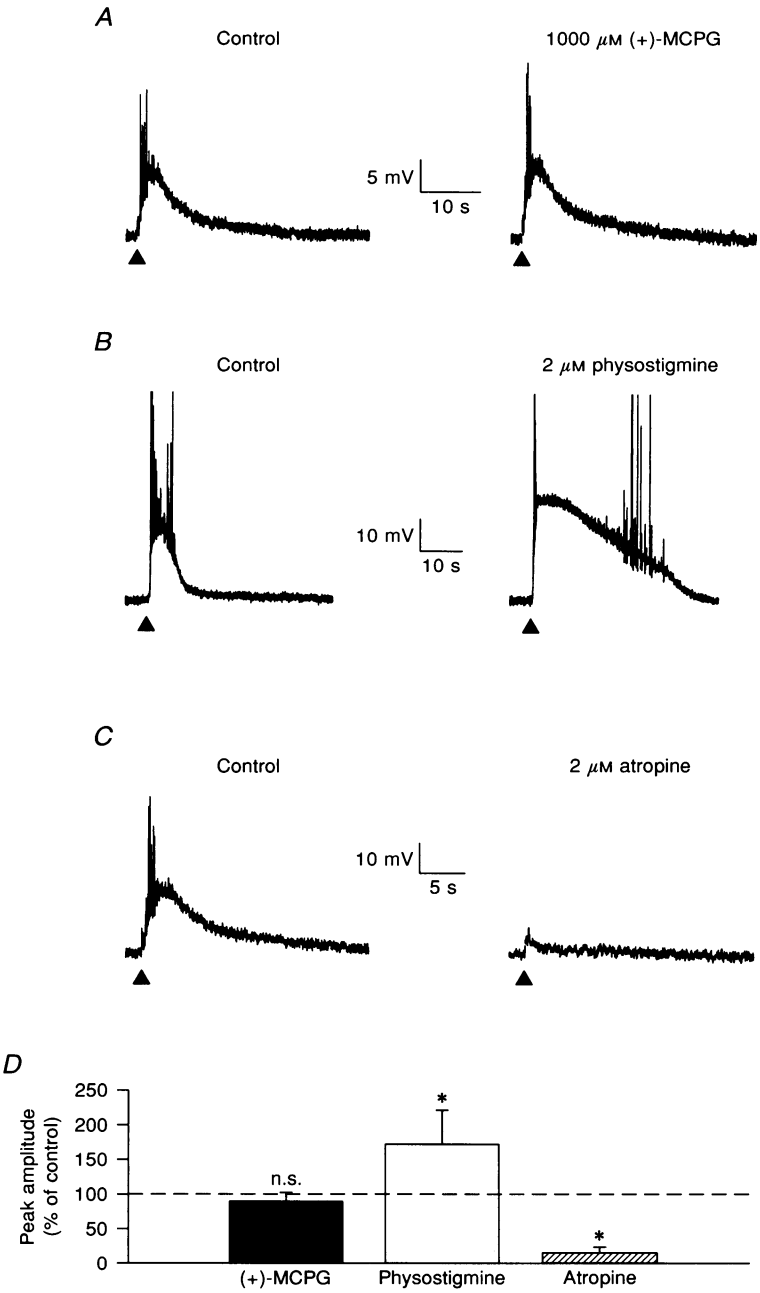

Figure 2. Effects of (+)-MCPG, physostigmine and atropine on the slow EPSP

In *A–C*, each trace is a single sweep illustrating the effects of 1000 μM (+)-MCPG, 2 μM physostigmine and 2 μM atropine, respectively, on slow EPSPs evoked in three separate neurones. The membrane potentials of these cells were −63, −64 and −63 mV, respectively. *D*, bar graph in which the pooled data for the peak amplitude of $EPSP_m$s recorded after 15–30 min in the presence of either (+)-MCPG (1000 μM; $n = 3$), physostigmine (2 μM; $n = 3$) or atropine (2 μM; $n = 3$) is expressed as a percentage of the mean value of the control $EPSP_m$s recorded over a 20–30 min baseline period prior to each drug application. Values are means ± S.E.M.; 100% is equivalent to no change. Note that whilst (+)-MCPG did not have a significant effect on the slow EPSP, physostigmine significantly enhanced the slow EPSP and atropine significantly inhibited the slow EPSP. $*P < 0{\cdot}05$; n.s., not significant (compared with control). In this and all subsequent figures, unless stated otherwise, traces are individual synaptic responses recorded intracellularly in response to a single stimulus delivered in the stratum oriens in the presence of 2–4 μM NBQX, 50 μM CGP 40116, 50 μM picrotoxin and 1 μM CGP 55845A. Each sweep was taken at the same membrane potential, which was achieved using DC injection to compensate for any drug-induced changes.

DPCPX alone caused a small but variable increase in the size of the $EPSP_m$ in three out of four neurones studied: the peak amplitude of the $EPSP_m$ in the presence of DPCPX was 122 ± 19% that of control ($n = 4$). In addition, in the presence of DPCPX, a stimulus that was previously sufficient to reduce spike frequency adaptation but was either subthreshold or just suprathreshold for evoking an $EPSP_m$ subsequently evoked a greatly enhanced $EPSP_m$ that had a peak amplitude of 8–10 mV ($n = 2$; Fig. 5*B*).

Effects of CADO on the reduction in spike frequency adaptation evoked by endogenous ACh

Stimulation that was subthreshold for activating the $EPSP_m$ caused a reduction in spike frequency adaptation in response to a depolarizing current step delivered 2 s after pathway stimulation ($n = 12$; Figs 5*B* and 6*A*). This reduction in spike frequency adaptation was inhibited by CADO (Fig. 6*B*) in a concentration-dependent manner (1–5 μM; $n = 3$) and was reinstated by subsequent application of DPCPX (0·2 μM;

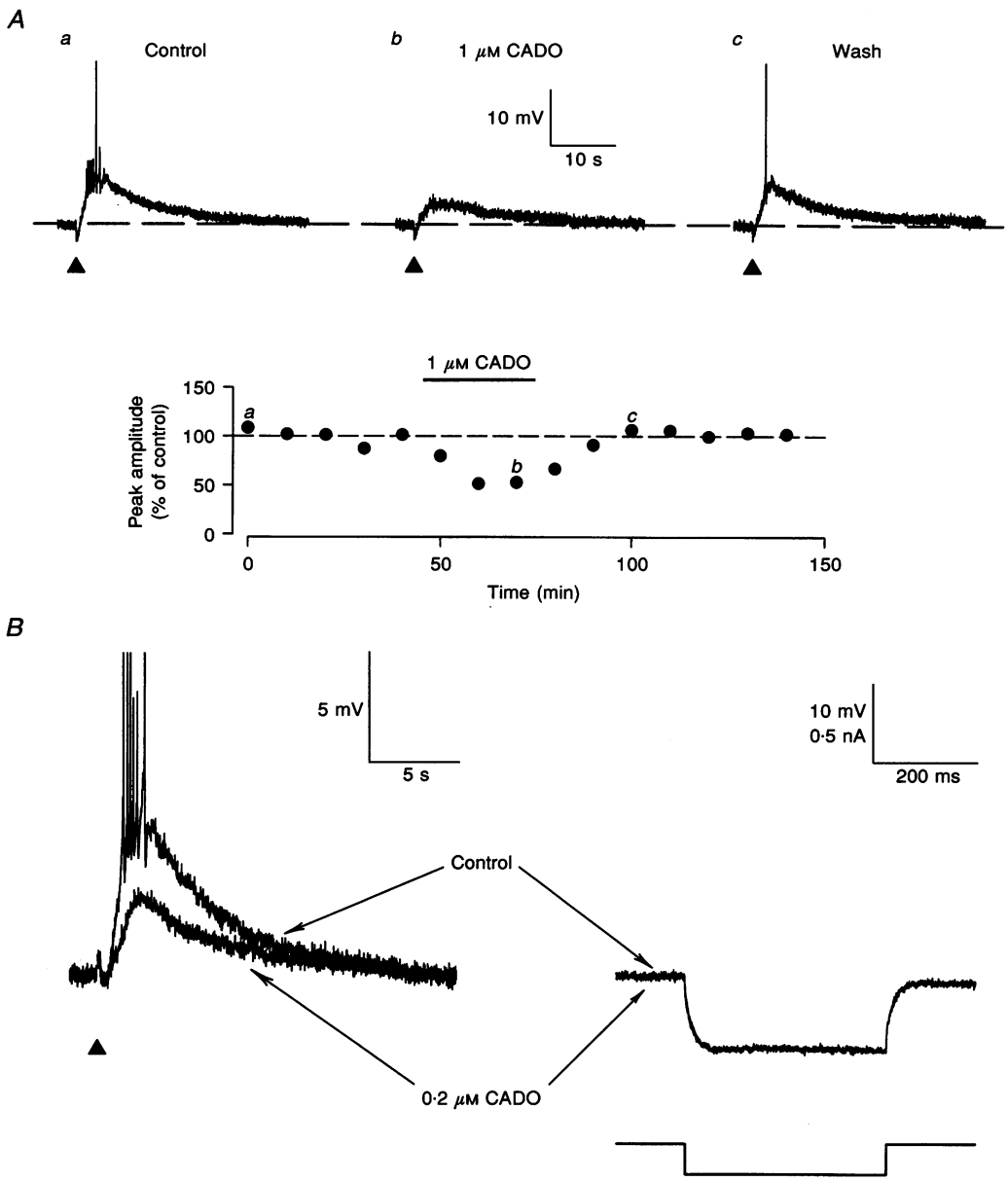

Figure 3. The effect of CADO on the $EPSP_m$

In *A*, synaptic traces are $EPSP_m$s, evoked in response to a single stimulus, recorded in control medium (*a*), in the presence of 1 μM CADO (*b*) and following washout (*c*). The graph shows a plot of the peak amplitudes of successive $EPSP_m$s normalized to the mean peak amplitude of the five $EPSP_m$s prior to CADO application *versus* time for a single experiment. CADO was applied for the time indicated by the bar. *a*, *b* and *c* refer to the synaptic traces illustrated above the graph. *B*, superimposed traces of $EPSP_m$s (left) and responses to a −0·2 nA current step (right) in the presence and absence of 0·2 μM CADO. Note that 0·2 μM CADO caused a substantial reduction in the $EPSP_m$ without affecting the input resistance of the cell. The membrane potential of both cells was −64 mV.

$n = 3$; Fig. 6*C*). In two neurones, in the combined presence of CADO and DPCPX, a small $EPSP_m$ was evoked by the previously subthreshold stimulus. This $EPSP_m$, along with the reduction in spike frequency adaptation, was abolished by subsequent application of atropine (1 μM; Fig. 6*D*).

Effects of an adenosine kinase inhibitor on the $EPSP_m$

The increase in the $EPSP_m$ that was caused by DPCPX alone suggested that endogenous adenosine within the slice was capable of tonically activating the adenosine A_1 receptors which inhibit the $EPSP_m$. Therefore, we next examined whether it was possible to potentiate this tonic effect by impairing the breakdown of adenosine. To do this we investigated the effects of the selective adenosine kinase inhibitor 5-IT which raises extracellular adenosine levels by preventing the breakdown of adenosine into adenosine monophosphate (AMP) (Pak, Haas, Decking & Schrader, 1994). 5-IT (10 μM) caused a postsynaptic hyperpolarization of 2–8 mV, a decrease in input resistance of 5–15% and a $74 \pm 10\%$ depression of the $EPSP_m$ ($n = 4$; Fig. 7*B*). In

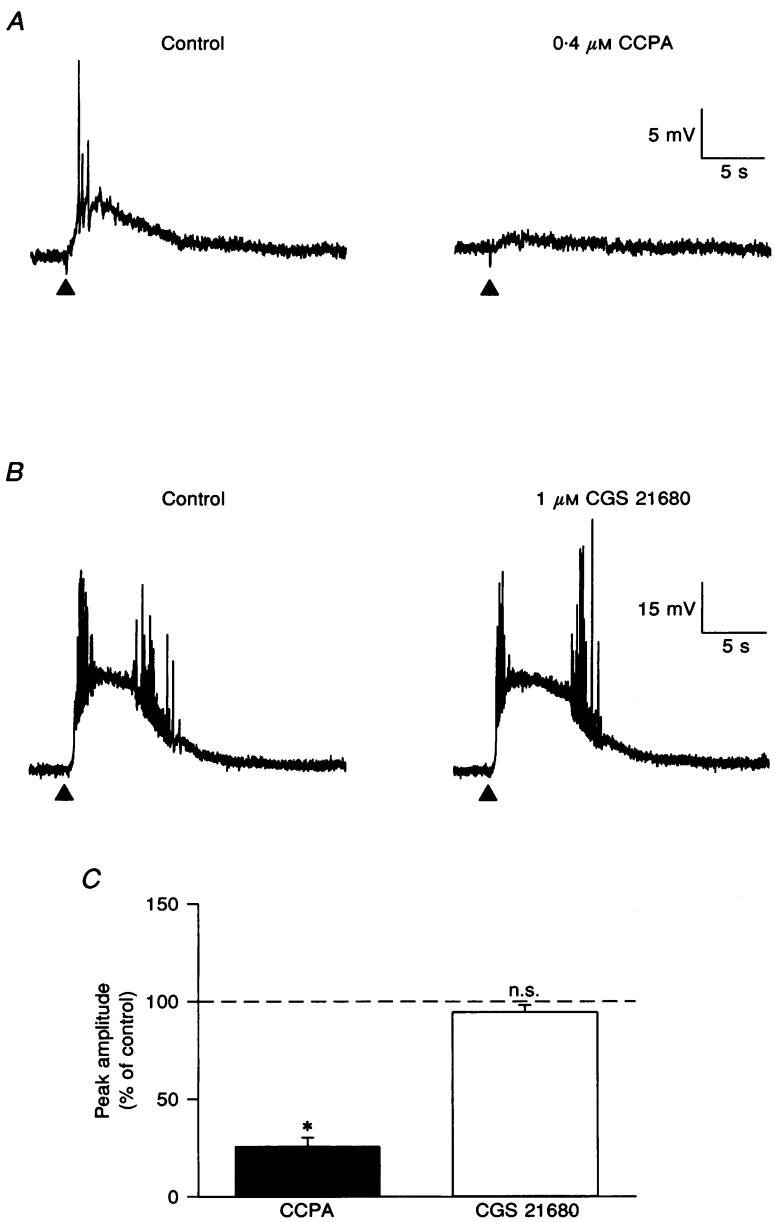

Figure 4. Effects of subtype-selective adenosine receptor agonists on the $EPSP_m$

In *A* and *B*, traces illustrate the respective effects of 0·4 μM CCPA and 1 μM CGS 21680 on $EPSP_m$s evoked in separate neurones. The membrane potentials of these cells were −63 and −64 mV, respectively. *C*, bar graph in which pooled data for the effects of CCPA (0·4 μM; $n = 4$) and CGS 21680 (0·5 μM; $n = 3$) on the $EPSP_m$ are plotted as described in Fig. 2*D*. Note that whilst CGS 21680 did not have a significant effect, CCPA significantly inhibited the $EPSP_m$. Means ± S.E.M.; * $P < 0{\cdot}05$; n.s., not significant (compared with control).

three of these neurones, subsequent application of DPCPX (0·2 μM) completely reversed these effects (Fig. 7*A*).

Is the adenosine-mediated depression of mAChR-mediated postsynaptic responses pre- or postsynaptic?

Having established a depressant action of adenosine on postsynaptic mAChR-mediated responses evoked by afferent stimulation, we addressed next whether these effects were mediated pre- or postsynaptically. Initially, we compared the concentration–response relationships for the depression of the $EPSP_m$ with those of known post- and presynaptic adenosine A_1 receptor-mediated effects, i.e. membrane hyperpolarization (Fig. 8*A*; Greene & Haas, 1985; Gerber, Greene, Haas & Stevens, 1989) and depression of glutamate AMPA/kainate receptor-mediated EPSPs (Fig. 8*B*; Lupica, Proctor & Dunwiddie, 1992), respectively. As illustrated in Fig. 8*C*, CADO was more potent at inhibiting the $EPSP_m$ than it was at inhibiting pure AMPA/kainate receptor-mediated EPSPs or causing postsynaptic hyperpolarization. The respective IC_{50} values and EC_{50} value for these effects were 0·3, 0·8 and 3·0 μM (Fig. 8*C*). In addition, the logistic fit for the depressant action of CADO on the $EPSP_m$ more closely paralleled that for its presynaptic depressant action on glutamate-mediated EPSPs.

If CADO was acting presynaptically to depress both the $EPSP_m$ and the reduction in spike frequency adaptation evoked by subthreshold stimulation then it should not affect the respective postsynaptic responses evoked by carbachol. To maximize the probability of observing an effect of CADO on carbachol-induced postsynaptic responses, we used concentrations of carbachol that (i) were close to the EC_{50} values reported for causing postsynaptic depolarization and

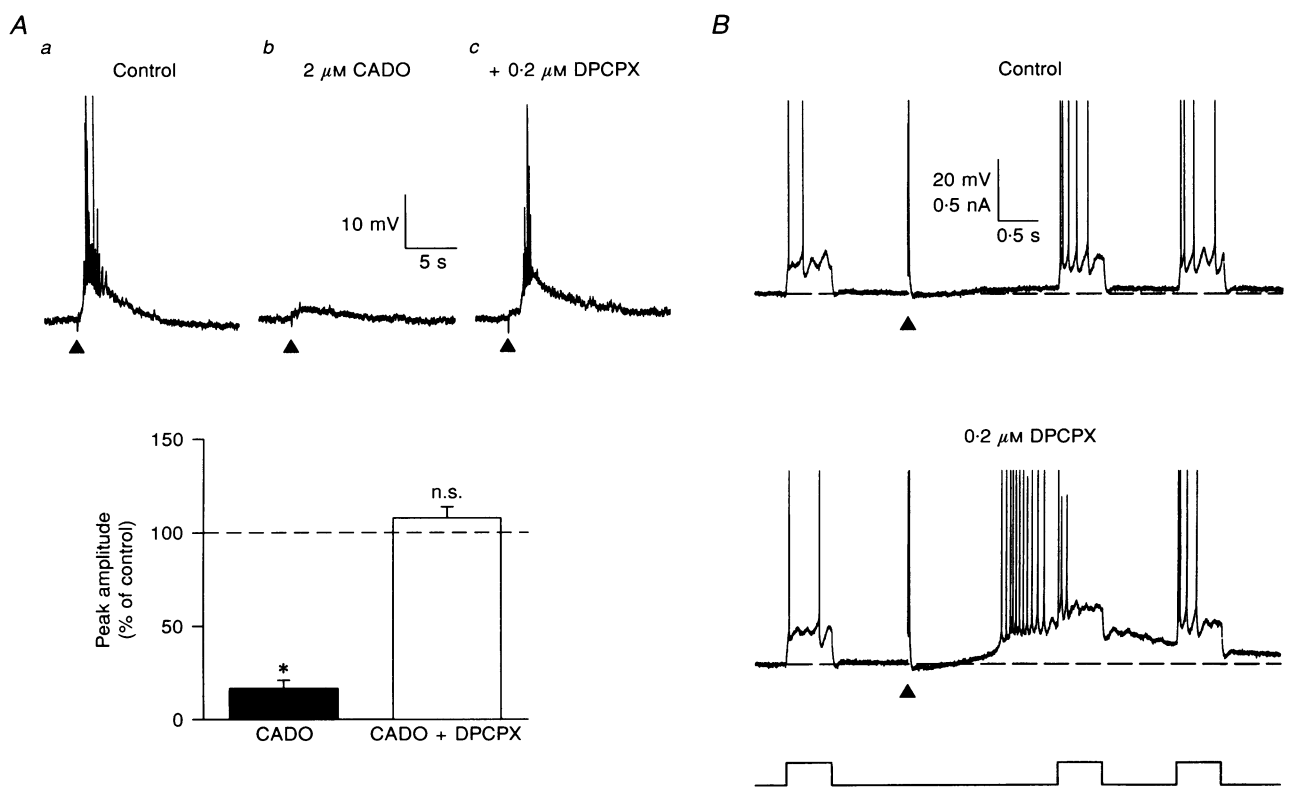

Figure 5. Effects of DPCPX on the $EPSP_m$ and the CADO-induced depression of the $EPSP_m$

In *A*, synaptic traces are $EPSP_m$s recorded in control medium (*a*), in the presence of 2 μM CADO (*b*) and in the combined presence of 2 μM CADO and 0·2 μM DPCPX (*c*). The membrane potential of the cell was −64 mV. The bar graph illustrates pooled data for the effects of 1 μM CADO ($n = 8$) and 1 μM CADO + 0·2 μM DPCPX ($n = 4$) on the $EPSP_m$. Each value was calculated as described for Fig. 2*D*. Note that CADO significantly depressed the $EPSP_m$ and that responses fully recovered in the additional presence of both CADO and DPCPX. Means ± S.E.M.; $*P < 0·05$; n.s., not significant. In *B*, traces are continuous records of the membrane potential of a single cell in which depolarizing current steps (+0·2 nA, 600 ms) were delivered 1 s prior to, and 2 and 3·5 s after pathway stimulation at an intensity just suprathreshold for activating the $EPSP_m$ in medium containing the four glutamate and GABA receptor antagonists (top trace) and following the addition of 0·2 μM DPCPX (middle trace). The bottom trace represents the current injected into the cell. The membrane potential of the cell was −65 mV. Note that in control medium spike frequency adaptation was reduced following pathway stimulation. After addition of DPCPX this intensity of stimulation now additionally activated a substantial $EPSP_m$.

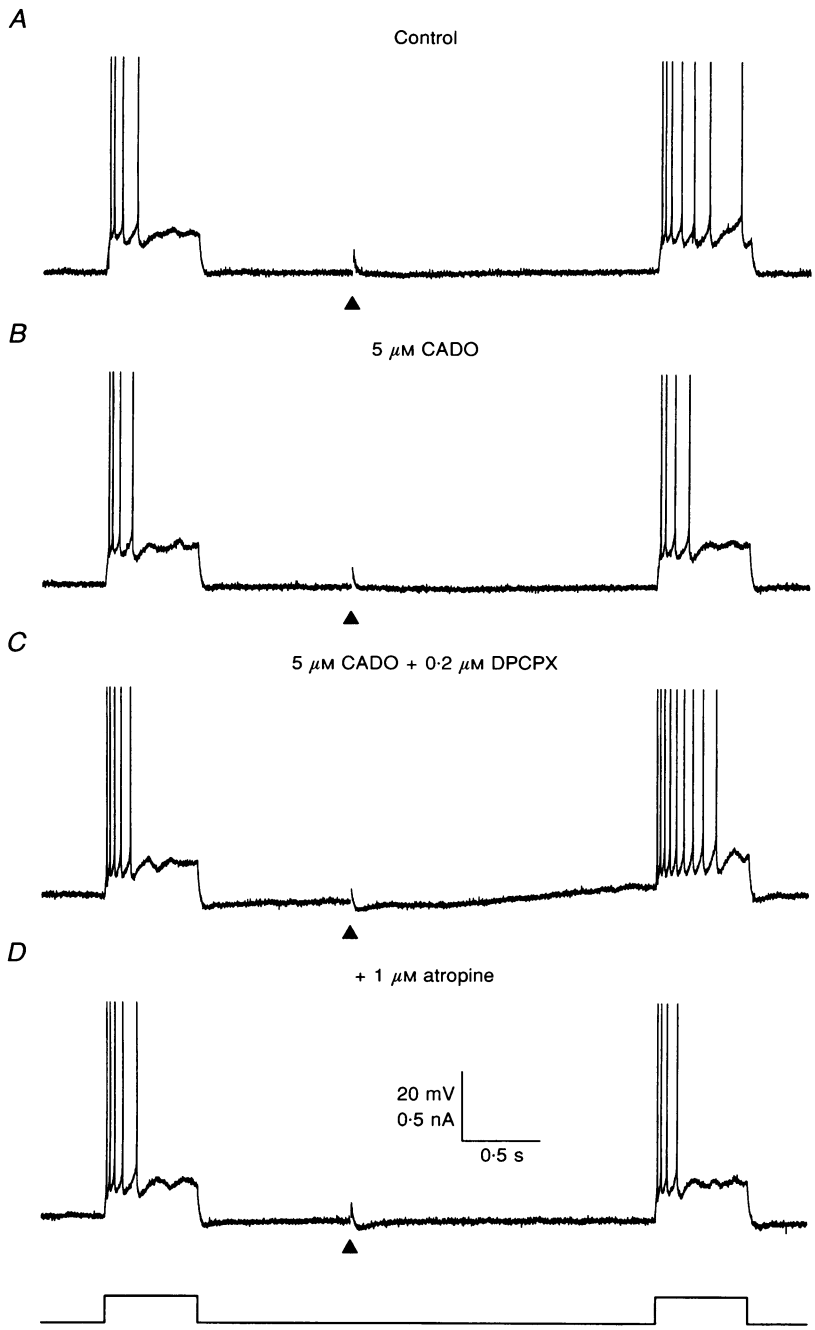

Figure 6. CADO depressed the reduction in spike frequency adaptation evoked by pathway stimulation that was subthreshold for activating the $EPSP_m$

Traces were generated as described in Fig. 5*B* except that only the response to the first depolarizing current step after afferent stimulation is illustrated. The responses were obtained in medium containing the four glutamate and GABA receptor antagonists (*A*), in the additional presence of 5 μM CADO (*B*), in the combined presence of 5 μM CADO and 0·2 μM DPCPX (*C*) and following subsequent addition of 1 μM atropine (*D*). The point of pathway stimulation is indicated by the arrowheads and the trace at the bottom of the figure illustrates the points at which current was injected into the cell. Note that in control medium spike frequency adaptation was reduced following pathway stimulation, that this reduction was abolished by CADO in a DPCPX-sensitive manner and that it was also completely inhibited by subsequent addition of atropine. If two current steps were delivered without intervening subthreshold afferent stimulation, the level of spike frequency adaptation evoked by the second step was not significantly different from that evoked by the first step ($n = 4$; $P > 0{\cdot}05$; not illustrated). Note that in the presence of CADO + DPCPX previously subthreshold pathway stimulation now evoked a small $EPSP_m$ that was abolished by the subsequent addition of atropine. The initial membrane potential of the neurone was −66 mV.

reduction in spike frequency adaptation (Madison *et al.* 1987), and (ii) produced responses similar to those evoked by afferent stimulation (see Fig. 10). In addition, we used concentrations of CADO that were near-maximal for inhibiting the $EPSP_m$ and the reduction in spike frequency adaptation evoked by subthreshold stimulation (see Figs 6 and 8*C*).

In a first series of experiments, we tested the effect of CADO on the postsynaptic depolarization and increase in input resistance evoked by brief bath applications of carbachol (3 μM for 30–60 s). In four neurones, repeated application of carbachol caused consistent and reversible depolarizations that were associated with increases in input resistance (Figs 9*A* and 10*A*). In these same neurones, addition of CADO (1 μM) caused a hyperpolarization (1–5 mV) that was associated with a small decrease in input resistance (4–7%). However, CADO did not have a significant effect on the carbachol-induced depolarizations and increases in input resistance ($n = 4$; $P > 0{\cdot}05$; Fig. 9*A*). In five other neurones, these postsynaptic effects of carbachol were abolished by atropine (1–5 μM; not illustrated). These results contrast with those for the $EPSP_m$, which was depressed significantly by both CADO (1 μM, $P < 0{\cdot}05$; Fig. 10*A*) and atropine (1–5 μM, $P < 0{\cdot}05$; Fig. 2*C*).

In a second series of experiments, we investigated the effects of CADO and carbachol on spike frequency adaptation

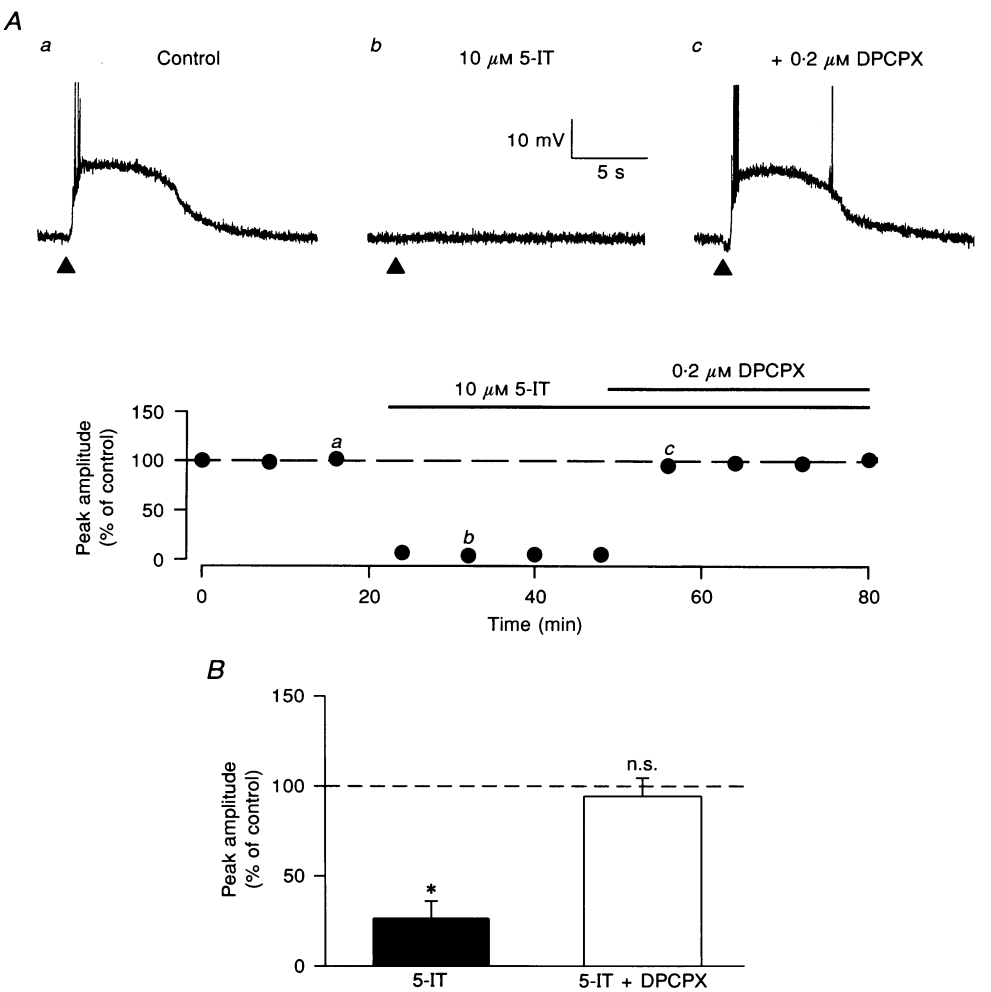

Figure 7. The effects of an inhibitor of adenosine kinase on the $EPSP_m$

A, the bottom graph is similar to that illustrated in Fig. 3 and shows the peak amplitude of successive $EPSP_m$s, for a single experiment, *versus* time to illustrate the depressant effect of 10 μM 5-IT on the $EPSP_m$ and its reversal by 0·2 μM DPCPX. The duration of application of each drug is indicated by the bars above the graph. The synaptic traces (top) are representative $EPSP_m$s recorded in control (*a*), in the presence of 5-IT (*b*) and in the presence of 5-IT + DPCPX (*c*) taken at the time points indicated. The membrane potential of this neurone was −66 mV. *B*, bar graph illustrating pooled data for the effects of 5-IT (10 μM; $n = 4$), and 5-IT + 0·2 μM DPCPX ($n = 3$) on the $EPSP_m$. The values plotted were calculated as described in Fig. 2*D*. Note that 5-IT significantly depressed the $EPSP_m$ and that responses in the presence of both 5-IT and DPCPX were not significantly different from controls. Means ± S.E.M.; $*P < 0{\cdot}05$; n.s., not significant.

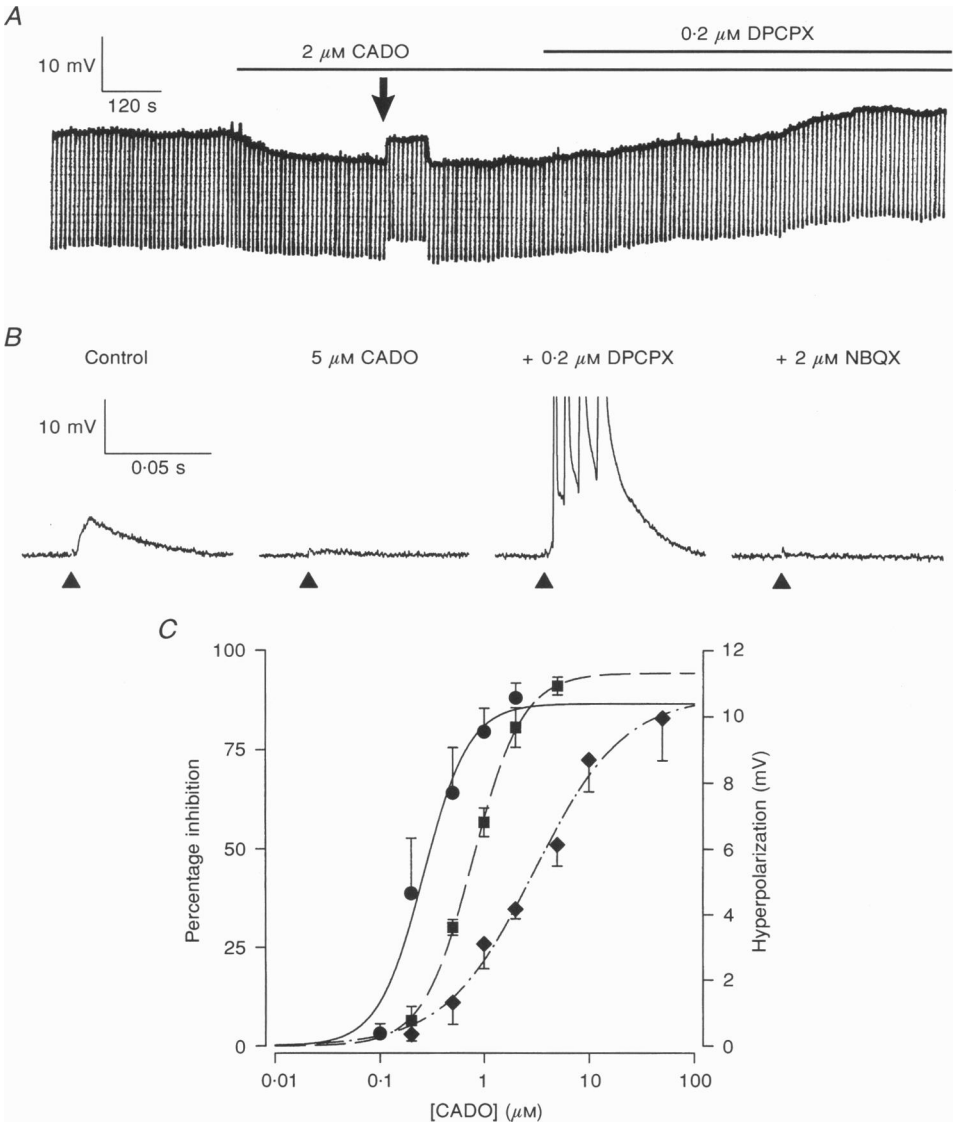

Figure 8. Comparison of the concentration–response relationships for CADO-induced depression of the $EPSP_m$ with those for postsynaptic hyperpolarization and depression of glutamate-mediated EPSPs

A, continuous chart record illustrating the effects of 2 μM CADO and 0·2 μM DPCPX on passive membrane properties of a CA1 neurone. The trace shows the membrane potential (thick line) and hyperpolarizing voltage responses (downward deflections) of the cell to constant current steps (−0·3 nA, 300 ms). The arrow indicates the point at which DC was injected for 90 s to reset the membrane potential of the cell to the level prior to CADO application. Note: (i) that CADO caused a hyperpolarization and a reduction in input resistance that was completely reversed by DPCPX, and (ii) that in the presence of DPCPX the membrane potential became more depolarized than that before any drug treatment, presumably indicating the presence of an endogenous adenosine tonus in the slice. The membrane potential of this neurone was −62 mV. In *B*, the responses are pure AMPA/kainate receptor-mediated EPSPs ($EPSP_A$s) recorded in the combined presence of 50 μM CGP 40116, 50 μM picrotoxin and 1 μM CGP 55845A. The traces, from left to right, are representative $EPSP_A$s recorded in control medium, in the presence of 5 μM CADO, in the combined presence of 5 μM CADO and 0·2 μM DPCPX and following subsequent addition of 2 μM NBQX. Note that DPCPX reversed the CADO-induced depression of the $EPSP_A$ and that in the presence of DPCPX the $EPSP_A$ gave rise to multiple action potentials. This increase in magnitude above control levels again presumably reflects the presence of an endogenous adenosine tonus in the slice. Each trace is an average of four successive responses evoked 15 s apart and the membrane potential of this neurone was −70 mV. *C*, plots of the percentage inhibition of $EPSP_m$s (●; data from 19 neurones) and of $EPSP_A$s (■; data from 4 cells) induced by CADO *versus* concentration of CADO. Superimposed on these plots is a plot of the magnitude of CADO-induced postsynaptic hyperpolarization, from a starting membrane potential of between −62 and −64 mV, *versus* concentration of CADO (◆; data from 18 neurones). Each point for each of the three plots is the mean value obtained from three to nine separate neurones and the error bars represent S.E.M. All data (Y) were fitted to the logistic expression: $Y = M(X^P/(X^P + K^P))$, where X is the concentration of CADO, M is the maximum effect, K is the IC_{50} or EC_{50} value and the power P determines the slope of the sigmoidal curve.

evoked in response to a depolarizing current step. Carbachol (0·5 μM) caused a small depolarization and a reduction in spike frequency adaptation that was reversible on washout ($n = 5$). Subsequent addition of CADO (5 μM) slightly, but not significantly, enhanced the level of spike frequency adaptation *per se* but did not significantly affect the reduction in spike frequency adaptation induced by a second application of carbachol ($n = 3$; Fig. 9*B*). In contrast, atropine abolished the carbachol-induced reduction in spike frequency adaptation ($n = 5$; not illustrated). These results differ from those in which spike frequency adaptation was reduced using subthreshold stimulation in that the latter was abolished by both CADO (5 μM; Fig. 10*B*) and atropine (1 μM).

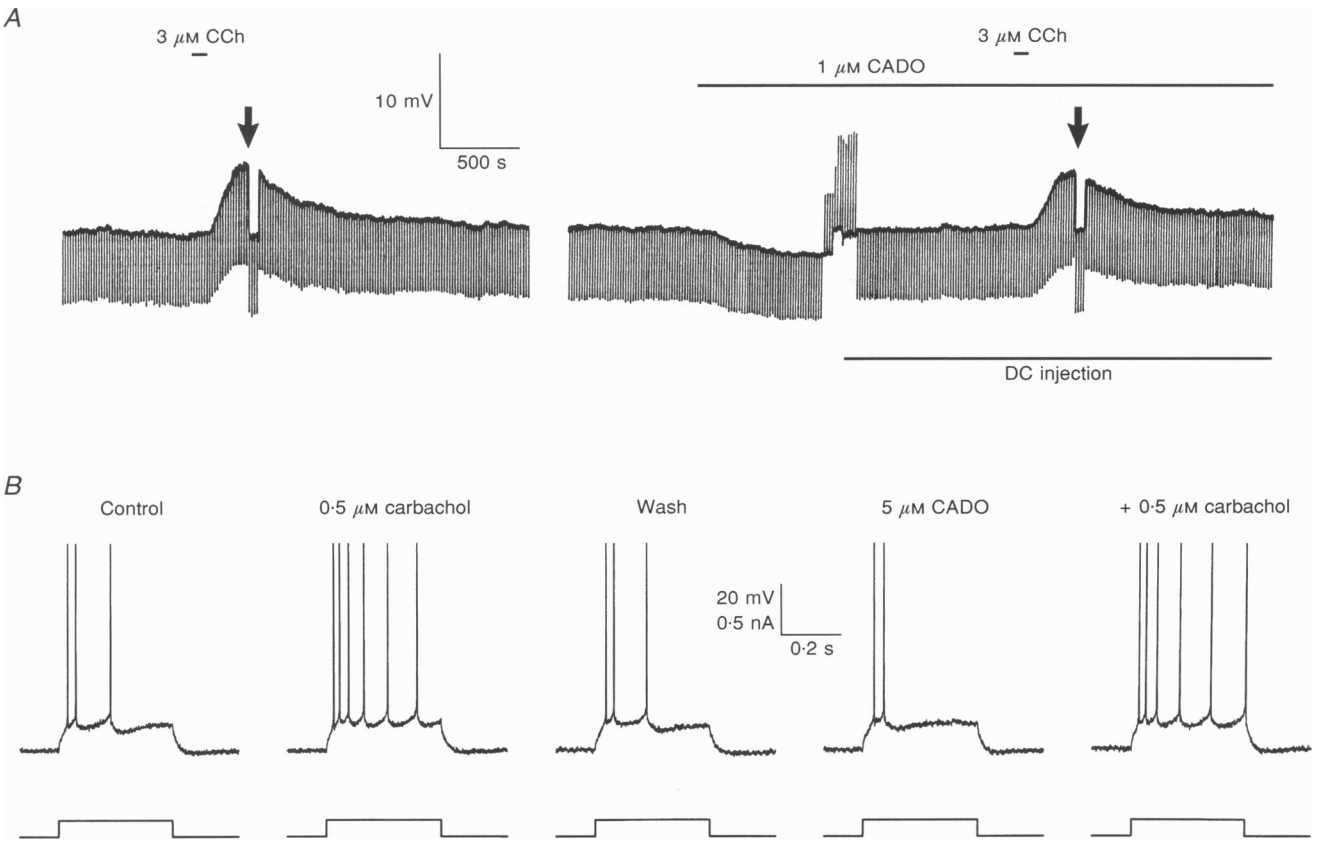

Figure 9. The effects of CADO on postsynaptic responses evoked by carbachol

A shows the effect of CADO (1 μM) on the change in membrane potential and input resistance evoked by carbachol (CCh; 3 μM). The trace is a chart record of membrane potential and cell input resistance, as described in Fig. 8, and illustrates the effects of carbachol on these parameters prior to and following CADO application. The arrows just after the end of each carbachol application indicate the points at which DC was injected for 60 s to reset the membrane potential of the cell to the level prior to the application of the agonist. The bar below the chart record indicates the period during which DC injection was used in the presence of CADO to restore the membrane potential of the cell to that prior to CADO application. This was kept constant for the period indicated by the length of the bar except for when the increase in input resistance evoked by carbachol was measured. The upward deflections just prior to and just after the point at which DC was applied in the presence of CADO represent voltage responses to depolarizing current steps (+0·3 nA, 300 ms). The bars above the chart record represent the periods during which carbachol and CADO were applied. The initial membrane potential of this neurone was −64 mV. *B*, responses (from left to right) evoked by a depolarizing current step (+0·2 nA, 400 ms) in control medium, in the presence of carbachol (0·5 μM), following washout, in the presence of CADO (5 μM) and in the combined presence of CADO (5 μM) and carbachol (0·5 μM). The membrane potential of the cell was maintained at −66 mV throughout the experiment by injecting DC through the recording electrode to compensate for the hyperpolarizing and depolarizing effects of CADO and carbachol, respectively. The trace at the bottom of the figure represents the time during which depolarizing current steps were injected via the recording electrode. Note that carbachol reduced spike frequency adaptation evoked during the depolarizing step and that this was unaffected by CADO.

DISCUSSION

The mAChR-mediated EPSP

mAChR-mediated EPSPs have been recorded previously from CA1 pyramidal neurones most commonly in response to high frequency bursts of stimuli, e.g. 20–100 Hz for 0·5–1·0 s (Cole & Nicoll, 1984; Madison *et al.* 1987; Segal, 1988; Pitler & Alger, 1990; Azouz *et al.* 1994). These EPSPs were often evoked in the presence of acetylcholinesterase inhibitors which prolong the effective synaptic concentration of ACh. In addition, ionotropic glutamate and GABA receptor antagonists were not added to the perfusion medium and, therefore, mAChR-mediated EPSPs were

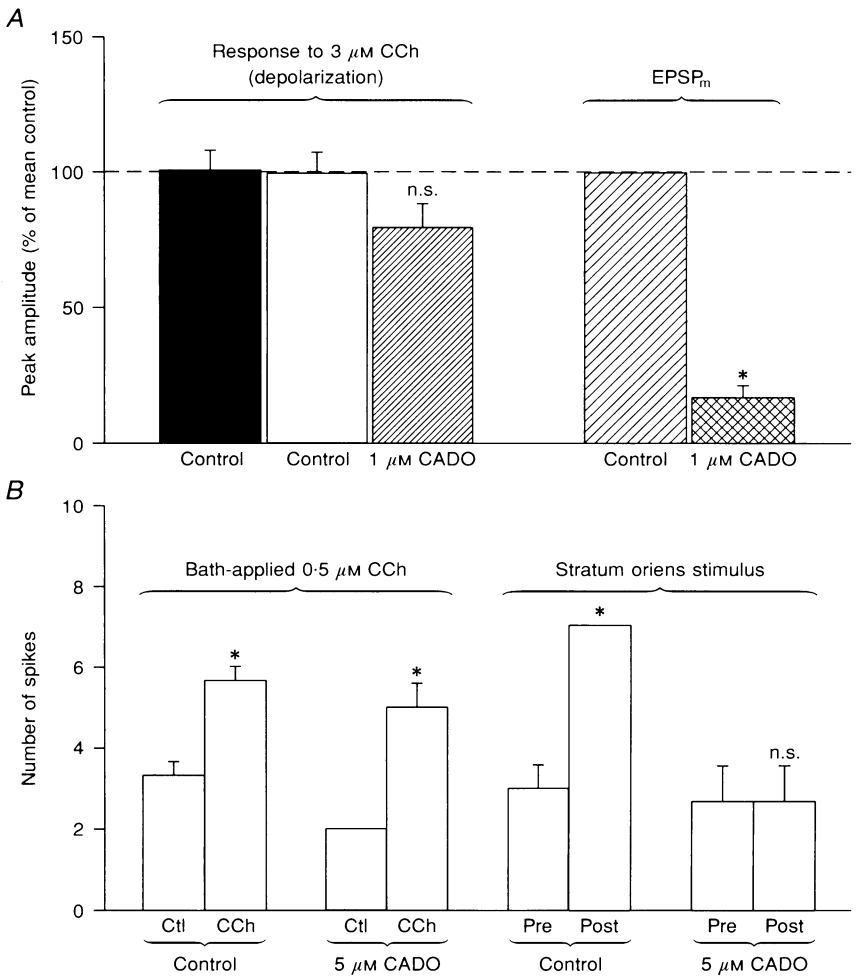

Figure 10. Comparison of the effects of CADO on postsynaptic mAChR-mediated responses evoked by endogenous ACh with those evoked by carbachol

A, pooled data (left) for the peak amplitude of the two carbachol (3 μM)-induced depolarizations prior to the application of CADO (Control) and one depolarization in the presence of 1 μM CADO plotted as a percentage of the mean value for the responses prior to CADO application ($n = 4$; data as generated in Fig. 9*A*); and pooled data (right) for the peak amplitude of the EPSP$_m$ evoked in the presence of 1 μM CADO plotted as a percentage of control ($n = 8$; data generated as in Fig. 3). Note that the same concentration of CADO depressed the EPSP$_m$ to a much greater extent than it did the carbachol-induced depolarization. The small reduction in the size of the carbachol-induced depolarization in the presence of CADO is not statistically significant and can be explained largely on the basis of the decrease in input resistance induced by CADO in this group of cells. In contrast, CADO significantly depressed the EPSP$_m$. *B*, pooled data (left) for the number of action potentials fired during a depolarizing step in the absence (Ctl) and presence (CCh) of 0·5 μM carbachol plotted for control medium and for medium containing 5 μM CADO ($n = 3$; data generated as in Fig. 9*B*); and pooled data (right) for the number of action potentials fired during a depolarizing step 1 s before (Pre) and 2 s after (Post) a subthreshold stimulus in the absence and presence of 5 μM CADO ($n = 3$; data generated as in Fig. 6). Statistical significance was tested for CCh *versus* Ctl and Post *versus* Pre data for each of the paired data sets. Note that CADO completely inhibited the increase in number of action potentials fired following subthreshold afferent stimulation in stratum oriens without affecting the increase evoked by carbachol application. Means ± S.E.M.; * $P < 0·05$; n.s., not significant.

superimposed on glutamate- and GABA-mediated synaptic potentials. Here we have shown that a slow EPSP can be evoked by a single stimulus in the presence of ionotropic glutamate and GABA receptor antagonists without the need for acetylcholinesterase inhibitors. This EPSP is mediated by activation of mAChRs since it was blocked by atropine and unaffected by (+)-MCPG. Its waveform varied considerably between neurones and could not be readily correlated with differences in any of the other electrophysiological characteristics of the neurones in which it was recorded. One notable difference, however, between the $EPSP_m$ recorded in this study and those recorded previously was its large magnitude, even when evoked using just a single stimulus. The reason for this is unclear but it is possible that the amino acid receptor antagonists may relieve an inhibitory influence on the activation of this synaptic potential. Alternatively, in some cells, a number of voltage-activated conductances may boost the apparent magnitude of the $EPSP_m$ (Fraser & MacVicar, 1996). However, preliminary observations that the Ca^{2+} channel antagonist nitrendipine (10 μM) has little or no effect on the $EPSP_m$ suggest that a Ca^{2+}-dependent plateau potential does not necessarily contribute to the $EPSP_m$. Whatever the case, the response kinetics of the $EPSP_m$ were, in general, similar to those reported for $EPSP_m$s evoked by high frequency bursts of stimuli (Cole & Nicoll, 1984; Gähwiler & Brown, 1985; Pitler & Alger, 1990) as well as pure spontaneous and evoked metabotropic glutamate receptor-mediated EPSPs (Charpak & Gähwiler, 1991; Bianchi & Wong, 1995). As with these EPSPs, the $EPSP_m$ described here was accompanied by a reduction in spike frequency adaptation. This effect was mimicked by physostigmine, presumably because this drug increased extracellular levels of spontaneously released ACh by inhibiting acetylcholinesterase activity (Cole & Nicoll, 1984; Azouz *et al.* 1994). A feature of the $EPSP_m$, however, that hampered its investigation was that it could be evoked reproducibly only every 5–10 min. As such, relatively long intracellular impalements were required to obtain meaningful data. This contrasts with metabotropic glutamate receptor-mediated EPSPs which can be evoked reproducibly every 30–60 s (Gerber, Lüthi & Gähwiler, 1993). The reason(s) for the protracted fatigue of the $EPSP_m$ is unclear but by comparison with previous studies it appears to be independent of the stimulation protocol used to evoke it, i.e. a high frequency train *versus* a single stimulus (Cole & Nicoll, 1984; Pitler & Alger, 1990).

Adenosine receptor-mediated depression of postsynaptic responses to synaptically released ACh

To date, few studies have addressed the mechanisms by which mAChR-mediated synaptic transmission is regulated. In one study, the neuropeptide galanin was reported to depress the $EPSP_m$ in the ventral hippocampus by 60% via a presynaptic mechanism (Dutar, Lamour & Nicoll, 1989). Here we have provided compelling evidence that adenosine also inhibits the $EPSP_m$ and that, in addition, it prevents the reduction in spike frequency adaptation mediated by synaptic activation of mAChRs. No distinction was made between dorsal and ventral hippocampus in the present study and, as such, these depressant actions of adenosine are likely to operate throughout the entire CA1 region of the hippocampus. Pharmacological analysis of these effects revealed that they were mediated by adenosine A_1 receptors and that activation of these receptors was capable of abolishing both the $EPSP_m$ and synaptically induced mAChR-mediated reduction in spike frequency adaptation. This is analogous to the situation at glutamate synapses where adenosine A_1 receptors also completely inhibit both AMPA and NMDA receptor-mediated synaptic transmission (Thompson *et al.* 1992; De Mendonça & Ribeiro, 1993). The IC_{50} value for the CADO-induced depression of the $EPSP_m$ is slightly less than that observed for inhibition of AMPA receptor-mediated EPSPs but is similar to that for NMDA receptor-mediated EPSPs (De Mendonça & Ribeiro, 1993; Dunwiddie & Diao, 1994). This possibly reflects the greater need for control of cholinergic and NMDA receptor-mediated synaptic inputs because of their much greater influence on postsynaptic excitability. Indeed, a small degree of adenosinergic inhibition is active tonically as suggested by the enhancement of the $EPSP_m$ by DPCPX. This tonus is greatly enhanced when the metabolism of adenosine is impaired by 5-IT, as is also reported for glutamate synapses (Pak *et al.* 1994). However, the source of the endogenous adenosine tonus is unclear. One possibility is that it is due, at least in part, to non-specific accumulation of adenosine due to cell damage resulting from slice preparation (Thompson *et al.* 1992). Another possibility is that adenosine is released following afferent stimulation (Mitchell, Lupica & Dunwiddie, 1993), e.g. from GABAergic interneurones (Manzoni, Manabe & Nicoll, 1994). Whatever the case, there is considerable evidence that the level of extracellular adenosine in slices is comparable to that in the CNS *in vivo* (Zetterström, Vernet, Ungerstedt, Tossman, Jonzon & Fredholm, 1982; Fredholm, Dunwiddie, Bergman & Lindström, 1984; Dunwiddie & Diao, 1994). As such, it is likely that in the intact animal cholinergic synaptic inputs are tonically inhibited by circulating levels of adenosine.

Locus of the adenosine A_1 receptor-mediated depression of the $EPSP_m$

Since adenosine A_1 receptors are found in abundance both pre- and postsynaptically in the CA1 region of the hippocampus, it is possible that adenosine inhibits mAChR-mediated postsynaptic responses by activation of one or both populations of these receptors (Greene & Haas, 1985; Gerber *et al.* 1989). That the concentration–response relationship for the CADO-induced depression of the $EPSP_m$ most closely paralleled that for the depression of the AMPA/kainate receptor-mediated EPSP, but not that for the postsynaptic hyperpolarization, indirectly favours a presynaptic locus of the adenosine A_1 receptor-mediated depression. In this respect, at least three additional lines of evidence suggest the involvement of a presynaptic depressant

mechanism: (i) CADO can depress the $EPSP_m$ even at concentrations that do not affect postsynaptic passive membrane properties, (ii) CADO does not significantly inhibit carbachol-induced postsynaptic responses, and (iii) both the $EPSP_m$ and mAChR-mediated postsynaptic reduction of spike frequency adaptation evoked by subthreshold stimulation, which result from the inhibition of distinct K^+ conductances (Madison *et al.* 1987), are inhibited by CADO. In addition, neurochemical data from both hippocampal synaptosomes and slices strongly indicate the existence of a presynaptic adenosine A_1 receptor-mediated inhibition of [^{3}H]ACh release (Cunha, Milusheva, Vizi, Ribeiro & Sebastião, 1994). When taken together these data provide convincing evidence that a significant proportion of the adenosine A_1 receptor-mediated inhibition of the $EPSP_m$ and associated reduction in spike frequency adaptation occurs by presynaptic inhibition of ACh release. Such a mechanism has previously been proposed for cholinergic synapses at the neuromuscular junction where activation of adenosine A_1 receptors inhibits the average number of quanta of ACh released from the nerve terminal without affecting the size of each individual quantum (Ginsborg & Hirst, 1972; Silinsky, 1984).

It is conceivable, however, that adenosine may also act postsynaptically to depress mAChR-mediated postsynaptic responses. In this respect, a direct interaction between the transduction mechanisms activated by adenosine A_1 receptors and mAChRs seems unlikely as adenosine A_1 receptors classically couple to the G-protein G_i which inhibits adenylate cyclase activity and neither the $EPSP_m$ nor the mAChR-mediated reduction in spike frequency adaptation are affected by cAMP mimetics (Madison *et al.* 1987; R. A. Morton, unpublished observations) or antagonists of protein kinase A (Pedarzani & Storm, 1993). However, negative interactions between adenosine A_1 receptors and the M_1 mAChR-mediated inhibition of the M-current have been reported in the superior cervical ganglion (Connolly & Stone, 1995). Despite this, the M-current is unlikely to account for the actions of synaptically activated muscarinic receptors in the hippocampus (Madison *et al.* 1987) even though pyramidal neurones are thought to express both the M_1 and M_3 mAChR subtypes (Dutar & Nicoll, 1988; Pitler & Alger, 1990; Segal & Fisher, 1992). These receptors classically couple through the pertussis-toxin insensitive $G_{q/11}$ family of G-proteins to phospholipase C (PLC), the activity of which can, in some cells, e.g. aorta, be inhibited by adenosine A_1 receptors. However, it is unclear whether activation of PLC fully accounts for mAChR-mediated depolarization and reductions in spike frequency adaptation in the hippocampus (Muller & Misgeld, 1986; Dutar & Nicoll, 1988; Colino & Halliwell, 1993). Whatever the case, the inability of CADO to abolish carbachol-induced responses would suggest that this mechanism or a membrane-delimited interaction between the adenosine A_1 receptor-mediated signal transduction mechanism(s) and the mAChR-coupled K^+ conductances is unlikely to account for the depressant effects of adenosine A_1 receptors on the $EPSP_m$ and reduction in spike frequency adaptation in response to subthreshold stimulation (see Fig. 10*B*). However, adenosine, by activating postsynaptic K^+ conductances, will restrict the magnitude of the $EPSP_m$ recorded at the soma, to some extent, by the shunting of membrane currents as well as hyperpolarization towards the reversal potential of the $EPSP_m$ (Cole & Nicoll, 1984).

Implications for adenosine receptor-mediated inhibition of the $EPSP_m$

The established role of adenosine in regulating glutamate but not GABA-mediated synaptic transmission has implicated this neuromodulator as a neuroprotective agent (Thompson *et al.* 1993). The present study identifies another dimension of adenosine-mediated regulation of synaptic excitability in the hippocampal CA1 region, viz. inhibition of mAChR-mediated synaptic responses. This regulation is likely to be of relatively minor importance under normal physiological conditions as the septohippocampal cholinergic synaptic input is important in learning and memory formation *in vivo* (Decker & McGaugh, 1991; Hasselmo & Bower, 1993). However, during seizures or hypoxic and ischaemic episodes, when extracellular adenosine levels are raised, adenosine A_1 receptors may restrict cholinergic synaptic transmission to levels that are below that which is damaging to neurones.

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