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Evidence that the presynaptic A_{2a}-adenosine receptor of the rat motor nerve endings is positively coupled to adenylate cyclase

P. Correia-de-Sá[1], J. A. Ribeiro[1,2]

[1] Laboratory of Pharmacology, ICBAS, University of Oporto, Portugal
[2] Laboratory of Pharmacology, Gulbenkian Institute of Science, Oeiras, Portugal

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Abstract. The action of the A_{2a}-adenosine analogue, CGS 21680C, on electrically evoked [^{3}H]acetylcholine ([^{3}H]-ACh) release, and its interaction with forskolin (an activator of adenylate cyclase), MDL 12,330A (an irreversible inhibitor of adenylate cyclase), rolipram (an inhibitor of cyclic AMP specific phosphodiesterase), dibutyryl- (db-cAMP) and 8-bromo- (8-Br-cAMP) cyclic AMP analogues (substances that mimic intracellular actions of cyclic AMP), were investigated using rat phrenic nerve-hemidiaphragm preparations.

CGS 21680C facilitated [^{3}H]-ACh release. Forskolin (but not 1,9-dideoxy forskolin), rolipram, db-cAMP and 8-Br-cAMP also increased evoked neurotransmitter release in a concentration-dependent manner. When the evoked [^{3}H]-ACh release that is dependent on stimulation of the adenylate cyclase/cyclic AMP transduction system was supramaximally stimulated by these compounds, CGS 21680C (3 nmol/l) could not further increase [^{3}H]-ACh release. Phosphodiesterase inhibition with low concentrations ($\leqslant 30$ μmol/l) of rolipram significantly potentiated the augmenting effect of CGS 21680C (1 nmol/l) on evoked [^{3}H]-ACh release. MDL 12,330A (an irreversible inhibitor of adenylate cyclase) decreased evoked [^{3}H]-ACh release. The irreversible blocking action of MDL 12,330A on [^{3}H]-ACh release was overcome by by-passing cyclase activation with db-cAMP and 8-Br-cAMP, but could not be overcome with FSK or CGS 21680C. The inhibitory effect of MDL 12,330A on evoked [^{3}H]-ACh release was not mimicked by nifedipine.

It is concluded that the increase in [^{3}H]-ACh release caused by CGS 21680C results from activation of an A_{2a}-adenosine receptor positively linked to the adenylate cyclase/cyclic AMP system.

Correspondence to: J. A. Ribeiro, Laboratory of Pharmacology, Gulbenkian Institute of Science, P-2781 Oeiras, Portugal

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Introduction

That the selective A_2-adenosine agonist CGS 21680C (Hutchinson et al. 1989) enhances [^{3}H]-acetylcholine ([^{3}H]-ACh) release from the phrenic nerve endings in a xanthine-sensitive manner has been described (Correia-de-Sá et al. 1991). This effect was interpreted to be a result of A_2-adenosine receptor activation. As CGS 21680C is effective in very low concentrations (1–10 nmol/l) (see Correia-de-Sá et al. 1991), this receptor is probably an A_{2a} subtype. In most preparations, this receptor operates by increasing cyclic AMP accumulation, but in spite of the A_2-selectivity of CGS 21680C, this compound does not stimulate adenylate cyclase in some systems (Cushing et al. 1991; Sheldon and Eichberg 1993). It therefore seemed of interest to determine whether the excitatory effect of CGS 21680C on [^{3}H]-ACh release from motor nerve terminals results from stimulation of the adenylate cyclase/cyclic AMP system. Because direct measurement of intraneuronal variations of cyclic AMP accumulation is not possible in this preparation, a pharmacological approach was designed. Interactions between CGS 21680C and substances that activate, e.g. forskolin (FSK) (Seamon et al. 1981), or inhibit, e.g. MDL 12,330A (formerly named RMI 12,330A) (Guellaen et al. 1977), adenylate cyclase, were investigated. We also stimulated cyclic AMP accumulation with the non-xanthine inhibitor of phosphodiesterases, rolipram (ROL) (Beavo and Reifsnyder 1990). Interactions between enzymatically stable cyclic AMP analogues, e.g. dibutyryl cyclic AMP (db-cAMP) and 8-bromo-cyclic AMP (8-Br-cAMP), that easily permeate cell membranes and mimic the actions of intracellular cyclic AMP (Henion et al. 1967), and CGS 21680C were also examined.

A brief account of some of the results has already been published (Correia-de-Sá et al. 1992).

Methods

[^{3}H]-Acetylcholine release. The experiments were done with left phrenic nerve-hemidiaphragm preparations from Wistar rats of either sex and of about 200 g in weight. Innervated muscle strips of about 0.5 cm width were mounted in Sylgard-lined organ baths of 3 ml capacity. Evoked transmitter release was assessed by measuring evoked tritium outflow from preparations labelled with [^{3}H]-choline by the method of Wessler and Kilbinger (1986). The preparations were superfused (3 ml min^{-1}; 37° C) with Tyrode solution continuously gassed with 95% O_2 and 5% CO_2 and containing (mmol/l): NaCl 137, KCL 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001. After a 30 min equilibration period, the perfusion was stopped and the nerve endings were labelled by exposing the tissue, for 40 min, to 1 µmol/l [^{3}H]-choline (specific activity 2.5 µCi nmol^{-1}). During this labelling period, the phrenic nerve was electrically stimulated at 1 Hz with supramaximal rectangular pulses of 40 µs duration. Immediately after the end of the labelling period, the preparations were again superfused (15 ml min^{-1}) and the nerve stimulation stopped. From this time onwards, hemicholinium-3 (10 µmol/l) was present in the Tyrode solution to prevent uptake of choline. After a 60 min washout period, the perfusion was stopped and 3 ml samples were collected every 3 min by emptying and refilling the organ bath. Aliquots (1 ml) of the incubation medium were added to 6 ml of Packard Insta Gel II scintillator and their tritium content was measured in a Beckman model LS 3801 scintillation spectrometer.

Stimulation parameters. Transmitter release was evoked by phrenic nerve stimulation at 5 Hz for 3 min with supramaximal rectangular pulses of 40 µs duration. In some of the experiments, the stimulation pulse width was adjusted (increasing stimulus pulse duration to 1 ms while keeping the amplitude and the train length constant, see Correia-de-Sá and Ribeiro 1994) in order to obtain reference-release (S_1) values similar to those obtained in the presence of the release-modulating compounds (namely forskolin and rolipram) used to interact with CGS 21680C (see Table 3). The increase in pulse duration (1 ms) enhanced evoked [^{3}H]-ACh release by about 25%, i.e., evoked [^{3}H]-ACh release during the first stimulation period (S_1 average) increased from $30 \pm 2 \times 10^3$ dpm/g ($n = 19$) to $38 \pm 4 \times 10^3$ dpm/g ($n = 12$) of wet weight of the preparation when the stimulation pulse width increased from 40 µs to 1 ms. Two or three stimulation periods were used. These were at 12–15 min (S_1), at 39–42 min (S_2) and at 66–69 min (S_3) after the end of the washout period (zero time). Note that the electrical stimulation of the phrenic nerve increases the release of [^{3}H]-ACh (which is abolished in the absence of calcium ions or by 0.3 µmol/l tetrodotoxin) whereas the output of [^{3}H]-choline remains unchanged (Wessler and Kilbinger 1986). Therefore, evoked [^{3}H]-ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (cf. Correia-de-Sá et al. 1991). The basal outflow of tritium declined slightly during the whole collection period (cf. Wessler 1989). The basal tritium outflow during the stimulation period was obtained by calculating the arithmetic mean of the [^{3}H]-contents of two samples before stimulation and two samples reaching the pre-stimulation level after stimulation.

Drug interactions. Test drugs were added 15 min before S_2 or S_3 and were then present up to the end of the experiments. Their effects were expressed by comparing the ratio of the tritium content of the sample collected during the second stimulation period (S_2) to the tritium content of the sample collected during the first stimulation period (S_1), or the ratio of the tritium content of the sample collected during the third stimulation period (S_3) to the tritium content of the sample collected during the first stimulation period (S_1), with the corresponding ratio obtained under control conditions. For convenience, these ratios will be referred to as S_2/S_1 and S_3/S_1. When a given drug supramaximally activates an intracellular messenger system, a second substance, applied in its presence, cannot further stimulate the same system, but is still able to activate a different system within the same cell. We took advantage of this general phenomenon to study the interactions between CGS 21680C and substances that are able to activate maximally or inhibit the adenylate cyclase/cyclic AMP transduction system. Therefore, when testing the ability of maximally effective concentrations of forskolin (3 µmol/l), 1,9-dideoxy forskolin (3 µmol/l), rolipram (300 µmol/l) and 8-Br-cAMP (3 µmol/l to modify the effect of CGS 21680C (3 nmol/l), these drugs were present in both S_1 and S_2, i.e., they were applied 15 min before, and were immediately washed out after, each stimulation period. Under these conditions, S_2/S_1 ratios, obtained when forskolin (3 µmol/l), 1,9-dideoxy forskolin (3 µmol/l), rolipram (300 µmol/l) and 8-Br-cAMP (3 mM) were present, were not statistically ($P > 0.05$) different from the S_2/S_1 ratio obtained in the absence of drugs (see Table 1). CGS 21680C was only applied 15 min before S_2 together with forskolin, 1,9-dideoxy forskolin, rolipram or 8-Br-cAMP. Since changes in the magnitude of the evoked tritium outflow in the presence of these drugs could complicate the interpretation of the results, the effect of CGS 21680C in the presence of these drugs was compared (1) to the effect of CGS 21680C per se under the same stimulation conditions, and (2) to the effect of CGS 21680C in adjusted stimulation conditions (1 ms pulse duration) that reproduced an increase (about 25%) in reference (S_1) evoked release similar to that obtained with forskolin (3 µmol/l) or rolipram (300 µmol/l). MDL 12,330A (1–10 µmol/l) was added to the bathing solution either 15 min before S_2 and then removed from the bath (S_2-washout) or, 15 min before S_2 and remained in the bathing solution until the end of the experiments (S_2-S_3), whereas CGS 21680C (3 nmol/l), forskolin (3 µmol/l), 8-Br-cAMP (3 mmol/l) and db-cAMP (3 mmol/l) were applied only 15 min before S_3. When nifedipine was used, it was applied 15 min before S_2, and care was taken to protect the incubation solutions and the chamber from light. When testing the ability of the A_1-adenosine antagonist, 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX, 2.5 nmol/l), to modify the effect of CGS 21680C, the xanthine was present in the bathing solution from the beginning of the release period (time zero) until the end of the experiments, whereas CGS 21680C was applied 15 min before S_2. The osmotic influence of cyclic AMP analogues, when applied in the millimolar concentration range, and the effect of the butyrate radicals present in the most effective cyclic AMP analogue tested (dibutyryl cyclic AMP), on evoked tritium outflow were assessed by investigating the effects of sucrose (3 mmol/l) and butyrate (3–6 mmol/l) applied 15 min before S_2, in parallel experiments. Drugs, at the concentrations to be tested, were added to the bath by emptying the bath and refilling it with 3 ml of the drug solution.

Drugs. 8-Bromo-cyclic AMP (8-Br-cAMP), *n*-butyric acid, choline chloride, dibutyryl cyclic AMP (db-cAMP), 1,9-dideoxy forskolin (1,9-ddFSK), forskolin (FSK), hemicholinium-3, nifedipine, sucrose (Sigma); 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX) (Research Biochemicals Inc.); [methyl-^{3}H]-choline chloride (ethanol solution, 80 Ci mmol^{-1}) (Amersham); CGS 21680C (2-[*p*-(2-carboxyethyl) phenethylamino]-5′-N-ethylcarboxamide adenosine) was a gift from Dr. M. Williams of Ciba-Geigy (USA); rolipram (4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone (4-RS)) was a gift from Schering AG, Berlin (Germany); MDL 12,330A (N-(as-2-phenylcyclopentyl) azacyclo-tridecan-2-imine hydrochloride) was a gift from Merrell Dow Pharmaceuticals Inc. (USA). Forskolin: 20 mmol/l stock solution in absolute ethanol; 1,9-dideoxy forskolin and MDL12330A: 20 mmol/l stock solutions in dimethylsulphoxide (DMSO); rolipram: 200 mmol/l stock solution in DMSO; DPCPX: 5 mmol/l stock solution in 99% DMSO/1% 1M NaOH (v/v) and nifedipine: 10 mmol/l stock solution kept protected from the light. Aqueous dilutions of these stock solutions were used. Appropriate solvent control experiments were made. No statistically significant differences between control experiments, made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v), were observed.

Statistics. Data are expressed as mean values ± SEM, and *n* is the number of experiments. The significance of a difference between the means

was calculated by Student's t test. P values of 0.05 or less were considered to represent significant differences.

Results

Forskolin, rolipram and cyclic AMP analogues

Concentration-response curves, for the enhancing effects of forskolin (1–10 μmol/l), rolipram (0.01–1 mmol/l), db-cAMP (0.3–3 mmol/l) and 8-Br-cAMP (0.1–3 mmol/l) on the electrically evoked release of [^{3}H]-ACh, are shown in Fig. 1. Each of these substances consistently increased evoked [^{3}H]-ACh release, measured as S_2/S_1 ratios and compared with the S_2/S_1 ratio obtained in control experiments. The concentrations needed to increase [^{3}H]-ACh release by 25% were about 3 μmol/l for forskolin, 200 μmol/l for rolipram, 630 μmol/l for 8-Br-cAMP and 675 μmol/l for db-cAMP. Forskolin and rolipram, in low concentrations, were more effective than the cyclic AMP analogues, db-cAMP and 8-Br-cAMP; however, the maximal increases caused by forskolin, rolipram or 8-Br-cAMP, ranged from 30 to 49%, values which were much smaller than that caused by db-cAMP (99±14%, $n = 4$). At high concentrations, there is a difference between the effects of the two cyclic AMP analogues on evoked [^{3}H]-ACh release from motor nerve endings. To test whether this results from the butyrate moiety of the db-cAMP molecule we studied the effects of butyrate alone and in combination with 8-Br-cAMP. Butyrate, 3 and 6 mmol/l, applied 15 min before S_2, increased evoked [^{3}H]-ACh release from phrenic motor nerve endings by 15% ($n = 1$) and 26±4% ($n = 3$), respectively. In the presence of 8-Br-cAMP (3 mmol/l), the augmenting effect of butyrate (6 mmol/l, 26±4%, $n = 3$) was added to the increase induced by 8-Br-cAMP (3 mmol/l, 49±1, $n = 3$), i.e. when 8-Br-cAMP (3 mmol/l)+butyrate (6 mmol/l) were applied 15 min before S_2, the evoked [^{3}H]-ACh release was increased by 69±8% ($n = 3$). In addition, parallel experiments were done to evaluate the osmotic influence of the drugs, when applied at high concentrations (3 mmol/l), on evoked [^{3}H]-ACh release. An isosmotic solution of sucrose (3 mmol/l), applied 15 min before S_2, did not significantly change evoked tritium outflow (S_2/S_1 ratio: 0.83±0.04, $n = 3$).

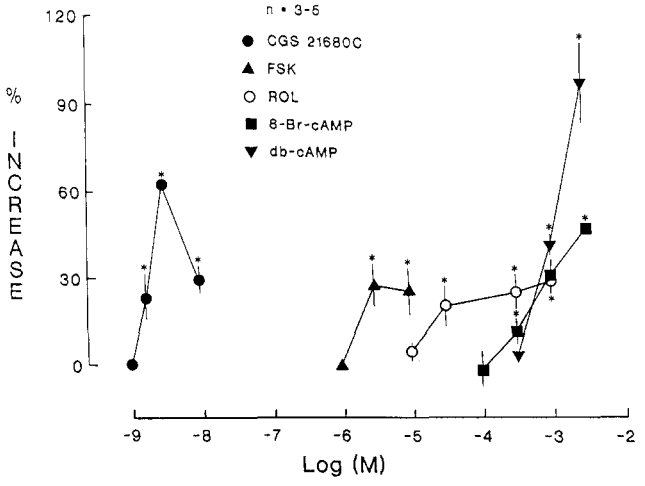

Fig. 1. Concentration-response curves for the effect of CGS 21680C, forskolin (FSK), rolipram (ROL), 8-Br-cAMP and db-cAMP on electrically evoked tritium outflow from rat phrenic nerve-diaphragm preparations loaded with [^{3}H]-choline. *Ordinate:* percentage increases in the S_2/S_1 ratio relative to the S_2/S_1 ratio in the control experiment. Zero percent represents identity between the two ratios. Average S_2/S_1 ratio in control experiments: 0.81±0.03 ($n = 8$). Average evoked tritium ouflow during S_1: $29\pm2\times10^3$ dpm/g wet weight of preparation. *Abscissa*: drug concentration (log mol/l). Drugs were applied 15 min before the end of S_2 and remained in the bath up to the end of the experiments. Each point is the mean of 3–5 experiments. The vertical bars represent ±SEM, and are shown when they exceed the symbols in size. * $P<0.05$ (Student's t test); differences from zero percent

Table 1. Effect of test drugs on electrically evoked [^{3}H]-ACh release from rat phrenic nerve endings

Control (no drug in S_1 or S_2)	S_2/S_1 ratios	
	0.81±0.03 (8)	
	Drug in S_2	Drug in S_1 and S_2
1,9-ddFSK (3 μmol/l)	0.78±0.03 (3)	0.78±0.01 (3)
FSK (3 μmol/l)	1.03±0.01 (4)*	0.79±0.03 (3)
ROL (300 μmol/l)	1.03±0.05 (4)*	0.75±0.05 (3)
8-Br-cAMP (3 mmol/l)	1.21±0.01 (3)*	0.80±0.04 (3)
db-cAMP (3 mmol/l)	1,54±0.08 (3)*	0.81±0.01 (3)

S_1, Tritium content (dpm/g wet weight of tissue) of sample 1 (stimulation period 1); S_2, tritium content (dpm/g wet weight of tissue) of sample 2 (stimulation period 2). 1,9-ddFSK, 1,9-dideoxy forskolin; FSK, forskolin; ROL, rolipram; db-cAMP, dibutyryl cyclic AMP or 8-Br-cAMP, 8-bromo-cyclic AMP were added to the bathing solution 15 min before S_2 (drug in S_2) or 15 min before each stimulation period with intermediate washout (drug in S_1 and S_2). Similar electrical stimulation parameters (supramaximal amplitude, 5 Hz, 3 min) were used in each set of experiments
Significance of difference from control S_2/S_1 ratio: * $P<0.05$, Student's t-test

The effect of forskolin seems to be specific, since its analogue, 1,9-dideoxy forskolin, which does not activate the catalytic subunit of adenylate cyclase (Laurenza et al. 1989), virtually, did not modify evoked [^{3}H]-ACh release (see Table 1) when applied at a concentration of 3 μmol/l.

As shown in Table 1, no statistically significant ($P > 0.05$) differences were found between S_2/S_1 ratios obtained under control conditions and those obtained after treatment with 1,9-dideoxy forskolin (3 μmol/l), forskolin (3 μmol/l), rolipram (300 μmol/l), db-cAMP (3 mmol/l) or 8-Br-cAMP (3 mmol/l) applied 15 min before, and immediately washed out after, each stimulation period, i.e., when present in both S_1 and S_2. However, the corresponding evoked tritium outflow was increased by about the same proportion as that observed when these drugs were applied 15 min before the second period of stimulation (see Table 2).

CGS 21680C

The concentration-response curve for the facilitatory effect of CGS 21680C (1–10 nmol/l) on evoked [^{3}H]-ACh

Table 2. Increase in the electrically evoked [^{3}H]-ACh release from rat phrenic nerve endings induced by CGS 21680C: influence of forskolin, 1,9-dideoxy forskolin, rolipram and 8-Br-cAMP

Drugs in S_1 and S_2	Evoked [^{3}H]-ACh release in S_2 (10^3 dpm/g)	
	Control	+CGS 21680C (3 nmol/l, S_2)
Control	23±1 (8)	38±1 (4)
FSK (3 μmol/l)	29±2 (4)*	39±5 (4)
1,9-ddFSK (3 μmol/l)	24±2 (3)	41±2 (3)
ROL (300 μmol/l)	28±3 (4)*	27±4 (3)**
8-Br-cAMP (3 mmol/l)	33±2 (3)*	36±4 (3)

FSK (forskolin), 1,9-ddFSK (1,9-dideoxy forskolin), ROL (rolipram) or 8-Br-cAMP (8-bromo-cyclic AMP) were added to the bathing solution 15 min before each stimulation period with intermediate washout (drugs in S_1 and S_2). CGS 21680C (3 nmol/l) was applied 15 min before S_2

Significant increase (*) ($P<0.05$, Student's t test) when compared with the average evoked tritium outflow in S_2 under control conditions

Significant decrease (**) ($P<0.05$, Student's t test) when compared with the effect of CGS 21680C in the absence of FSK, 1,9-ddFSK, ROL and 8-Br-cAMP, respectively

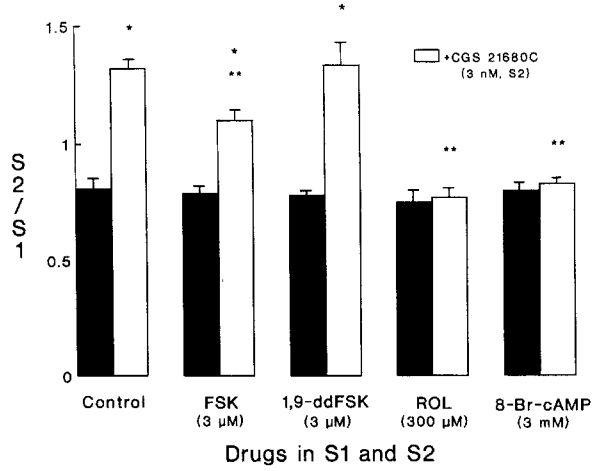

Fig. 2. Facilitatory effect of CGS 21680C, in the presence of supramaximal concentrations of forskolin (FSK), 1,9-dideoxy forskolin (1,9-ddFSK), rolipram (ROL) and 8-Br-cAMP, on evoked tritium outflow from rat phrenic nerve-diaphragm preparations loaded with [^{3}H]-choline. FSK (3 μmol/l), 1,9-ddFSK (3 μmol/l), ROL (300 μmol/l) and 8-Br-cAMP (3 mmol/l) were added to the bathing solution 15 min before each stimulation period, i.e. they were present in both S_1 and S_2; CGS 21680C (3 nmol/l) was applied 15 min before S_2 (*open columns*). *Ordinates*: evoked tritium outflow expressed as S_2/S_1 ratios. Values obtained in control experiments are also shown for comparison. Each column represents pooled data from 3–8 experiments. The *vertical bars* represent ±SEM. * and ** $P<0.05$ (Student's t test); differences from S_2/S_1 values in the absence of CGS 21680C and differences from the effect of the same concentration of CGS 21680C in the absence of FSK, ROL and 8-Br-cAMP, respectively

release is also shown in Fig. 1 for comparison. CGS 21680C (3 nmol/l), as previously described (Correia-de-Sá et al. 1991), maximally increased evoked [^{3}H]-ACh release by 63±2% ($n=4$), without modifying the basal tritium outflow. A higher concentration (10 nmol/l) of CGS 21680C also increased evoked tritium outflow ($P<0.05$), but the increase was smaller (30±5%, $n=4$) than that observed with 3 nmol/l. Pretreatment of the preparations with the A_1-adenosine receptor antagonist, 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), at a concentration (2.5 nmol/l) virtually devoid of effect on tritium outflow, significantly ($P<0.05$) increased the enhancing effect of CGS 21680C (10 nmol/l) on [^{3}H]-ACh release up to an amount (64±10%, $n=4$) similar to that observed with a concentration of 3 nmol/l. Since the excitatory effect of CGS 21680C (3 nmol/l) was not markedly changed by DPCPX (2.5 nmol/l), it is likely that high concentrations of CGS 21680C activate both A_{2a}-excitatory and A_1-inhibitory adenosine receptors, which are known to be present in the rat phrenic motor nerve endings (Correia-de-Sá et al. 1991).

In Fig. 2, the results of the interactions between CGS 21680C and forskolin, 1,9-dideoxy forskolin, rolipram or 8-Br-cAMP are summarized. Forskolin (3 μmol/l), 1,9-dideoxy forskolin (3 μmol/l), rolipram (300 μmol/l) and 8-Br-cAMP (3 mmol/l) were added to the bathing solutions 15 min before each stimulation period, i.e. they were present in both S_1 and S_2, whereas CGS 21680C (3 nmol/l) was applied only in S_2. When forskolin, rolipram or 8-Br-cAMP were applied at the above concentrations, which maximally increased evoked [^{3}H]-ACh release, the augmenting effect of CGS 21680C (3 nmol/l) was markedly reduced. In contrast, the facilitatory effect of CGS 21680C (3 nmol/l) was not significantly ($P>0.05$) changed after pretreatment with 1,9-dideoxy forskolin (3 μmol/l). As shown in Table 2, the absolute amounts of evoked tritium outflow induced by CGS 21680C during S_2, determined after pretreatment of the preparations, with forskolin (3 μmol/l), 1,9-dideoxy forskolin (3 μmol/l), rolipram (300 μmol/l) or 8-Br-cAMP (3 mmol/l), were not higher than those obtained in the absence of these drugs. The relative attenuation of the enhancing effect of CGS 21680C was more clear cut in the presence of rolipram or 8-Br-cAMP than in the presence of forskolin. Forskolin (3 μmol/l) attenuated the effect of CGS 21680C (2 or 3 nmol/l) to a similar extent, i.e., CGS 21680C 2 nmol/l increased [^{3}H]-ACh release by 24±8% ($n=4$) and 3 nmol/l by 63±2% ($n=4$) in the absence of, and by 0±5% ($n=3$) and 35±8% ($n=4$) in the presence of, forskolin (3 μmol/l), respectively. In addition, no greater increase in evoked tritium outflow was observed when CGS 21680C (3 nmol/l) was applied together with forskolin (3 μmol/l) 15 min before S_2. Under these conditions, where there was no treatment with forskolin during S_1, CGS 21680C (3 nmol/l)+forskolin (3 μmol/l) increased evoked [^{3}H]-ACh release by 53±3% ($n=4$), a value that is not significantly ($P>0.05$) different from the effect of CGS 21680C (3 nmol/l) alone (63±2%, $n=4$).

In contrast, neither attenuation of, nor significant ($P>0.05$) differences between, the dose-response curves for the enhancing effect of CGS 21680C (1–3 nmol/l) were found, when this substance was applied to preparations where the stimulation pulse duration was increased to 1 ms. This was done in order to generate increases in

Table 3. Influence of increases in electrically evoked [^{3}H]-ACh release on the facilitatory effect of CGS 21680C: comparison between increases induced by forskolin or rolipram and an increase due to a longer duration of the stimulation pulse

Conditions for S_1 and S_2	Average tritium content of S_1 (10^3 dpm/g)	S_2/S_1 ratios	
		Control	+ CGS 21680C (3 nmol/l, S_2)
40 μs pulse duration	30±2 (19)	0.81±0.03 (8)	1.31±0.03 (4)
+ FSK (3 μmol/l, in S_1 and S_2)	37±3 (7)*	0.78±0.01 (3)	1.10±0.03 (4)**
+ ROL (300 μmol/l, in S_1 and S_2)	38±4 (6)*	0.75±0.05 (3)	0.77±0.04 (3)**
1 ms pulse duration	38±4 (12)*	0.81±0.02 (3)	1.26±0.08 (3)

[^{3}H]-ACh release was elicited by two electrical nerve stimulation trains (5 Hz frequency, each for 3 min) with 40 μs or 1 ms pulse duration. Forskolin (FSK) and rolipram (ROL) were added to the bathing solution 15 min before each stimulation period (5 Hz frequency, 3 min, 40 μs pulse duration) with intermediate washout (drugs in S_1 and S_2). CGS 21680C (3 nmol/l) was applied 15 min before S_2

*: Significant increase ($P<0.05$, Student's t test) when compared with the average evoked tritium outflow during S_1 obtained under control conditions (5 Hz frequency, 3 min, 40 μs pulse duration)

**: Significant difference ($P<0.05$, Student's t test) when compared with the facilitatory effect of CGS 21680C under control conditions (5 Hz frequency, 3 min, 40 μs pulse duration)

reference (S_1) evoked [^{3}H]-ACh release similar to those caused either by forskolin (3 μmol/l) or rolipram (300 μmol/l) (Table 3). These increases were approximately 25% more than those obtained when the preparations were stimulated with 40 μs pulses. In the presence of a supramaximal concentration of CGS 21680C (3 nmol/l), S_2/S_1 ratios were 1.31±0.03 ($n=4$) and 1.26±0.08 ($n=3$) when the phrenic nerves were stimulated with pulses of 40 μs and 1 ms duration, respectively (see Table 3). The increase in tritium outflow caused by increasing stimulation pulse duration to 1 ms (S_2 average: $30\pm2\times10^3$ dpm/g; $n=4$), was further enhanced in the presence of CGS 21680C by approximately the same proportion as that obtained with pulses of 40 μs duration (S_2 average: $23\pm1\times10^3$ dpm/g; $n=8$), i.e. the S_2 average obtained in the presence of CGS 21680C (3 nmol/l) was $44\pm2\times10^3$ dpm/g ($n=4$) with pulses of 1 ms and $38\pm1\times10^3$ dpm/g ($n=4$) with pulses of 40 μs.

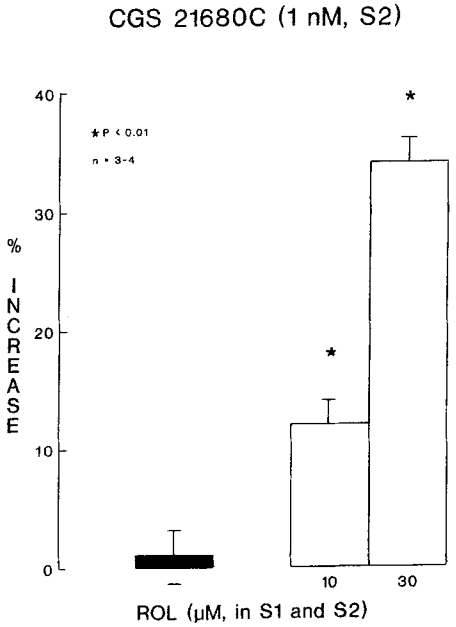

Fig. 3. Submaximal phosphodiesterase inhibition by rolipram (ROL) potentiates the facilitatory effect of CGS 21680C on evoked [^{3}H]-ACh release from rat phrenic nerve endings. ROL (10 and 30 μmol/l) was added to the bathing solution 15 min before each stimulation period (*white columns*); CGS 21680C (1 nmol/l) was applied 15 min before S_2. *Ordinates* are percentage increases in the S_2/S_1 ratio relative to the S_2/S_1 ratio in control experiments. Zero percent represents identity of the two ratios. Average S_2/S_1 ratio in control experiments: 0.81±0.03 ($n=8$). Each column represents pooled data from 3–4 experiments. The *vertical bars* represent ±SEM. * $P<0.01$ (Student's t test); differences from the effect of CGS 21680C (1 nmol/l) in the absence of ROL (*black column*)

It is known that increasing the intracellular concentration of cyclic AMP, by inhibiting phosphodiesterases, potentiates the physiological effects of substances that stimulate adenylate cyclase activity, provided that the system is not saturated. In view of this, experiments were done in which the effect of CGS 21680C, applied at a concentration (1 nmol/l) which did not modify tritium outflow from the phrenic motor nerve endings (see Fig. 3), was tested in preparations pretreated (applied in both S_1 and S_2) with submaximal concentrations (10 and 30 μmol/l) of rolipram (see Fig. 1). Under these conditions, pretreatment with rolipram did not significantly modify S_2/S_1 ratio values if compared to those obtained in control experiments, i.e., the S_2/S_1 ratio obtained with rolipram (10 μmol/l) was 0.81±0.02 ($n=2$), and that with rolipram (30 μmol/l) was 0.79±0.04 ($n=3$). Figure 3 shows that rolipram (10 μmol/l) significantly ($P<0.01$) potentiated the augmenting effect of CGS 21680C (1 nmol/l) by 12±2% ($n=3$) and rolipram (30 μmol/l) did so by 34±2% ($n=3$).

MDL 12,330A

The inhibitor of adenylate cyclase, MDL 12,330A, decreased evoked [^{3}H]-ACh release in a concentration-dependent manner. This inhibitory effect was greater when the incubation time was longer (Fig. 4). A 47±6% ($n=3$) decrease in evoked [^{3}H]-ACh release during S_3 was still observed when MDL 12,330A (10 μmol/l) was added 15 min before S_2 and then removed from the bathing solution. Therefore, the inhibitory effect of

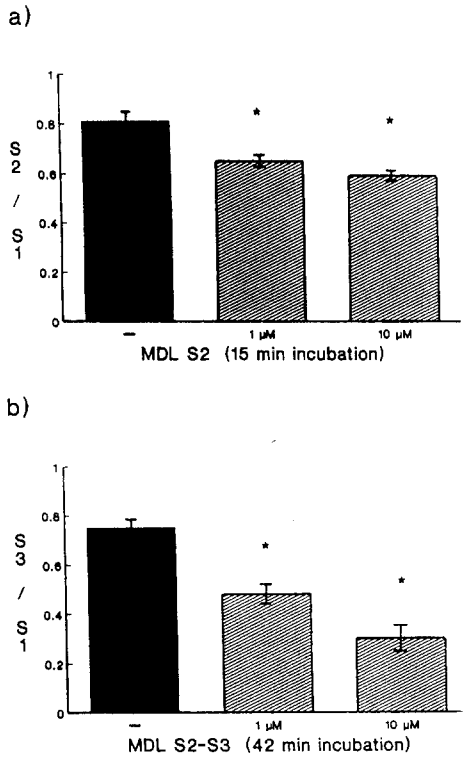

Fig. 4. Inhibitory effect of MDL 12,330A on electrically evoked [^{3}H]-ACh release from rat phrenic nerve endings. MDL 12,330A (1 and 10 μmol/l) was added 15 min before S_2 and remained in the bathing solution until the end of S_3. Average evoked tritium outflow during S_1: $31 \pm 3 \times 10^3$ dpm/g wet weight of preparation. Values obtained in control experiments are also shown for comparison. Each column represents pooled data from 3–11 experiments. The *vertical bars* represent ±SEM. *$P < 0.05$ (Student's t test); differences from the control ratio in the absence of MDL 12,330A

MDL 12,330A (10 μmol/l) was studied in relation to S_3. However, the inhibitory effect of MDL 12,330A (10 μmol/l), which is still evident in S_3 after its washout, was abolished by CGS 21680C (3 nmol/l), but was only partially prevented by forskolin (3 μmol/l), when these drugs were applied 15 min before S_3 (Table 4). This suggests that re-activation of the cyclase can overcome its blockade by MDL 12,330A. MDL 12,330A (10 μmol/l), present in both S_2 and S_3, induced a strong inhibition of the evoked [^{3}H]-ACh release during S_3, and completely blocked the facilitatory effects of CGS 21680C (3 nmol/l) and forskolin (3 μmol/l) (Table 4). In contrast, both of the cyclic AMP analogues, 8-Br-cAMP (3 mmol/l) and db-cAMP (3 mmol/l), completely reversed the inhibition induced by MDL 12,330A (10 μmol/l) applied in both S_2 and S_3 (Table 4).

To find out if this inhibitory effect on neurotransmitter relase was due to adenylate cyclase inhibition, and not to potent L-type channel blockade, by MDL 12,330A (Rampe and Triggle 1990), the L-type channel blocker, nifedipine (1–10 μmol/l), was tested. The S_2/S_1 ratio in the presence of nifedipine (1 μmol/l), applied 15 min before S_2, was 0.85 ± 0.05 ($n = 3$), and in the presence of nifedipine (10 μmol/l), was 0.84 ± 0.01 ($n = 4$). These

Table 4. Actions of CGS 21680C, forskolin, 8-Br-cAMP and db-cAMP on the inhibitory effect of MDL 12,330A on electrically evoked [^{3}H]-ACh released from rat phrenic nerve endings

	S_3/S_1 ratios	
Control	0.75 ± 0.03 (8) S_2 + washout	$S_2 - S_3$
MDL 12,330A (10 μmol/l)	0.48 ± 0.04 (3)	0.30 ± 0.05 (4)
+ CGS 21680C (3 nmol/l, S_2)	0.83 ± 0.05 (6)*	0.36 ± 0.10 (4)
+ FSK (3 μmol/l, S_2)	0.64 ± 0.01 (3)*	0.30 ± 0.06 (3)
+ 8-Br-cAMP (3 mmol/l, S_2)	–	0.83 ± 0.04 (3)*
+ db-cAMP (3 mmol/l, S_2)	–	0.82 ± 0.06 (3)*

MDL 12,330A (10 μmol/l) was added 15 min before S_2 and then omitted from the bathing solution (S_2 + washout) or its presence was maintained until the end of S_3 ($S_2 - S_3$). CGS 21680C, FSK, 8-Br-cAMP or db-cAMP were added 15 min before and were present during the collection of S_3. Average evoked tritium outflow for S_1: $29 \pm 3 \times 10^3$ dpm/g wet weight of tissue

*: Significant increase ($P < 0.05$, Student's t test) when compared with the S_3/S_1 ratio obtained with MDL 12,330A in the absence of CGS 21680C, FSK, 8-Br-cAMP or db-cAMP

S_2/S_1 ratios were not statistically different ($P > 0.05$) from those obtained in the control experiments.

Discussion

The present results show that facilitation of electrically evoked neurotransmitter release by maximal activation of the cyclic AMP pathway with substances that directly activate adenylate cyclase, e.g. forskolin (Seamon et al. 1981), that inhibit cAMP-specific phosphodiesterase, e.g. rolipram (Schwabe et al. 1976) and that mimic the intracellular effects of cyclic AMP, e.g. 8-Br-cAMP (Henion et al. 1967), all prevented further increases in [^{3}H]-ACh release from rat phrenic motor nerve terminals evoked by the A_{2a} selective adenosine receptor agonist, CGS 21680C (Hutchinson et al. 1989). However, when used in subsaturating concentrations, synergism was observed between the facilitatory actions of the phosphodiesterase inhibitor, rolipram, and of the A_{2a}-agonist, CGS 21680C, in agreement with Sutherland's postulates (see e.g. Siggins 1978). The enhancing effect of CGS 21680C was not evident when adenylate cyclase was irreversibly blocked by MDL 12,330A (Hunt and Evans 1980).

We have confirmed previous findings (e.g. Markstein et al. 1984; Sebastião and Ribeiro 1990) that the adenylate cyclase activator, forskolin (Seamon et al. 1981), can increase transmitter release. The enhancement of neuromuscular transmission caused by forskolin, as well as its ability to attenuate the augmenting effect of the A_{2a}-adenosine receptor agonist, CGS 21680C, at the rat neuromuscular junction, might be related to its ability to activate adenylate cyclase, since the effective concentrations of forskolin are within the range of those that have been shown to increase cyclic AMP levels in neural tissues (Seamon et al. 1981; Daly et al. 1982). Furthermore, the forskolin analogue that is inactive towards adenylate cyclase, 1,9-dideoxy forskolin, and which retains the

other non-cyclic AMP dependent, effects of forskolin (Laurenza et al. 1989), when used at the same concentration as that of forskolin, neither modified the evoked [^{3}H]-ACh release nor the facilitatory effect of CGS 21680C. Additionally, forskolin did not increase evoked [^{3}H]-ACh release when adenylate cyclase was irreversibly blocked by MDL 12,330A (Hunt and Evans 1980). The finding that supramaximal activation of adenylate cyclase by forskolin did not completely prevent the augmenting effect of CGS 21680C, contrasts with the more pronounced attenuation obtained either with rolipram or 8-Br-cAMP. These observations agree with the results obtained in previous studies (Correia-de-Sá and Ribeiro 1993), where it was demonstrated that the facilitatory effect of submaximal concentrations of forskolin on evoked [^{3}H]-ACh release is greatly potentiated by A_{2a}-adenosine receptor activation with CGS 21680C; an effect, probably, related to the recruitment of G_s proteins, which facilitates the coupling of forskolin to the adenylate cyclase (Alousi et al. 1991).

MDL 12,330A irreversibly decreased the evoked tritiated neurotransmitter release from the rat motor nerve endings at concentrations similar to those that caused an irreversible decrease in the amplitude of the evoked endplate potentials in the frog neuromuscular junction (Sebastião and Ribeiro 1990). This irreversible blockade prevented the facilitation of [^{3}H]-ACh release induced by forskolin but not that induced by 8-Br-cAMP or by db-cAMP. It is likely that these analogues by-pass cyclase activation and directly mimic the intracellular actions of cyclic AMP and, thus, overcome the blockade of [^{3}H]-ACh release by MDL 12,330A. Since the L-type calcium channel blocker, nifedipine, when used in effective concentrations (Miller 1987), did not modify the evoked acetylcholine release, it is highly probable that the inhibitory effect of MDL 12,330A is mainly due to adenylate cyclase inhibition and not to L-type channel blockade (Rampe and Triggle 1990). It is unlikely that either phosphodiesterase inhibition or ATPase inhibition, attributable to MDL 12,330A when used in high concentrations (Grupp et al. 1980; Hunt and Evans 1980), could produce changes in evoked neurotransmitter release, since these actions would enhance, rather than inhibit, acetylcholine release (see e.g. Ginsborg and Jenkinson 1976). Supramaximal activation of adenylate cyclase by forskolin attenuated, and the irreversible blockade of the cyclase by MDL 12,330A, completely blocked the facilitatory effect of CGS 21680C on evoked [^{3}H]-ACh release. This suggests that the facilitation of transmitter release induced by the activation of A_{2a}-adenosine receptors depends on the stimulation of adenylate cyclase.

Multiple forms of phosphodiesterases have been characterized in nervous tissue (Davis 1984; Strada et al. 1984). Rolipram is a selective inhibitor of calcium-independent, cyclic AMP-specific, type IV phosphodiesterase and has no adenosine receptor affinity (Schwabe and Trost 1980). From the present results, it appears that, at least the phosphodiesterase type IV isozyme is present in the rat motor nerve endings and regulates the intracellular cyclic AMP levels, since inhibition of this enzyme significantly enhanced evoked [^{3}H]-ACh release. The finding that rolipram, in concentrations that maximally increase evoked [^{3}H]-ACh release, prevented the excitatory effect of CGS 21680C, suggests that both drugs are acting on the same self-limiting pathway through an increase in the intracellular cyclic AMP level. The fact that submaximal inhibition of cyclic AMP-specific phophodiesterase by rolipram (≤ 30 μmol/l) potentiated the augmenting effect of CGS 21680C applied at a concentration that had no effect on neurotransmitter release, further indicates that cyclic AMP is involved in the A_{2a}-adenosine receptor-mediated facilitation of ACh release. CGS 21680C (3 nmol/l) increased evoked tritium outflow more potently than rolipram (300 μmol/l), and pretreatment of the preparations with rolipram (300 μmol/l) completely abolished this effect of CGS 21680C. These results could indicate that the persistent accumulation of intracellular cyclic AMP, as a result of phosphodiesterase blockade, can decrease the sensitivity of the adenosine receptor for its A_{2a}-agonist, or, alternatively, can inhibit receptor coupling to the adenylate cyclase amplification mechanism. It is worth noting that both processes can regulate the efficacy of the adenosine A_{2a} receptor-coupled cyclic AMP system. Recently, Premont et al. (1992) demonstrated that the catalytic activity of several subtypes of adenylate cyclase can be inhibited by cyclic AMP-dependent protein kinase (PKA) phosphorylation.

Exogenously applied, the lipophilic cyclic AMP derivative, db-cAMP, has been found to facilitate neurotransmission in the rat diaphragm (Goldberg and Singer 1969). In the present work, also done with rat phrenic nerve-hemidiaphragm preparations loaded with [^{3}H]-choline, we found that both db-cAMP and 8-Br-cAMP increased acetylcholine release, thus confirming the idea that increasing the intracellular accumulation of cyclic AMP analogues stimulates calcium influx into the nerve terminal (Ribeiro and Sá-Almeida 1984) through direct activation of cyclic AMP-dependent protein kinases (Simon et al. 1973), thus facilitating neurotransmitter release (Katz 1969). Surprisingly, at high concentration (3 mmol/l), the dibutyryl-derivative was much more effective than 8-Br-cAMP in stimulating neurotransmitter release. Since db-cAMP (3 mmol/l) was unable to saturate the release process, it might indicate that butyrate groups (several mmol/l) could be partially responsible for the increase in evoked neurotransmitter release. Butyrate itself, increased evoked [^{3}H]-ACh release from phrenic motor nerve endings, indicating that db-cAMP is not the best tool to mimic the intracellular actions of cyclic AMP. In the presence of 8-Br-cAMP, butyrate had no effect further than that resulting from its summation with the effect of 8-Br-cAMP. This further supports the conclusion that the greater effect of db-cAMP results from the butyrate moiety of its molecule. The finding that the facilitatory effect of CGS 21680C was no longer observed in the presence of the highest concentration of 8-Br-cAMP tested, suggests that activation of the cyclic AMP-dependent protein kinase (PKA) is involved in the enhancement of evoked neurotransmitter release by the A_{2a}-adenosine analogue, CGS 21680C.

The present findings that CGS 21680C enhanced evoked neurotransmitter release more efficiently than forskolin, rolipram or 8-Br-cAMP, suggest that (1) receptor coupled adenylate cyclase activation stimulates cyclic AMP-induced facilitation of neurotransmitter release more efficiently than direct activation of its catalytic subunit (cf. Seamon et al. 1981; Alousi et al. 1991), and (2) the rate limiting step that regulates the cyclic AMP transduction system might involve adenylate cyclase (cf. Premont et al. 1992).

Synergism frequently occurs between stimuli that activate different mechanisms of transduction (Rasmussen and Barrett 1984; Schepp et al. 1990) but not between stimuli that, by themselves, maximally activate the same mechanism. The present observations show that supramaximal activation of the adenylate cyclase/cyclic AMP transduction system by substances that directly activate adenylate cyclase, e.g. forskolin, inhibit cyclic AMP-specific phosphodiesterases, e.g. rolipram, or mimic the cyclic AMP activation of protein kinase A, e.g. 8-Br-cAMP, all attenuated the effect of the A_{2a}-adenosine receptor agonist. These effects did not depend on the amount of neurotransmitter released in the presence of the drugs, since in experiments where the pulse duration was increased to obtain amounts of neurotransmitter release similar to those obtained with forskolin, rolipram or the cyclic AMP analogues, the facilitatory effect of CGS 21680C (1–3 nmol/l) was virtually unchanged. It was also shown that facilitation by CGS 21680C summed with the relative increase in [^{3}H]-ACh release evoked by 1 ms pulses when compared to the release induced by 40 μs pulses. This indicates that (1) maximal activation of A_{2a}-adenosine receptors does not saturate the release mechanism; (2) the sensitivity of the A_{2a}-adenosine receptors to agonists, namely to CGS 21680C, is not changed by increasing the stimulus intensity or the amount of acetylcholine released; and (3) co-activation of different transducing mechanisms can interact cooperatively to increase transmitter release. Those results, taken together with the observation that CGS 21680C was not able to overcome the irreversible blockade caused by the adenylate cyclase inhibitor MDL 12,330A, suggest that the excitatory presynaptic A_{2a}-adenosine receptors of the rat motor nerve terminals are positively coupled to the adenylate cyclase/cyclic AMP transduction system.

The present findings also suggest that increases in intracellular cyclic AMP accumulation are associated with increases in [^{3}H]-ACh release, and that decreases in cyclic AMP accumulation are associated with decreases in actylcholine release, in agreement with the results obtained by other authors who have reported facilitation of neuromuscular transmission by cyclic AMP analogues (Goldberg and Singer 1969). The inhibitory effect of the adenylate cyclase blocker, MDL 12,330A, on evoked [^{3}H]-ACh release, also indicates that there is a basal production of cyclic AMP that tonically facilitates the release of acetylcholine during repetitive nerve stimulation. This might result from the activation of facilitatory presynaptic adenylate cyclase-coupled receptors by endogenous substances that are released and/or produced at the synaptic cleft, e.g. adenosine and calcitonin gene related peptide (CGRP) (Correia-de-Sá and Ribeiro 1994). Since, in these experiments, uptake of choline was blocked by hemicholinium-3 (10 μmol/l), thus inhibiting the synthesis of acetylcholine (Takagi et al. 1970), changes in evoked [^{3}H]-ACh release were exclusively due to facilitation or inhibition of the neuronal release process.

In conclusion, the present results indicate that the augmenting effect of the A_{2a}-selective adenosine receptor agonist, CGS 21680C, on acetylcholine release from motor nerve endings, is a consequence of stimulation of the adenylate cyclase/cyclic AMP system. A direct proof that cyclic AMP mediates the action of CGS 21680C awaits the development of methods to detect variations in the concentrations of cyclic nucleotides in isolated motor nerve terminals.

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