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ADENOSINE A_{2A} RECEPTOR FACILITATION OF HIPPOCAMPAL SYNAPTIC TRANSMISSION IS DEPENDENT ON TONIC A_1 RECEPTOR INHIBITION

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Abstract—Adenosine tonically inhibits synaptic transmission through actions at A_1 receptors. It also facilitates synaptic transmission, but it is unclear if this facilitation results from pre- and/or postsynaptic A_{2A} receptor activation or from indirect control of inhibitory GABAergic transmission. The A_{2A} receptor agonist, CGS 21680 (10 nM), facilitated synaptic transmission in the CA1 area of rat hippocampal slices (by 14%), independent of whether or not GABAergic transmission was blocked by the $GABA_A$ and $GABA_B$ receptor antagonists, picrotoxin (50 μM) and CGP 55845 (1 μM), respectively. CGS 21680 (10 nM) also inhibited paired-pulse facilitation by 12%, an effect prevented by the A_{2A} receptor antagonist, ZM 241385 (20 nM). These effects of CGS 21680 (10 nM) were occluded by adenosine deaminase (2 U/ml) and were made to reappear upon direct activation of A_1 receptors with N^6-cyclopentyladenosine (CPA, 6 nM). CGS 21680 (10 nM) only facilitated (by 17%) the K^+-evoked release of glutamate from superfused hippocampal synaptosomes in the presence of 100 nM CPA. This effect of CGS 21680 (10 nM), in contrast to the isoproterenol (30 μM) facilitation of glutamate release, was prevented by the protein kinase C inhibitors, chelerythrine (6 μM) and bisindolylmaleimide (1 μM), but not by the protein kinase A inhibitor, H-89 (1 μM). Isoproterenol (30 μM), but not CGS 21680 (10–300 nM), enhanced synaptosomal cAMP levels, indicating that the CGS 21680-induced facilitation of glutamate release involves a cAMP-independent protein kinase C activation. To discard any direct effect of CGS 21680 on adenosine A_1 receptor, we also show that in autoradiography experiments CGS 21680 only displaced the adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentyladenosine ([^{3}H]DPCPX, 0.5 nM) with an EC_{50} of 1 μM in all brain areas studied and CGS 21680 (30 nM) failed to change the ability of CPA to displace DPCPX (1 nM) binding to CHO cells stably transfected with A_1 receptors.

Our results suggest that A_{2A} receptor agonists facilitate hippocampal synaptic transmission by attenuating the tonic effect of inhibitory presynaptic A_1 receptors located in glutamatergic nerve terminals. This might be a fine-tuning role for adenosine A_{2A} receptors to allow frequency-dependent plasticity phenomena without compromising the A_1 receptor-mediated neuroprotective role of adenosine.

Key words: glutamate, PKC, hippocampus, synaptosomes, PKA.

In several areas of the CNS, adenosine plays an important role as a neuromodulator. Particularly in the hippocampus, the most highly expressed A_1 receptors are responsible for the inhibitory effects mediated by adenosine in synaptic transmission and neuronal excitability (Dunwiddie and Masino, 2001). In recent years, however, it has been demonstrated that low concentrations of the adenosine A_{2A} receptor agonist, CGS 21680, trigger facilitatory responses, suggesting the existence of facilitatory adenosine A_{2A} receptors (for a review see Sebastião and Ribeiro, 1996). In the hippocampus, adenosine may therefore exert a dual regulation of synaptic transmission via these two subtypes of receptors (Cunha, 2001).

The mechanism by which activation of adenosine A_1 receptors leads to inhibition of transmitter release is reasonably well understood (Thompson et al., 1992; Ambrósio et al., 1997). By contrast, the way in which A_{2A} receptors affect neurotransmission is less clear. It has been proposed that A_{2A} receptors could presynaptically facilitate glutamate release (Cunha et al., 1997; Li and Henry, 1998), but some electrophysiologically

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Abbreviations: ADA, adenosine deaminase; CGP 55845, 3-*N*-[1-(*S*)-(3,4-dichlorophenyl)ethyl]amino-2-(*S*)-hydroxypropyl-*P*-benzylphosphinic acid; CGS 21680, 2-[4-(2-*p*-carboxyethyl)phenylamino]-5′-*N*-ethylcarboxamidoadenosine; CHO, Chinese hamster ovary; CPA, N^6-cyclopentyladenosine; DMSO, dimethylsulfoxide; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EDTA, ethylenediaminetetra-acetate; fEPSP, field excitatory postsynaptic potential; H-89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]5-isoquinolinesulfonamide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); HPLC, high-performance liquid chromatography; LTP, long-term potentiation; PKA, protein kinase A; PKC, protein kinase C; PPF, paired-pulse facilitation; Rolipram, [4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone]; R-PIA, N^6-*R*-phenylisopropyladenosine; ZM 241385, 4-(2-{7-amino-2(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol.

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recorded A_{2A} receptor-mediated effects in hippocampal slices may be attributed to a postsynaptic site of action (Li and Henry, 1998; O'Kane and Stone, 1998). Also, since there is a tight A_1/A_{2A} receptor interaction (Dixon et al., 1997; Lopes et al., 1999a), it is unclear whether the function of A_{2A} receptors is to directly control neurotransmitter release (Sebastião and Ribeiro, 1996) or, alternatively, to attenuate the tonic inhibitory action of A_1 receptors (Lopes et al., 1999a). Finally, since excitatory synaptic transmission in the hippocampus is under GABAergic control (Buckmaster and Soltesz, 1996), it is possible that A_{2A} receptor-mediated facilitation results from interference with the GABAergic system (Cunha and Ribeiro, 2000a), as proposed in other brain areas (Phillis, 1998; Edwards and Robertson, 1999).

We therefore investigated if the action of adenosine A_{2A} receptors in the rat hippocampus depends on GABAergic function or if it is a direct effect on glutamatergic nerve terminals controlling the release of neurotransmitter. We also tested if the facilitatory effects observed when A_{2A} receptors are activated mainly result from an attenuation of tonic A_1 receptor responses.

EXPERIMENTAL PROCEDURES

Drugs

N^6-Cyclopentyladenosine (CPA), 1,3-dipropyl-8-cyclopentyladenosine (DPCPX) and CGS 21680 were from RBI (Natick, MA, USA), chelerythrine, bisindolylmaleimide and H-89 were from Calbiochem (Darmstadt, Germany), isoproterenol, propanol, picrotoxin and N^6-*R*-phenylisopropyladenosine (R-PIA) were from Sigma (St. Louis, MO, USA), ZM 241385 was from Tocris Cookson (Bristol, UK), [^{3}H]DPCPX (specific activity 110.6 Ci/mmol) was from DuPont NEN (Stevenage, Hertfordshire, UK) and [^{3}H]glutamate (specific activity 45 Ci/mmol) was from Amersham (Buckinghamshire, UK). Rolipram was provided by Schering and CGP 55845 was supplied by Ciba Geigy. All cell culture solutions were from Gibco.

CPA, CGS 21680, chelerythrine, bisindolylmaleimide, H-89 and ZM 241385 were made up to a 5 mM stock solution in dimethylsulfoxide (DMSO) and rolipram was made up in a 50 mM stock solution in DMSO. DPCPX and CGP 55485 were made up into 5 mM stocks in 99% DMSO and 1% NaOH 1 M and picrotoxin was made up to a 50 mM stock solution in ethanol. Aqueous dilution of these stock solutions was made daily.

Electrophysiological recordings of hippocampal synaptic transmission

Field excitatory postsynaptic potentials (fEPSPs) were recorded in the CA1 area of hippocampal slices obtained from male Wistar rats (5–6 weeks old; Harlan Iberica, Barcelona, Spain), handled according to the European guidelines (86/609/EEC), as previously described (e.g. Cunha et al., 1994). A cut was made to separate the CA1 from the CA3 region to prevent propagation of epileptiform activity. The intensity of the stimulus was adjusted to evoke a fEPSP with an amplitude of 0.7–1 mV without appreciable population spike contamination and responses were quantified as the initial slope of the averaged fEPSPs (Anderson and Collingridge, 1997). In all of the experiments, the data were analyzed as mean percentage change in response slope when compared with responses obtained during the control period.

To elicit paired-pulse facilitation, the Schaffer pathway was stimulated twice with 50-ms inter-pulse interval and the synaptic facilitation was quantified as the ratio (P_2/P_1) between the slopes of the fEPSP elicited by the second (P_2) and the first (P_1) stimuli.

[^{3}H]Glutamate release from hippocampal nerve terminals

The evoked release of [^{3}H]glutamate was adapted from Lonart et al. (1998), following a methodology similar to that used to study the release of GABA and acetylcholine from rat hippocampal nerve terminals (Cunha et al., 1997; Cunha and Ribeiro, 2000a). Basically, the synaptosomes were labelled with [^{3}H]glutamate (0.2 μM) during 5 min at 37°C, layered over Whatman GF/C filters and superfused (flow rate: 0.8 ml/min) with Krebs solution of the following composition (in mM): 124 NaCl, 3 KCl, 26 $NaHCO_3$, 1.25 NaH_2PO_4, 1 $MgSO_4$, 2 $CaCl_2$ and 10 glucose, gassed with a 95% O_2–5% CO_2 mixture. The synaptosomes were stimulated with 28 mM K^+ (isomolar substitution of Na^+ by K^+ in the Krebs superfusion solution) at 3 and 9 min after starting sample collection (S_1 and S_2) and test drugs were added 2 min before S_2 onwards. The amount of radioactivity recovered in the effluent of the evoked release peak was mostly glutamate, since high-performance liquid chromatography (HPLC) separation of the effluent samples (see Cunha and Ribeiro, 2000a) showed that $65 \pm 6\%$ ($n = 4$) of total radioactivity in samples of the synaptosomal superfusate collected in basal conditions and $89 \pm 4\%$ ($n = 4$) of total radioactivity in samples collected upon K^+ stimulation was recovered in the glutamate peak. The evoked release of [^{3}H]glutamate was essentially Ca^{2+}-dependent since omission of Ca^{2+} in the Krebs solution from 2 min before S_2 onwards essentially abolished the K^+-evoked tritium release ($n = 4$). Thus, we considered that the evoked release of tritium in the present experimental conditions corresponds to a Ca^{2+}-dependent release of [^{3}H]glutamate.

When we evaluated the changes of the effect of a drug by a modifier, this modifier was applied 15 min before the beginning of sample collection period and was present during S_1 and S_2. When present during S_1 and S_2, CPA (100 nM), chelerythrine (6 μM), H-89 (1 μM) or propanol (30 μM) did not significantly ($P > 0.05$) alter the S_2/S_1 ratio as compared with the S_2/S_1 ratio obtained in control conditions (no added drug), and bisindolylmaleimide (1 μM) also failed to appreciably modify the S_2/S_1 ratio.

Autoradiography in brain slices

In order to examine interactions of CGS 21680 with A_1 receptors we first used quantitative autoradiography essentially as described (Parkinson and Fredholm, 1992). In brief, coronal sections of rat brain were preincubated with adenosine deaminase (ADA), washed, and incubated for 2 h at room temperature with 0.5 nM [^{3}H]DPCPX and increasing (5–5000 nM) concentrations of CGS 21680 and 1 or 10 mM $MgCl_2$. After washing, sections were dried and apposed, together with microscales, to Hyperfilm (Amersham) for 3 weeks. Quantitation was performed using a MCID M4 system and data analyzed using GraphPad Prism.

Radioligand binding in transfected CHO cells

Chinese hamster ovary (CHO) cells (CHO-K1 cells; CCL61, American Type Culture Collection, Rockville, MD, USA) stably transfected with human adenosine A_1 or human A_{2A} receptors were grown adherent and maintained as described by Klotz et al. (1998). The whole cell binding assays were performed as previously described (Gerwins et al., 1990). For saturation curves, [^{3}H]DPCPX (0–10 nM) was incubated with 150 000–250 000 cells (counted by the Trypan Blue exclusion method) in a final volume of 300 μl in an incubation solution containing Dulbecco's modified Eagle's medium (DMEM) buffered with 20 mM HEPES, pH 7.4 (Gerwins et al., 1990). All samples were assayed in duplicate. The incubation was for 2 h at room temperature (20–25°C). Competition curves of the A_1 receptor antagonist, [^{3}H]DPCPX, by the A_1 receptor agonist, CPA, were performed in the absence and in the presence of the A_{2A} receptor agonist, CGS 21680. The whole cell suspension (150 000–250 000 cells/well) was incubated with [^{3}H]DPCPX (1 nM) and 10 different concentrations of the displacer, CPA (ranging from 0.1 nM to 1 μM), in DMEM/HEPES, pH 7.4 solution in a final volume of 300 μl. The remaining conditions were the same as described above. The specific binding was obtained by subtracting the total binding from non-specific binding, which was measured in the presence of R-PIA (20 μM) and represented only 10% of total binding. The IC_{50} values were converted into K_i values by non-linear fitting of the semi-logarithmic curves derived from the competition curves. An F-test ($P<0.05$) was used to determine whether the curves were best fitted by a one or two independent binding site equation. Protein determination was by Bradford method using Bio-Rad reagent.

cAMP assays in hippocampal nerve terminals

Hippocampal synaptosomes were prepared as described above and resuspended in 1 ml gassed Krebs solution also containing 2 U/ml ADA and 50 μM rolipram. A 90-μl synaptosomal aliquot was warmed at 37°C for 15 min and then incubated at 37°C for 4 min with gassed Krebs containing ADA and rolipram (control) or with this modified Krebs containing various concentrations of isoproterenol (30 μM) or CGS 21680 (30–300 nM) without or with CPA (100 nM) in the absence or in the presence of chelerythrine (6 μM) or H-89 (1 μM). The treated synaptosomes were then inactivated by boiling for 10 min in 1 ml of 50 mM Tris/4 mM EDTA, pH 7.6. The levels of cAMP in the supernatants obtained after sonication and centrifugation ($14\,000\times g$, 10 min, 4°C) were quantified with a radioimmunoassay kit (Amersham), as previously described (Lopes et al., 1999b), and protein measured according to Peterson (1977).

Statistics

The values presented are mean ± S.E.M. of n experiments. To test the significance of the effect of a drug versus control, a paired Student's t-test was used. When making comparisons from different sets of experiments with control, a one-way analysis of variance (ANOVA) was used, followed by a Dunnett's test. $P\leq 0.05$ was considered to represent a significant difference.

RESULTS

Effect of A_{2A} receptor activation upon blockade of GABAergic transmission

The release of several neurotransmitters (excitatory and inhibitory) contributes to the overall response that is analyzed in a fEPSP (Thompson et al., 1992). We first investigated if the result of activation of A_{2A} receptors on extracellular electrophysiological recordings was dependent on inhibitory GABAergic transmission. In this set of experiments, the superfusion of hippocampal slices with the A_{2A} receptor agonist, CGS 21680 (10 nM), caused a 14.4 ± 1.9% ($n=4$, $P<0.05$) facilitation, reversible upon washout, of synaptic transmission in Schaffer fiber/CA1 pyramid synapses (Fig. 1A), a response previously shown to be mediated by adenosine A_{2A} receptors since it was prevented by the A_{2A} receptor antagonist, ZM 241385 (Cunha et al., 1997; Cunha and Ribeiro, 2000b). The addition of the $GABA_A$ receptor antagonist, picrotoxin (50 μM), plus the $GABA_B$ receptor antagonist, CGP 55845 (1 μM), caused a 26.3 ± 2.4% ($n=3$) facilitation of fEPSP slope, as a consequence of removing the GABAergic inhibitory action. However, the facilitatory effect of CGS 21680 (10 nM) on synaptic transmission remained intact after this GABAergic blockade (Fig. 1). Facilitation of fEPSP slope caused by CGS 21680 (10 nM) was 16.3 ± 2.7% ($n=3$, $P<0.05$) in the absence and 17.9 ± 3.8% ($n=3$, $P<0.05$) in the presence of picrotoxin (50 μM) plus CGP 55845 (1 μM). This indicates that the defined A_{2A} receptor-mediated facilitation of synaptic transmission is independent of GABAergic transmission and that led us to investigate the effect of CGS 21680 on the glutamatergic transmission.

Effect of A_{2A} receptor activation on paired-pulse facilitation

When two consecutive pulses are applied to the afferent Schaffer fibers with an interval of 50 ms, the fEPSP response to the second pulse is increased as a result of presynaptic calcium accumulation. Modification of this phenomenon, called paired-pulse facilitation (PPF), is an indication of a presynaptic action in the control of glutamatergic nerve terminals (Wu and Saggau, 1994). When PPF is increased by a drug it suggests an inhibition of glutamate release and when PPF is decreased it suggests a facilitation of glutamate release (Thompson et al., 1992). As shown in Fig. 2, CGS 21680 (10 nM) inhibited by 12.3 ± 1.4% ($P<0.05$, $n=5$) PPF while simultaneously facilitating the fEPSP slope. In three of these experiments, we found that ZM 241385 (20 nM) blocked the ability of CGS 21680 to inhibit PPF. By itself, ZM 241385 (20 nM) was devoid of measurable effects on PPF. This is a strong suggestion that the studied A_{2A} receptor agonist acts presynaptically, facilitating glutamate release and therefore facilitating the fEPSP response.

Effect of A_{2A} receptor activation on glutamate release

To directly demonstrate that the action of the A_{2A} receptor agonist occurs at the presynaptic level, we studied its effect on glutamate release from nerve terminals. When hippocampal synaptosomes, previously loaded with [^{3}H]glutamate, were stimulated for 30 s with 28 mM K^+, they released tritium in a Ca^{2+}-dependent manner that is mostly [^{3}H]glutamate, as assessed by HPLC (see Experimental procedures). Two periods of chemical stimulation (S_1 and S_2), separated by a 6-min interval, produced a similar evoked tritium release (Fig. 3A, B), with an S_2/S_1 ratio of 0.89 ± 0.02 ($n=15$). As illustrated in Fig. 3A, application of CGS 21680 (10 nM) 2 min before the second stimulation period (S_2) failed to modify the K^+-evoked release of tritium. The S_2/S_1 ratio was of 0.75 ± 0.07 in control conditions and of 0.71 ± 0.13 when CGS 21680 was present ($n=4$). This lack of effect of CGS 21680 (10 nM) is not due to a general inability to facilitate the evoked release of glutamate from hippocampal nerve terminals, since the β-adrenergic receptor agonist, isoproterenol (30 μM), facilitated the evoked release of glutamate (Fig. 3C, D). This effect of isoproterenol was antagonized by the β-ad-

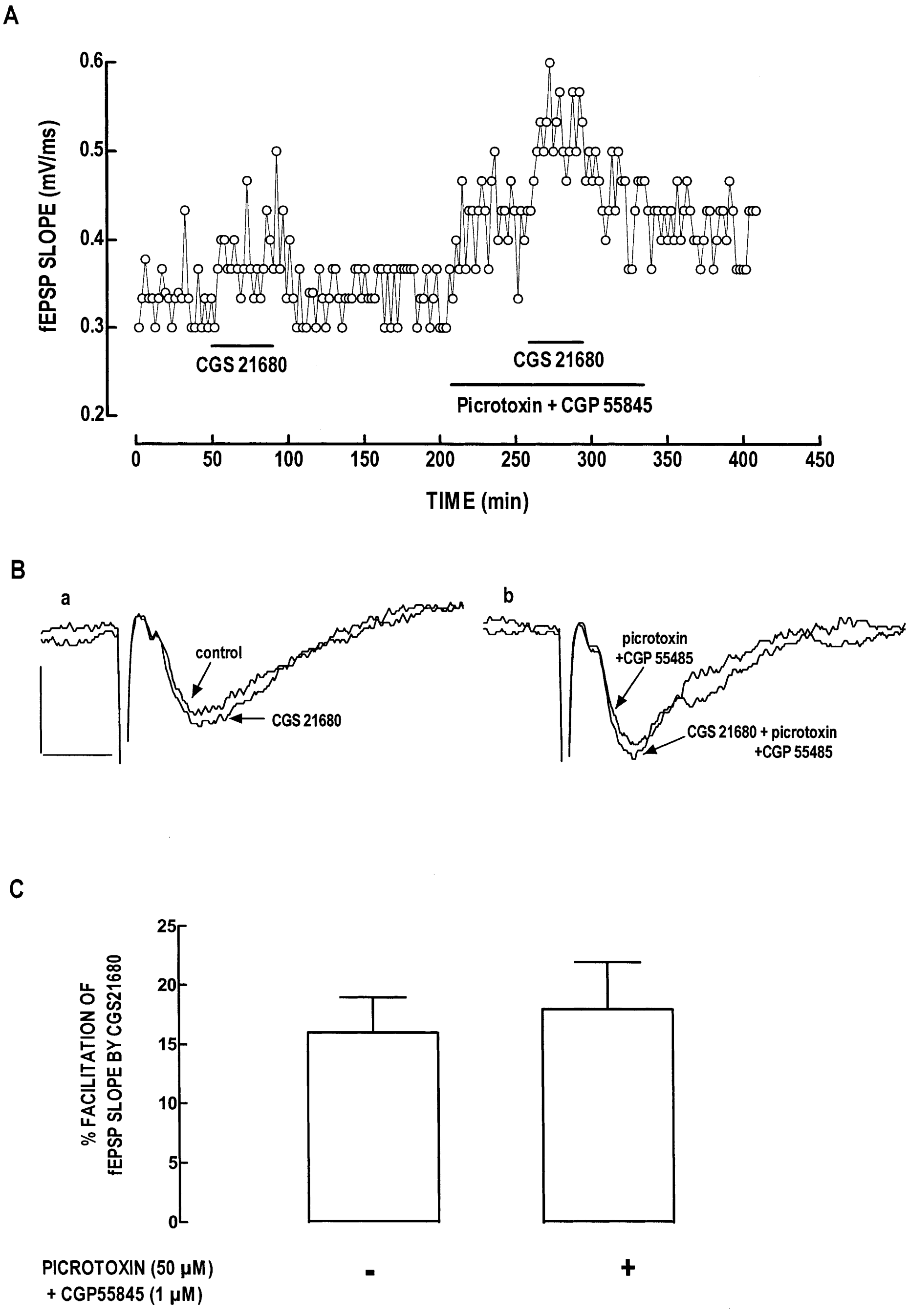

Fig. 1. Comparison of the effects of the adenosine A_{2A} receptor agonist, CGS 21680 (10 nM), on synaptic transmission in rat hippocampal slices in the absence and in the presence of the $GABA_A$ receptor antagonist, picrotoxin (50 μM), and of the $GABA_B$ receptor antagonist, CGP 55845 (1 μM). (A) Averages of the slopes of eight consecutive fEPSPs recorded from the CA1 area of a hippocampal slice, which was first superfused with CGS 21680 (10 nM), then washed out, then with picrotoxin (50 μM) and CGP 55845 (1 μM), then with CGS 21680 (10 nM) in the presence of picrotoxin (50 μM) and CGP 55845 (1 μM), as indicated by the upper horizontal bars. The superimposed fEPSPs presented in B were obtained in (a) before adding any drug to the superfusion solution and 20–24 min after application of CGS 21680 (10 nM), and in (b) 20–24 min after adding picrotoxin (50 μM) and CGP 55845 (1 μM) and 20–24 min after application of CGS 21680 (10 nM) in the presence of picrotoxin (50 μM) and CGP 55845 (1 μM). Scale bars = 500 μV, 5 ms. (C) The ordinates represent the average percentage increase of fEPSP slope caused by CGS 21680 (10 nM) in the absence ($n=3$, control fEPSP slope of 0.42 ± 0.03 μV/ms) and in the presence of picrotoxin (50 μM) and CGP 55845 (1 μM) ($n=3$, control fEPSP slope of 0.43 ± 0.03 μV/ms).

renergic receptor antagonist propanol (30 μM, $n=4$). To exclude that the lack of effect of CGS 21680 on the evoked release of glutamate might result from an insufficient period of equilibration of CGS 21680, we tested the effect of CGS 21680 (10 nM) applied 6 min before S_2. Under these conditions, which were used to show the facilitatory effect of CGS 21680 (10 nM) on the evoked release of acetylcholine and of GABA from the same

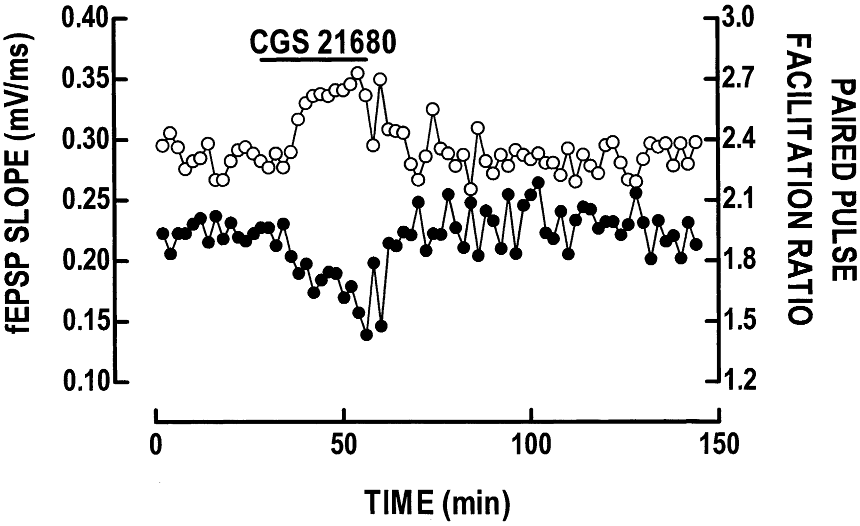

Fig. 2. Representative experiment illustrating the effects of the adenosine A_{2A} receptor agonist, CGS 21680 (10 nM), on synaptic transmission and on PPF recorded in the same experiment in rat hippocampal slices. The open symbols are the averages of the slopes of eight consecutive fEPSPs recorded from the CA1 area of a hippocampal slice that was superfused with CGS 21680 (10 nM) as indicated in the upper bar. The filled symbols represent the average of eight PPF (50-ms interval) of fEPSPs, quantified as the ratio (P_2/P_1) between the slope of the response to the second stimulus (P_2) and the slope of the response to the first stimulus (P_1).

preparation (Cunha et al., 1997; Cunha and Ribeiro, 2000a), CGS 21680 (10 nM) was still devoid of effects on the evoked release of glutamate ($n = 2$, data not shown). Finally, we tested the effect of a higher concentration of CGS 21680. At a concentration of 30 nM, CGS 21680 was still devoid of effects on the evoked release of glutamate (S_2/S_1 ratio: control $= 0.82 \pm 0.14$; test $= 0.85 \pm 0.13$, $n = 5$) and higher concentrations of CGS 21680 were not tested since they were previously shown to produce a lower or no effect, possibly due to desensitization of A_{2A} receptors (e.g. Cunha et al., 1997; Cunha and Ribeiro, 2000a).

Role of tonic A_1 receptor-mediated inhibition for A_{2A} receptor facilitation

The lack of effect of the tested A_{2A} receptor agonist alone on the evoked release of glutamate from superfused hippocampal synaptosomes was in marked contrast to its effect on PPF in hippocampal slices. One hypothesis to be tested was that the facilitation of synaptic transmission mediated by CGS 21680 depends on attenuation of a tonic A_1 receptor-mediated inhibition, since we reported earlier that A_{2A} receptor activation reduces A_1 receptor responses (Lopes et al., 1999a). In hippocampal slices, the selective A_1 receptor antagonist, DPCPX (20 nM), facilitated by $21 \pm 3\%$ ($n = 3$, $P < 0.05$) fEPSP slope, indicating that endogenous adenosine is tonically activating inhibitory A_1 receptors to depress synaptic transmission (e.g. Dunwiddie and Diao, 1994). In contrast, DPCPX (20 nM, applied 2 or 6 min before S_2) was devoid of effects on the evoked release of glutamate from hippocampal synaptosomes (S_2/S_1 ratio of 0.92 ± 0.08 in the absence and 0.91 ± 0.09 in the presence of 20 nM DPCPX, $n = 4$). This indicates the absence of a tonic A_1 receptor-mediated control of the evoked release of glutamate, possibly due to the fast washout of extracellular adenosine upon superfusion of the diluted synaptosomes. Indeed, such dilution of agonist explains the lack of effect of many different antagonists of presynaptic neuromodulatory systems when tested in superfused nerve terminals (see Nicholls, 1989).

To investigate if the lack of effect of CGS 21680 on the evoked release of glutamate might be due to this absence of tonic A_1 receptor-mediated inhibition, we tested the effect of CGS 21680 (10 nM) in the presence of an A_1 receptor agonist, CPA (100 nM), that has previously been shown to inhibit the evoked release of glutamate from rat hippocampal synaptosomes (Ambrósio et al., 1997). In the presence of CPA (100 nM), present during S_1 and S_2, addition of CGS 21680 (10 nM) 2 min before S_2 now facilitated the evoked release of glutamate (Fig. 4A), an effect prevented by the A_{2A} receptor antagonist, ZM 241385 (20 nM) ($n = 4$) (Fig. 4A).

To further demonstrate that the tested A_{2A} receptor agonist only facilitates glutamatergic transmission if a tonic A_1 receptor inhibition is present, we compared the effect of CGS 21680 (10 nM) both on synaptic transmission and on PPF upon removing endogenous adenosine with the use of ADA (which converts adenosine into its inactive metabolite inosine) and then re-admitting A_1 receptor activation with the use of the non-metabolizable and selective A_1 receptor agonist, CPA, still in the presence of ADA. The concentration of ADA tested (2 U/ml) has been shown earlier to be effective in removing endogenous adenosine (Sebastião et al., 2000). As illustrated in Fig. 4B, ADA (2 U/ml) caused a $18.5 \pm 1.5\%$ ($n = 3$, $P < 0.05$) facilitation of fEPSP slope, consistent with its ability to remove a tonic inhibition of synaptic transmission by endogenous adenosine. In the presence of ADA (2 U/ml), CGS 21680 (10 nM) failed to modify synaptic transmission (Fig. 4B). Still in the presence of ADA (2 U/

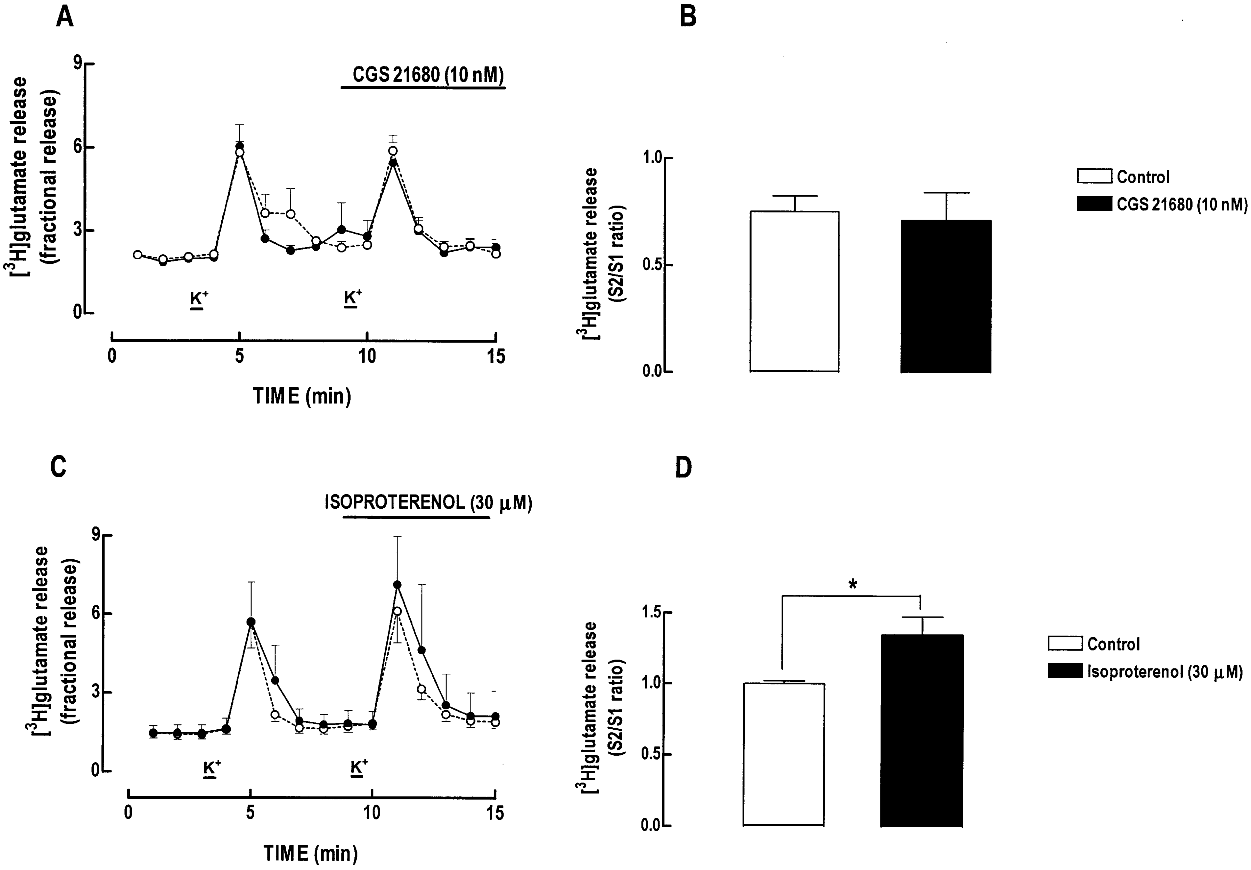

Fig. 3. Lack of effect of the adenosine A_{2A} receptor agonist, CGS 21680 (10 nM), and facilitation by the β-receptor agonist, isoproterenol (30 μM), of glutamate release from rat hippocampal synaptosomes. (A, C) Time course of tritium release that was confirmed to be essentially [^{3}H]glutamate release. The preparation was challenged with two periods of stimulation with 20 mM K^+ (S_1 and S_2), as indicated by the bars above the abscissa. The open symbols represent tritium release from a control chamber, to which no drug was added, and the filled symbols represent the tritium release of the test chamber, to which either CGS 21680 (10 nM; in A) or isoproterenol (30 μM; in C) was added through the superfusate, as indicated by the upper bar. (B, D) Average effect of CGS 21680 (10 nM; in B) or isoproterenol (30 μM; in D). The results are mean ± S.E.M. of four experiments. *$P < 0.05$.

ml), CPA (6 nM) caused a 44.3 ± 2.3% ($n = 3$, $P < 0.05$) inhibition of fEPSP slope, an effect previously shown to be mediated by A_1 receptors (Alzheimer et al., 1991; Sebastião et al., 2000). Finally, upon direct activation of A_1 receptors with CPA still in the presence of ADA, we now observed that CGS 21680 (10 nM) facilitated the fEPSP slope by 17.1 ± 1.0% ($n = 3$, $P < 0.05$). This ability of CGS 21680 to facilitate synaptic transmission likely results from a presynaptic site of action, since the effects of CGS 21680 (10 nM) on PPF were a mirror image of the effects of CGS 21680 on fEPSP slope. In fact, as illustrated in Fig. 4B, CGS 21680 (10 nM) was devoid of effects on PPF in the presence of ADA (2 U/ml, which by itself decreased PPF by 13.7 ± 1.0%, $n = 3$, $P < 0.05$) but caused a 13.1 ± 1.7% ($n = 3$, $P < 0.05$) inhibition of PPF in the simultaneous presence of ADA (2 U/ml) and CPA (6 nM, which by itself caused a 29.1 ± 0.4% facilitation of PPF, $n = 3$, $P < 0.05$).

Effect of CGS 21680 on adenosine A_1 receptor binding

The above results would be compatible with a direct interaction of CGS 21680 with A_1 receptors. However, in most binding studies CGS 21680 has been shown to have a very weak effect on binding of either agonists or antagonists at A_1 receptors. Previous results had shown that binding of [^{3}H]CGS 21680 to rat brain is strongly magnesium-dependent (Johansson et al., 1992), but most previous studies on A_1 binding of CGS 21680 had used only low concentrations of this cation. We therefore performed studies of CGS 21680 displacement of the A_1 receptor antagonist radioligand [^{3}H]DPCPX at 1 and 10 mM Mg concentration. As seen from the results in Table 1, CGS 21680 was a weak displacing agent in all brain regions using the lower magnesium concentration, in confirmation of previous results. IC_{50} values were close to 1 μM in all brain regions studied. The same was true in cortex, caudate–putamen and the CA3 region of hippocampus at 10 mM magnesium. However, in the CA1 region, 30% (95% confidence interval: 9–46%, $n = 3$) of the [^{3}H]DPCPX binding was displaced by CGS 21680 with an IC_{50} value of 44 nM.

To further explore if low nanomolar concentrations of CGS 21680 could directly interfere with A_1 receptors, we tested the effects of CGS 21680 in heterologously expressed A_1 receptors. We have carried out these experi-

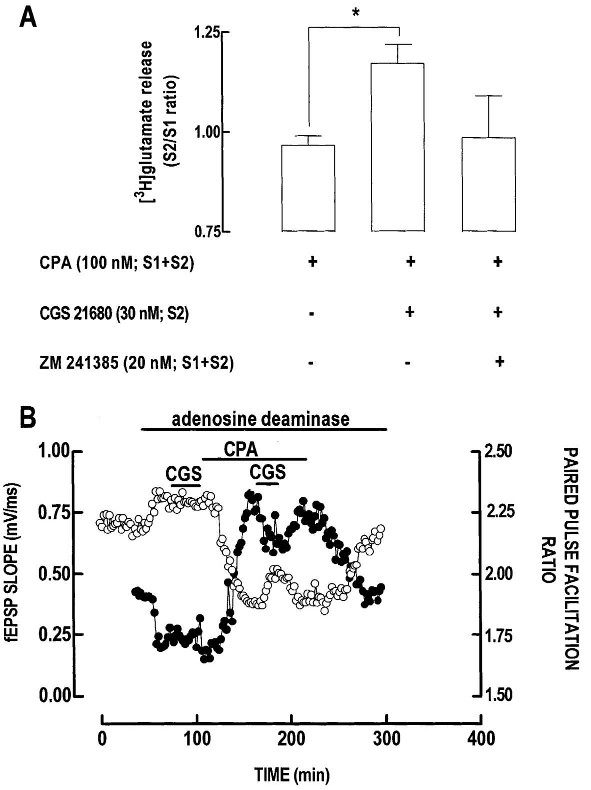

Fig. 4. Facilitatory effect of the A_{2A} receptor agonist, CGS 21680 (10 nM), on the evoked release of glutamate from hippocampal nerve terminals in the presence of the A_1 receptor agonist, CPA (100 nM) (A), and comparison of the effect of CGS 21680 (10 nM) on synaptic transmission and PPF in rat hippocampal slices in the absence and presence of A_1 receptor activation (B). (A) The ordinates represent tritium release, which is a good measure of [^{3}H]glutamate release, from rat hippocampal synaptosomes, measured as the ratio between two pulses of stimulation with 20 mM K^+ (S_1 and S_2). CGS 21680 (10 nM), added 2 min before S_2, facilitated the evoked tritium outflow when CPA (100 nM) was present during S_1 and S_2, and this effect was prevented by the A_{2A} receptor antagonist, ZM 241385 (20 nM). Thus, application of CGS 21680 in the presence of ZM 241385 and CPA did not produce a statistically significant increase in glutamate release compared to CPA alone. The results are mean ± S.E.M. of four to six experiments. $^*P < 0.05$. (B) An experiment where fEPSPs (open symbols) and PPF (50-ms interval; filled symbols) were recorded in the same experiment in the CA1 area of a rat hippocampal slice which was first superfused with ADA (2 U/ml), then with CGS 21680 (10 nM) in the presence of ADA (2 U/ml). Then CGS 21680 was washed out and CPA (6 nM) was added, still in the presence of ADA (2 U/ml), and finally first CGS 21680 and then CPA were washed out, as shown in the horizontal bars.

ments using human A_1 and A_{2A} receptors, since no major differences were previously found in terms of the binding characteristics of the tools used (DPCPX, CPA and CGS 21680) to rat and human A_1 and A_{2A} receptors (see Klotz et al., 1998). CGS 21680 (30 nM) failed to modify the ability of CPA (0.1 nM–1 μM) to displace [^{3}H]DPCPX (1 nM) binding to CHO cells stably transfected with human A_1 receptors [in control condition, $K_i = 122.0$ nM (95% confidence interval: 52.0–288.0 nM, $n = 2$) and in the presence of 30 nM CGS 21680, $K_i = 61.0$ nM (95% confidence interval: 24.0–153.0 nM, $n = 2$)]. Both curves were best fitted by a one independent binding site equation. As a control for the selectivity of [^{3}H]DPCPX (1 nM) labelling of A_1 receptors, we confirmed that [^{3}H]DPCPX (0–10 nM) failed to bind to CHO cells stably transfected with A_{2A} receptors (maximal specific binding of 1.42 ± 0.26 fmol/10^6 cells, $n = 2$).

Transducing system operated by A_{2A} receptors to attenuate A_1 receptor-mediated inhibition

Although A_{2A} receptors are classically classified as being coupled to the G_S/adenylate cyclase/cAMP pathway (Fredholm et al., 1994), we have previous reports of the involvement of protein kinase C (PKC) in response to A_{2A} receptor agonists (Lopes et al., 1999a; Cunha and Ribeiro, 2000b). However, it is unclear if PKC activity is directly controlled by A_{2A} receptors or if it is a consequence of an increase of cAMP levels within nerve terminals once A_{2A} receptors are activated (Gubitz et al., 1996). We directly quantified the cAMP levels in hippocampal nerve terminals and compared the effects of CGS 21680 and of isoproterenol thereupon. As illustrated in Fig. 5A, isoproterenol enhanced cAMP levels in hippocampal nerve terminals, as previously shown to occur in rat cortical nerve terminals (Herrero and Sánchez-Prieto, 1996). In contrast to the effect of isoproterenol, CGS 21680 (10 nM), either in the absence or in the presence of CPA (100 nM), failed to modify cAMP levels (Fig. 5A). Since we had previously observed that the concentration range required to detect CGS 21680-induced cAMP increases is nearly 10-fold greater than that required to facilitate neurotransmitter release in other CNS preparations (Lopes et al., 1999b), we tested the effect of higher concentrations of CGS 21680 on cAMP levels. But, again, either with or without CPA (100 nM), CGS 21680 in concentrations of 100 nM or 300 nM failed to modify cAMP levels ($n = 2$ for each condition, data not shown). Note that activation of A_1 receptors failed to cause measurable changes in cAMP levels (Fig. 5A), although CPA (100 nM) decreased cAMP levels upon stimulation with 30 μM isoproterenol ($n = 2$, data not shown) in accordance with the known ability of A_1 receptors to decrease the evoked accumulation of cAMP (e.g. Dunwiddie and Fredholm, 1989).

Finally, we compared the effect of inhibitors of protein kinase A (PKA) or of PKC on the facilitatory effects of CGS 21680 (in the presence of 100 nM CPA) and of isoproterenol on the evoked release of glutamate. As illustrated in Fig. 5B, the facilitatory effect of CGS

Table 1. Potency of CGS 21680 to displace [^{3}H]DPCPX (0.5 nM) binding to coronal sections of rat brain in media containing different magnesium concentrations

Region	1 mM Mg^{2+}	10 mM Mg^{2+}
Cortex	1420 (844–2389)	1005 (614–1645)
Caudate–putamen	998 (562–1769)	531 (283–995)
Hippocampus CA3	988 (591–1650)	1321 (666–2653)
Hippocampus CA1	1125 (991–1277)	44 (8–240) (30% displacement) 1950 (1052–3616)

The IC_{50} values are presented in nM as mean (95% confidence interval) from three experiments.

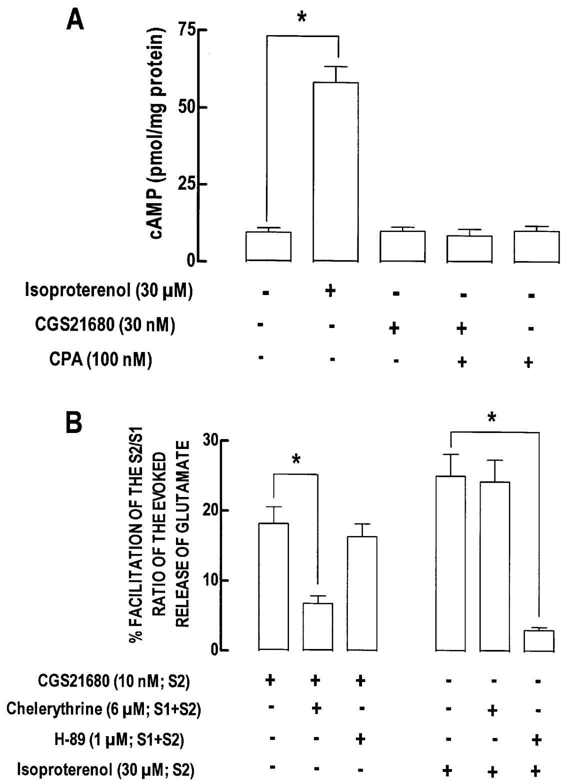

Fig. 5. β-Adrenergic receptor activation but not A_{2A} receptor activation enhanced the levels of cAMP in hippocampal nerve terminals and the A_{2A} receptor-induced facilitation, in contrast to the β-receptor-induced facilitation, of the evoked release of glutamate is dependent on PKC activation but not PKA activation. (A) The ability of isoproterenol (30 μM) to enhance the levels of cAMP in hippocampal nerve terminals, whereas CGS 21680 (30 nM), CPA (100 nM) or the simultaneous presence of CGS 21680 (30 nM) and CPA (100 nM) was devoid of effects. (B) The enhancement of the K^+-evoked release of [^{3}H]glutamate from hippocampal nerve terminals (subjected to two periods of stimulation with 20 mM K^+, S_1 and S_2) by CGS 21680 (30 nM, added 2 min before S_2 with 100 nM CPA present during S_1 and S_2) and by isoproterenol (30 μM, added 2 min before S_2). The PKC inhibitor, chelerythrine (6 μM, present during S_1 and S_2), but not the PKA inhibitor, H-89 (1 μM, present during S_1 and S_2), prevented the facilitation of the evoked release of glutamate by CGS 21680 (30 nM, in the presence of 100 nM CPA), whereas the facilitatory effect of isoproterenol (30 μM) was prevented by H-89 (1 μM), but not by chelerythrine (6 μM). The results are mean ± S.E.M. of four experiments. $*P < 0.05$.

21680 (10 nM), in the presence of 100 nM CPA, on the evoked release of glutamate was prevented by the PKC inhibitor, chelerythrine (6 μM), but not by the PKA inhibitor, H-89 (1 μM). This is in close agreement with the ability of different PKC inhibitors, but not of drugs affecting cAMP/PKA, to prevent CGS 21680-induced facilitation of hippocampal synaptic transmission. In two experiments we confirmed that another PKC inhibitor, bisindolylmaleimide (1 μM), also attenuated (68–92%) the facilitatory effect of CGS 21680 (10 nM), in the presence of 100 nM CPA, on the evoked release of glutamate. In contrast, the facilitatory effect of isoproterenol (30 μM) on the evoked release of glutamate was prevented by the PKA inhibitor, H-89 (1 μM), but not by the PKC inhibitor, chelerythrine (6 μM). These observations, together with the previous observation of the differential effects of CGS 21680 and of isoproterenol on cAMP levels, exclude the possibility that PKC activation might result from a rise in cAMP levels, since isoproterenol increased cAMP levels but increased glutamate release through a PKC-independent mechanism.

DISCUSSION

The present results show that the facilitation of hippocampal synaptic transmission mediated by a selective adenosine A_{2A} receptor agonist is not a direct effect, but instead results from a presynaptic attenuation of A_1 receptor response. This conclusion derives from the observed ability of the prototypical A_{2A} receptor agonist CGS 21680 to facilitate glutamate release and synaptic transmission as well as to inhibit PPF but only when a tonic A_1 receptor-mediated inhibition is present. In fact, these effects were abolished upon removal of endogenous extracellular adenosine.

For several reasons it is unlikely that the observed effects of CGS 21680 are due to a direct antagonism by CGS 21680 of the effects of adenosine at A_1 receptors. (1) Numerous studies have shown that CGS 21680 binds poorly to A_1 receptors with a K_i close to 1 μM. These findings, which were again replicated here, indicate that at the concentration used in the present experiments (10 nM) very few A_1 receptors would be occupied by CGS 21680. (2) There is no evidence that CGS 21680 could act as an A_1 receptor antagonist. In CHO cells heterologously expressing A_1 receptors, CGS 21680 failed to affect the displacement by an A_1 receptor agonist of the binding of the A_1 receptor antagonist, DPCPX. Also, DPCPX did not bind to heterologously expressed A_{2A} receptors. (3) The attenuation by CGS 21680 of tonic A_1 receptor inhibition observed in hippocampal slices and synaptosomes was prevented by low nanomolar concentrations of the selective A_{2A} receptor antagonist, ZM 241385, which only binds A_1 receptors at micromolar concentrations (Poucher et al., 1995).

Even though these and earlier observations strongly indicate that the effects of CGS 21680 are due to activation of A_{2A} receptors, alternative explanations cannot be excluded. It has been shown that most of the binding of CGS 21680 in hippocampus is to a site different from classical A_{2A} receptors (Johansson et al., 1993; Cunha et al., 1996, 1999; Lindström et al., 1996). These binding sites are characterized among others by being sensitive to DPCPX. In the present study we found that a small, but significant, proportion of the DPCPX binding sites only in the CA1 area of the hippocampus could be displaced by CGS 21680 in nanomolar concentrations. Thus, it may be that adenosine A_1 receptors, adenosine A_{2A} receptors or some other entity might display binding sites with a pharmacology that shows hybrid A_1/A_{2A} characteristics. It cannot be completely excluded that such an entity is partially responsible for the interactions observed here. Nevertheless, the most parsimonious interpretation is that the CGS 21680 facilitatory effects are dependent on A_1/A_{2A} receptor interaction. *In situ* hybridization studies have previously shown a co-expression, and receptor autoradiography indicates a co-localization of A_1 and A_{2A} receptors in the hippocampus (Cunha et al., 1994). In particular, both receptors are localized in nerve terminals of the hippocampus (Cunha et al., 1995). In addition, immunohistochemical studies with selective A_1 and A_{2A} receptor antibodies confirmed the localization of both receptors in hippocampal nerve terminals (Diáz-Hernandez et al. 2002). Finally, it has previously been reported that activation of A_{2A} receptors decreases the binding affinity of A_1 receptor agonists in a manner dependent on intracellular transducing systems and independent of the release of mediators (Dixon et al., 1997; Lopes et al., 1999a).

The observations that CGS 21680 causes parallel effects on synaptic transmission and PPF and is also able to facilitate glutamate release from isolated nerve terminals are strong indications that presynaptic A_{2A} receptors are responsible for the effects of CGS 21680 on synaptic transmission. However, it is important to note that other electrophysiological studies in hippocampal slices also identified responses apparently mediated by postsynaptic A_{2A} receptors (Li and Henry, 1998; O'Kane and Stone, 1998), making it possible that A_1/A_{2A} receptor interactions might also occur at the postsynaptic level. However, A_1 receptors control synaptic transmission in the hippocampus mostly by activation of presynaptic rather than postsynaptic A_1 receptors (Proctor and Dunwiddie, 1987). Therefore, it appears that the presynaptic component is the main locus of of A_1/A_{2A} receptor interaction involved in modulation of hippocampal synaptic transmission. A_{2A} receptors may not always require A_1 receptor-mediated inhibition to show an effect. There are several reports of A_{2A} receptor agonists modulating neurotransmitter release without requiring A_1 activation (Correia-de-Sá et al., 1991; Cunha et al., 1995; Cunha and Ribeiro, 2000a; Jin and Fredholm, 1997; Gubitz et al., 1996; Okada et al., 2001). Even at the glutamatergic synapses of the hippocampus, it is possible to reveal an A_{2A} receptor effect dependent on *N*-methyl-D-aspartate receptor activation (Nikbakht and Stone, 2001). Therefore, A_{2A} receptors can directly facilitate transmission or fulfill a fine-tuning role, acting as modulators of other neuromodulatory systems (Sebastião and Ribeiro, 2000), depending on the conditions and/or preparations, but it is not clearly understood what controls the ability of A_{2A} receptors to modulate directly or indirectly neurotransmitter release.

One hypothesis is that this different role of A_{2A} receptors in different systems may be related to activation of different transduction mechanisms. When A_{2A} receptors modulate neurotransmitter release independently of A_1 receptor function, the rise in cAMP appears to be a main requirement (Correia-de-Sá and Ribeiro, 1994; Gubitz et al., 1996; Okada et al., 2001). In contrast, the effects now reported appear to involve PKC recruitment independently of cAMP. In accordance with our previous findings in cortical slices from young rats (Lopes et al., 1999b), we were now also unable to see any variation in the levels of cAMP in hippocampal nerve terminals upon activation of A_{2A} receptors, while activation of the G_S/adenylate cyclase/cAMP coupled with β-adrenergic receptor increased cAMP levels. Furthermore, in contrast to the β-adrenergic receptor-mediated effect, the increase in the evoked release of glutamate mediated by A_{2A} receptors was prevented by two different inhibitors of PKC but not inhibitors of PKA, as previously described to occur for synaptic transmission (Cunha and Ribeiro, 2000a). These data further support the idea that A_1/A_{2A} receptor crosstalk in the hippocampus is PKC- but not PKA-dependent (Lopes et al., 1999a).

The present study also clearly excludes the involvement of an A_{2A} receptor effect on GABAergic transmission for the control of CA1 hippocampal synaptic transmission, in contrast with what has been shown to occur in other neuronal circuits in the CNS (Phillis, 1998; Edwards and Robertson, 1999; but see Kobayashi and Okada, 1999). Still, two possible roles for A_{2A} receptors on GABAergic systems need to be explored: (1) modulation of GABAergic transmission in different hippocampal areas, namely in the CA3 area where the density and effects of A_{2A} receptors are more pronounced (Cunha et al., 1994; Lopes et al., 1999b; Gonçalves et al., 1997) and (2) synchronization of hippocampal circuits, A_{2A} receptors eventually being located in GABAergic neurons that control other GABAergic neurons (see Buckmaster and Soltesz, 1996). Both hypotheses are compatible with the lack of involvement in GABAergic control by A_{2A} receptors in monosynaptic glutamatergic circuits in the Schaffer fiber/CA1 pyramid synapses.

The physiological relevance of this proposed A_{2A} receptor-mediated attenuation of A_1 receptors in the control exerted by adenosine of hippocampal CA1 synaptic transmission is probably dependent on the origin of the adenosine released into the synaptic cleft. Adenosine released as such through the bi-directional adenosine transporters leads to a predominant A_1 receptor activation, whereas the formation of adenosine from released adenine nucleotides leads to a preferential activation of facilitatory adenosine receptors (reviewed in Cunha, 2001). One might speculate that only in situations where A_1 receptors are not fully activated and when there is a large release of adenine nucleotides, mainly ATP, will this proposed A_{2A} receptor-mediated attenuation of A_1 receptor-mediated inhibition be operative. One such situation might be upon high frequency firing of Schaffer fibers, since long-term potentiation (LTP)-like stimulation induces a large release of adenine nucleotides (Wieraszko et al., 1989). It is well known that adenosine A_1 receptors modulate LTP (de Mendonça and Ribeiro, 1997) and the intensity of this effect is similar to that observed under basal stimulation conditions in spite of the larger transient release of purines that occurs (discussed in Cunha, 2001). The observation that adenosine A_{2A} receptor antagonists depress CA1 hippocampal LTP (e.g. Fujii et al., 2000) is in support of a role for a tonic A_{2A} receptor activation in LTP. Likewise, both A_{2A} receptor blockade and receptor deletion in transgenic animals impair LTP in the mouse nucleus accumbens without altering basal synaptic transmission (D'Alcantara et al., 2001). In contrast, in pathological situations such as excitotoxicity, we anticipate that this proposed A_{2A} receptor-mediated attenuation of A_1 responses may have a discrete role.

CONCLUSION

The present results reveal the mechanism by which A_{2A} receptors cause a facilitation of synaptic transmission in the hippocampal CA1 area. The presently proposed A_{2A} receptor-mediated attenuation of A_1 receptor tonic inhibition may be a fine-tuning mechanism to allow frequency-dependent plasticity phenomena without compromising the A_1 receptor-mediated neuroprotective role of adenosine.

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