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Potentiation by tonic A_{2a}-adenosine receptor activation of CGRP-facilitated [^{3}H]-ACh release from rat motor nerve endings

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1 The effect of calcitonin gene-related peptide (CGRP) on [^{3}H]-acetylcholine ([^{3}H]-ACh) release from motor nerve endings and its interaction with presynaptic facilitatory A_{2a}-adenosine and nicotinic acetylcholine receptors was studied on rat phrenic nerve-hemidiaphragm preparations loaded with [^{3}H]-choline.

2 CGRP (100–400 nM) increased electrically evoked [^{3}H]-ACh release from phrenic nerve endings in a concentration-dependent manner.

3 The magnitude of CGRP excitation increased with the increase of the stimulation pulse duration from 40 μs to 1 ms, keeping the frequency, the amplitude and the train length constants. With 1 ms pulses, the evoked [^{3}H]-ACh release was more intense than with 40 μs pulse duration.

4 Both the nicotinic acetylcholine receptor agonist, 1,1-dimethyl-4-phenylpiperazinium, and the A_{2a} adenosine receptor agonist, CGS 21680C, increased evoked [^{3}H]-ACh release, but only CGS 21680C potentiated the facilitatory effect of CGRP. This potentiation was prevented by the A_{2a} adenosine receptor antagonist, PD 115,199.

5 Adenosine deaminase prevented the excitatory effect of CGRP (400 nM) on [^{3}H]-ACh release. This effect was reversed by the non-hydrolysable A_{2a}-adenosine receptor agonist, CGS 21680C.

6 The nicotinic antagonist, tubocurarine, did not significantly change, whereas the A_2-adenosine receptor antagonist, PD 115,199, blocked the CGRP facilitation. The A_1-adenosine receptor antagonist, 1,3-dipropyl-8-cyclopentylxanthine, potentiated the CGRP excitatory effect.

7 The results suggest that the facilitatory effect of CGRP on evoked [^{3}H]-ACh release from rat phrenic motor nerve endings depends on the presence of endogenous adenosine which tonically activates A_{2a}-adenosine receptors. Since both CGRP and A_{2a}-adenosine receptors are positively coupled to the adenylate cyclase/cyclic AMP system, cooperation between these receptors might occur at the second messenger transduction system level.

Keywords: Calcitonin gene-related peptide; A_{2a}-adenosine receptor; nicotinic acetylcholine receptor; adenosine deaminase; [^{3}H]-acetylcholine release; motor nerve terminals; rat diaphragm

Introduction

A_{2a}-adenosine receptors are present at rat phrenic motor nerve terminals enhancing the evoked release of acetylcholine (ACh) (Correia-de-Sá *et al.*, 1991). In a recent report, Correia-de-Sá & Ribeiro (1993) showed that facilitation of evoked [^{3}H]-ACh release from the phrenic-diaphragm preparation by an activator of the catalytic subunit of adenylate cyclase, forskolin, depends on A_{2a}-adenosine receptor activation by endogenous adenosine.

Calcitonin gene-related peptide (CGRP) is co-secreted with ACh at the motor endplates of mammals (Rodrigo *et al.*, 1985; Takami *et al.*, 1985), and increases ACh release apparently through stimulation of adenylate cyclase activity (Kobayashi *et al.*, 1987; Mullholland & Jaffer, 1990) increasing intracellular accumulation of cyclic AMP (Takami *et al.*, 1986). It therefore seemed of interest to investigate whether activation of A_{2a}-adenosine receptors by endogenous adenosine and selective A_{2a} receptor agonists affects the enhancement of [^{3}H]-ACh release caused by CGRP at phrenic motor nerve endings. Interactions between CGRP and nicotinic presynaptic receptors which also mediate facilitation of [^{3}H]-ACh release (Wessler *et al.*, 1986) were also examined.

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Methods

[^{3}H]-acetylcholine release

The experiments were carried out on rat phrenic nerve-hemidiaphragm preparations (8 mm width) from Wistar rats of either sex of about 200 g in weight. The procedures used for labelling the preparations and measuring evoked [^{3}H]-ACh release were described previously by Correia-de-Sá *et al.* (1991) with minor modifications. Briefly, the preparations were superfused (3 ml min^{-1}) in 3 ml organ baths at 37°C with Krebs solution continuously gassed with 95% O_2 and 5% CO_2, containing (mM): 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 during 40 min with 1 μM [^{3}H]-choline (specific activity 2.5 μCi nmol^{-1}) under electrical stimulation at 1 Hz with supramaximal rectangular pulses of 15 V and 40 μs duration. 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 μM) was present to prevent uptake of choline. After a 60 min period of washout, the perfusion was stopped, and 3 ml bath samples were collected every 3 min by emptying and refilling again the organ bath with the solution in use. Aliquots (1 ml) of the incubation medium were added to 6 ml of Packard Insta Gel II scintillator. Radioactivity was measured in a Beckman

model LS 3801 scintillation spectrometer. [^{3}H]-ACh release was evoked by electrical nerve stimulation at 5 Hz frequency with trains of 750 supramaximal rectangular pulses of 15 V and 40 μs or 1 ms duration, which were monitored with a Meguro model MO-1251A oscilloscope. Two stimulation periods were used: at 12 min (S_1) and at 39 min (S_2) after the end of washout (zero time). It was shown (see Wessler & Kilbinger, 1986) that [^{3}H]-ACh release evoked by electrical stimulation of the phrenic nerve with pulses of up to 500 μs duration is abolished in the absence of calcium ions or by tetrodotoxin (TTX). In the present work we confirmed that TTX (1 μM) abolished the evoked [^{3}H]-ACh release elicited by electrical stimulation pulses with durations between 40 and 500 μs; with pulses of 1 ms duration, the evoked [^{3}H]-ACh release in the presence of TTX (1 μM) persisted as 40 ± 7% ($n = 4$) of the control values. Moreover, electrical stimulation of the phrenic nerve increased only the release of [^{3}H]-ACh while the output of [^{3}H]-choline remained unchanged (Wessler & 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). Test drugs were added 15 min before S_2 and were present up to the end of the experiments. Their effects were expressed by the ratios S_2/S_1, i.e. the ratio between the evoked [^{3}H]-ACh release during the second stimulation period (in the presence of the test drug) and the evoked [^{3}H]-ACh release during the first stimulation period (without the test drug).

Drug interactions

When testing the ability of the nicotinic acetylcholine antagonist, (+)-tubocurarine (TC), the A_2-adenosine agonist, CGS 21680C, the A_2-adenosine antagonist, PD 115,199, the A_1-adenosine antagonist, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and adenosine deaminase (ADA) (which deaminates adenosine into the inactive derivative inosine) to modify the effect of CGRP, the preparations were pretreated with those drugs 15 min before each stimulation period and were washed out immediately after each stimulation period, i.e. they were present in both S_1 and S_2, whereas CGRP was present only in S_2. In the experiments where the nicotinic agonist, 1,1-dimethyl-4-phenylpiperazinium (DMPP), was used, shorter, but efficient, pretreatment periods of 3 min were carried out before S_1 and S_2, to avoid presynaptic nicotinic acetylcholine receptor desensitization (Wessler *et al.*, 1986); in these experiments CGRP was also present only in S_2. When the same drugs were present in both S_1 and S_2, the S_2/S_1 ratios were not appreciably different from those obtained in control conditions, i.e., without addition of drugs (S_2/S_1 values were 0.81 ± 0.03, $n = 8$, and 0.81 ± 0.02, $n = 3$, for trains of 40 μs and 1 ms pulse width, respectively, see Table 1). Averaged evoked [^{3}H]-ACh release during S_1 (S_1 average) in control conditions were 30.3 ± 1.7 × 10^3 d.p.m. g^{-1} (n = 19) and 37.8 ± 3.5 × 10^3 d.p.m. g^{-1} ($n = 21$) of wet weight of preparation, which corresponds to about 25% increase in [^{3}H]-ACh release when the stimulation pulse was enlarged from 40 μs to 1 ms, keeping the frequency and the train length constants (cf. Ohhashi & Jacobowitz, 1988). The concentrations of CGS 21680C (1 nM–3 μM), PD 115,199 (25–100 nM) and DPCPX (2.5–5 nM) used, when present only in S_2, have been tested previously, and their effects on evoked [^{3}H]-ACh release from rat phrenic motor nerve terminals described (see Correia-de-Sá *et al.*, 1991; 1992). The effects of DMPP (1–30 μM) and TC (1–10 μM) on [^{3}H]-ACh release were previously investigated (Wessler *et al.*, 1986), and their effects were also studied in the presently described experimental conditions; similar results were obtained (see Results). None of the compounds used to interact with CGRP modified in a measurable way the basal tritium outflow.

Drugs

Adenosine deaminase (ADA) (type II), rat cyclic calcitonin gene-related peptide (CGRP), choline chloride, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), hemicholinium-3, Sigmacote, tetrodotoxin (TTX), (+)-tubocurarine chloride USP grade (TC) (Sigma); CGS 21680C (2-[*p*-(2-carboxyethylphenethylamino]-5′-N-ethylcarboxamide adenosine), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (Res. Biochem. Inc.); [methyl-^{3}H]-choline chloride (ethanol solution, 80 Ci $mmol^{-1}$) (Amersham); PD 115,199 (N-(2-(dimethylamino)-ethyl)-N-methyl-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purine-8-yl)-benzene sulphonamide) was a gift from Dr R.F. Bruns of Park-Davis (U.S.A.). DPCPX was made up in a 5 mM stock solution in 99% dimethylsuphoxide (DMSO)/1% NaOH 1 M (v/v); PD 115,199 was made up in a 1 mM stock solution in 80% methanol/20% NaOH 1 M (v/v). All stock solutions were stored as frozen aliquots at − 20°C. Dilutions of these stock solutions and appropriate solvent controls were made.

Handling of calcitonin gene-related peptide (CGRP): CGRP is readily adsorbed onto plastic. To minimize losses, all plastiware used in handling CGRP (i.e. pipette tips, Eppendorf tubes, automatic pumps) were pretreated with Sigmacote,

Table 1 The influence of stimulation pulse width on the facilitation induced by calcitonin gene-related peptide (CGRP), adenosine deaminase (ADA), CGS 21680C, (+)tubocurarine (TC) and 1,1-dimethyl-4-piperazinium iodide (DMPP) on evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release

		40 μs	*1 ms*
		S_1 average (× 10^3 d.p.m. g^{-1})	
		30.3 ± 1.7 (19)	37.8 ± 3.5 (21)***
		S_2/S_1	
Control		0.81 ± 0.03 (8)	0.81 ± 0.02 (3)
CGRP	200 nM	0.86 ± 0.06 (3)	1.13 ± 0.01 (4)*,**
ADA	0.5 u ml^{-1}	1.00 ± 0.05 (5)*	0.72 ± 0.06 (3)**
	2.5 u ml^{-1}	1.03 ± 0.04 (2)*	1.11 ± 0.10 (3)*
CGS	2 nM	1.00 ± 0.02 (4)*	1.09 ± 0.09 (4)*
TC	0.1 μM	0.75 ± 0.03 (3)	0.82 ± 0.07 (4)
	1 μM	0.43 ± 0.10 (3)*	0.88 ± 0.05 (3)**
DMPP	1 μM	1.13 ± 0.05 (3)*	1.15 ± 0.08 (2)*

CGRP, ADA, CGS 21680C and TC were added 15 min before S_2 and remained in the bath solution until the end of the experiments. DMPP was added 3 min before and was washed out immediately after S_2. 750 supramaximal intensity, 5 Hz frequency and 40 μs or 1 ms duration were used.

*$P < 0.05$ (Student's t test) when compared to S_2/S_1 ratio obtained in control conditions; **$P < 0.05$ (Student's t test) when compared to S_2/S_1 ratio obtained with the same drug when 40 μs stimulation pulses were applied; ***$P < 0.01$ (Student's t test) as compared to S_1 evoked release when 40 μs pulses were applied.

which is a silicone solution water repellent that easily forms a tight, microscopically thin film on glass and plastic. Control experiments in which Sigmacote was used were not significantly different from those in its absence.

Statistics

The data are expressed as mean ± s.e.mean from n number of experiments. The significance of the differences was evaluated by Student's t test. $P<0.05$ were considered to represent a significant difference.

Results

Effects of CGRP

A typical effect of CGRP (200 nM) applied 15 min before S_2 is illustrated in Figure 1, where it is shown that the effect of CGRP was dependent upon the width of pulse stimulation. When pulse width was 40 μs, CGRP caused little or no effect on [^{3}H]-ACh release, but with a pulse width of 1 ms, CGRP markedly increased [^{3}H]-ACh release. Similar results on evoked [^{3}H]-ACh release with CGRP (100–800 nM) were obtained in fourteen other experiments where pulses of 1 ms duration were used (Figure 2). The effect of CGRP (100–400 nM) was concentration-dependent, and a concentration of 800 nM did not cause a greater increase in [^{3}H]-ACh release than that of 400 nM (see Figure 2). In three out of seven experiments, CGRP (200 nM) increased basal tritium outflow by 20.3 ± 1.9 × 10^3 d.p.m. g^{-1}. This facilitatory effect on spontaneous tritium outflow was observed immediately after CGRP (200 nM) application and lasted 6–9 min.

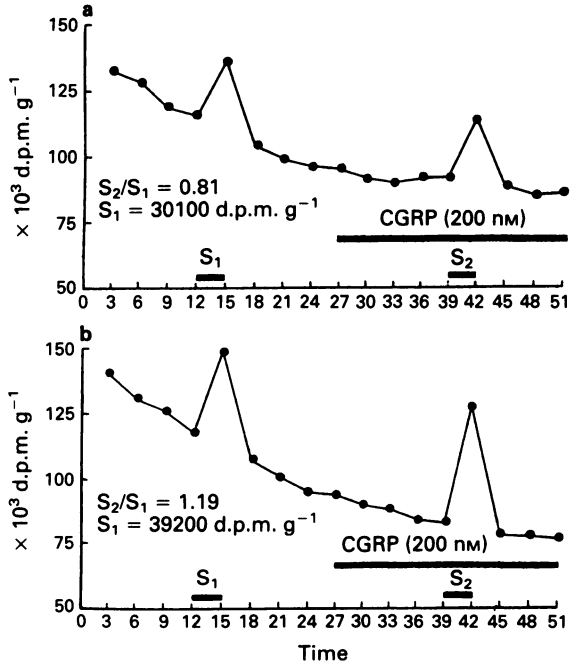

Figure 1 Effect of calcitonin gene-related peptide (CGRP, 200 nM) on tritium outflow from rat phrenic nerve terminals labelled with [^{3}H]-choline: influence of stimulation pulse width (40 μs and 1 ms). After the labelling and washout periods, [^{3}H]-acetylcholine ([^{3}H]-ACh) release was elicited by two electrical nerve stimulations with 750 pulses, 5 Hz frequency and (a) 40 μs and (b) 1 ms pulse width applied at the indicated times (S_1 and S_2). Tritium outflow was measured in samples collected every 3 min. CGRP was applied 15 min before S_2 and remained in the bath until the end of the experiments, as represented by the horizontal bar. S_1 averages and S_2/S_1 ratios were also given for comparison.

CGRP and A_{2a}-adenosine receptor activation

It is well established that adenosine is formed and accumulated extracellularly at neuromuscular junctions and that this accumulation of adenosine depends on the intensity of electrical stimulation (Cunha & Sebastião, 1993). As adenosine, through A_{2a}-adenosine receptor activation, can increase [^{3}H]-ACh release from phrenic nerve terminals (Correia-de-Sá *et al.*, 1991), we decided to investigate how inactivation of adenosine by adenosine deaminase (ADA) modifies the response to CGRP, as well as how the A_{2a} adenosine receptor agonist (CGS 21680C) interacts with CGRP.

ADA, the enzyme that hydrolyses adenosine into inosine, was applied 15 min before S_2 using stimulation pulse trains with 40 μs duration. In these conditions ADA increased the S_2/S_1 ratio to 1.00 ± 0.05 ($n = 5$) for ADA (0.5 u ml^{-1}) and to 1.03 ± 0.04 ($n = 2$) for ADA (2.5 u ml^{-1}) as compared with a control S_2/S_1 ratio of 0.81 ± 0.03 ($n = 8$) (Table 1). This effect of ADA is probably the consequence of removing the tonic inhibitory influence of adenosine as previously suggested (Ribeiro & Sebastião, 1987). When increasing the intensity of stimulation by increasing the duration of the pulse to 1 ms, ADA (0.5 u ml^{-1}) did not change or slightly decreased the ratio S_2/S_1 (0.72 ± 0.06, $n = 3$), as compared with the control (0.81 ± 0.02, $n = 3$), but in a higher amount ADA (2.5 u ml^{-1}) significantly increased [^{3}H]-ACh release up to 1.11 ± 0.10 ($n = 3$) (equivalent to 37% increase in [^{3}H]-ACh release when compared to control) (Table 1). The need to increase the ADA concentration to observe its excitatory effect on [^{3}H]-ACh release induced by wider stimulation pulses, probably resulted from greater extracellular adenine nucleotides/adenosine accumulation, in consequence of increasing stimulus intensity (Silinsky, 1975; Cunha & Sebastião, 1993).

To investigate the role of adenosine in the excitatory effect of CGRP on evoked [^{3}H]-ACh release, experiments were carried out in which the effect of this peptide, applied 15 min

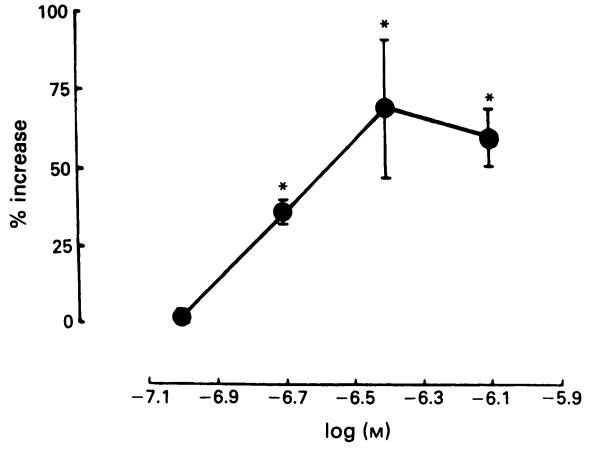

Figure 2 Concentration-response curve for the facilitatory effect of calcitonin gene-related peptide (CGRP) on electrically-evoked tritium outflow from phrenic-diaphragm preparations loaded with [^{3}H]-choline. Evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release was elicited by nerve stimulation with 5 Hz frequency and supramaximal intensity (15 V) 750 pulses of 1 ms width. The ordinates are percentage increases in the S_2/S_1 ratios as compared with the S_2/S_1 ratios in control experiments. Zero percent represents identity between the two ratios. Average S_2/S_1 ratio in control experiments: 0.81 ± 0.02 ($n = 3$). Average of evoked tritium outflow during S_1: 37.8 ± 3.8 × 10^3 d.p.m. g^{-1} of wet weight of preparation ($n = 15$). CGRP was 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 3–4 experiments. The vertical bars represent ± s.e.mean, and are shown when they exceed the symbols in size. *$P<0.05$ (Student's t test) when compared with zero percent.

before S_2 in a supramaximal concentration of 400 nM, was studied in the presence of ADA, applied in both S_1 and S_2 with a pulse duration of 1 ms. It was observed that ADA (2.5 u ml^{-1}) (S_1 average: $50.4 \pm 3.3 \times 10^3$ d.p.m. g^{-1}, $n = 4$) prevented the excitatory effect of CGRP (400 nM) (see Figure 3). This blockade seems to depend on the intensity of adenosine deamination, since a submaximal concentration of ADA (0.5 u ml^{-1}) (S_1 average: $35.9 \pm 4.0 \times 10^3$ d.p.m. g^{-1}, $n = 2$), applied in the same conditions, decreased the excitatory effect of CGRP (400 nM) by only 50% ($n = 2$, data not shown). Using the stable A_{2a}-selective adenosine analogue, CGS 21680C, in a concentration (2 nM) near its EC_{50} value for the increase in evoked [^{3}H]-ACh release (see Correia-de-Sá *et al.*, 1991), together with ADA (2.5 u ml^{-1}), in both S_1 and S_2 (S_1 average: $63.4 \pm 5.3 \times 10^3$ d.p.m. g^{-1}, $n = 4$), the facilitation induced by CGRP (400 nM), applied only in S_2, recovered to almost its value obtained in the absence of ADA (2.5 u ml^{-1}) + CGS 21680C (2 nM). The effect of CGRP (400 nM) was blocked by the A_2 adenosine antagonist, PD 115,199 (25 nM) (S_1 average: $37.0 \pm 2.1 \times 10^3$ d.p.m. g^{-1}, $n = 4$), and was greatly enhanced by the A_1 antagonist, DPCPX (2.5 nM) (S_1 average: $39.1 \pm 2.0 \times 10^3$ d.p.m. g^{-1}, $n = 4$). ADA (0.5–2.5 u ml^{-1}), CGS 21680C (2 nM), PD 115,199 (25 nM) and DPCPX (2.5 nM) applied in both S_1 and S_2 were virtually devoid of effects on the S_2/S_1 ratio.

CGRP (200 nM) applied in S_2 was tested during stimulation (40 μs pulse duration), in which on its own it was virtually devoid of effect on [^{3}H]-ACh release (see Figure 4). However, when using the A_{2a} agonist, CGS 21680C (2 nM, a concentration near its EC_{50} value), in both S_1 and S_2, the excitatory effect of CGRP (200 nM) became apparent. In these conditions, this peptide increased [^{3}H]-ACh release by $49 \pm 4\%$ ($n = 4$), which is a significantly ($P < 0.05$) higher effect than that obtained by increasing stimulus intensity (pulse width change from 40 μs to 1 ms) ($36 \pm 4\%$, $n = 4$). In both conditions, pretreatment with CGS 21680C (2 nM, 40 μs) (S_1 average: $36.6 \pm 1.9 \times 10^3$ d.p.m. g^{-1}, $n = 7$) and increase in pulse duration to 1 ms (S_1 average: $37.8 \pm 3.5 \times 10^3$ d.p.m. g^{-1}, $n = 21$), resulted in similar increase in evoked [^{3}H]-ACh release in both stimulation periods, when compared to control conditions with 40 μs pulse duration (S_1 average: $30.3 \pm 1.7 \times 10^3$ d.p.m. g^{-1}, $n = 19$). The potentiation of the effect of CGRP by CGS 21680C was prevented by applying the A_2 antagonist, PD 115,199 (25 nM), together with CGS 21680C (2 nM) (S_1 average: $28.0 \pm 1.8 \times 10^3$ d.p.m. g^{-1}, $n = 6$).

CGRP and nicotinic receptor activation

Increase in the intensity of electrical stimulation of the rat phrenic nerve enhances ACh release from the motor nerve terminals. This increase in ACh release could mediate a positive feed-back mechanism by activating nicotinic autoreceptors (Wessler *et al.*, 1986). The increase in pulse width from 40 μs to 1 ms increased evoked [^{3}H]-ACh release by about 25% (Table 1). To examine the role of presynaptic nicotinic receptor activation in the facilitatory effect of CGRP on the release of evoked [^{3}H]-ACh, we performed experiments with the nicotinic agonist, DMPP, to stimulate, and with the nicotinic antagonist, TC, to block these receptors. DMPP (0.3–1 μM) increased in a concentration-dependent manner [^{3}H]-ACh release when applied 3 min before S_2 and washed out immediately after S_2. A maximal increase in evoked [^{3}H]-ACh release of $40 \pm 3\%$ ($n = 4$) occurred with a

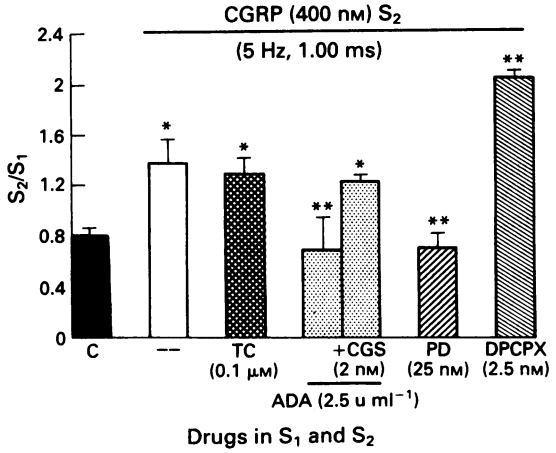

Figure 3 Effects of (+)-tubocurarine (TC), adenosine deaminase (ADA), CGS 21680C, PD 115,199 (PD) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) on the excitatory effect of calcitonin gene-related peptide (CGRP) on electrically-evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release from rat phrenic nerve endings loaded with 1 μM [^{3}H]-choline (specific activity 2.5 μCi $nmol^{-1}$). After labelling the preparations, hemicholinium-3 (10 μM) was present to prevent re-uptake of choline. TC (0.1 μM), ADA (2.5 u ml^{-1}), ADA (2.5 u ml^{-1}) + CGS 21680C (2 nM), PD 115,199 (25 nM) and DPCPX (2.5 nM) were added to the bath solution 15 min before and were immediately washed out after each stimulation period (S_1 and S_2) (5 Hz, 750 pulses, 1 ms pulse width); the S_2/S_1 ratios obtained under these conditions were not statistically different from the ratios obtained in control experiments (C, without any drug during S_1 and S_2) (S_2/S_1 ratio = 0.81 ± 0.02, $n = 3$). CGRP (400 nM) was applied 15 min before S_2. The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. Each column represents pooled data from 3–4 experiments. The vertical bars represent ± s.e.mean. *$P < 0.05$ (Student's t test) when compared to S_2/S_1 ratio in control conditions; **$P < 0.05$ (Student's t test) when compared with the effect of CGRP in the absence of drugs, respectively.

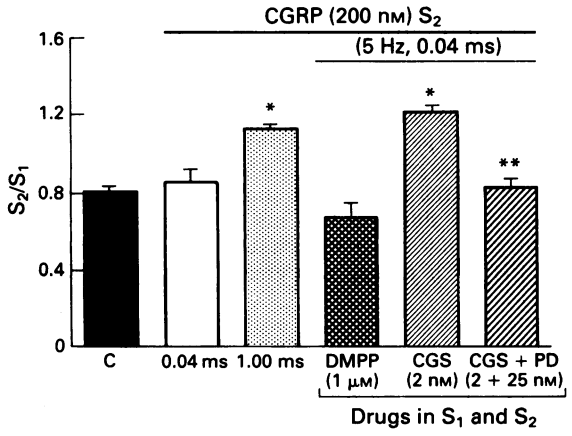

Figure 4 Stimulation pulse enlargement and activation of presynaptic A_{2a}-adenosine receptors, but not nicotinic cholinoceptors, potentiate the excitatory effects of calcitonin gene-related peptide (CGRP, 200 nM) on electrically evoked [^{3}H]-acetylcholine ([^{3}H]-ACh) release from rat phrenic nerve endings loaded with 1 μM [^{3}H]-choline (specific activity 2.5 μCi $nmol^{-1}$). After labelling the preparations, hemicholinium-3 (10 μM) was present to prevent re-uptake of choline. CGS 21680C (2 nM) with or without PD 115,199 (25 nM) were added 15 min before, whereas 1,1-dimethyl-4-piperazinium iodide (DMPP, 1 μM) was added 3 min before, and both were immediately washed out from the bath after each stimulation period (drugs in S_1 and S_2) (5 Hz, 750 pulses, 40 μs); in another set of experiments, evoked [^{3}H]-ACh release was induced by supramaximal intensity electrical stimulation (5 Hz, 750 pulses) with 1 ms pulse width, in both S_1 and S_2. The S_2/S_1 ratios obtained under those conditions were not statistically different from the ratio obtained in control experiments (C, without any drug during S_1 and S_2, 750 pulses, 40 μs) (S_2/S_1 ratio = 0.81 ± 0.03, $n = 8$). CGRP (200 nM) was applied 15 min before S_2. The ordinates represent evoked tritium outflow expressed by S_2/S_1 ratios. Each column represents pooled data from 3–8 experiments. The vertical bars represent ± s.e.mean. *$P < 0.05$ (Student's t test) when compared to S_2/S_1 ratio in control conditions; **$P < 0.05$ (Student's t test) when compared with the effect of CGRP in the presence of CGS 21680C.

concentration of DMPP of 1 μM, whilst a concentration of 10 μM was less effective (increase in [^{3}H]-ACh release of 20 ± 4%, $n = 2$). The facilitatory effects of DMPP on [^{3}H]-ACh release were not statistically different ($P > 0.05$) (Table 1), during electrical stimulations with pulses of 40 μs or 1 ms duration. DMPP (1 μM), applied 15 min before S_2, as we did with other compounds, did not facilitate [^{3}H]-ACh release, and in a concentration of 10 μM even decreased evoked [^{3}H]-ACh release by 20 ± 6% ($n = 2$), as previously described (see Wessler *et al.*, 1986). These authors suggested that this effect of DMPP results from desensitization of presynaptic nicotinic receptors by high agonist concentration and/or longer exposure time.

The nicotinic antagonist TC (0.1–10 μM) decreased electrically evoked [^{3}H]-ACh release in a concentration-dependent manner, when added to the bathing solution 15 min before S_2. The [^{3}H]-ACh released by electrical trains with pulses of 1 ms duration were less affected by TC than by trains with pulses with shorter duration (40 μs), i.e., S_2/S_1 ratios of 0.43 ± 0.10 ($n = 3$) and 0.88 ± 0.05 ($n = 3$) were found after applying TC (1 μM) 15 min before S_2 in 40 μs and 1 ms pulse width stimulating conditions, respectively (Table 1). Since a similar potency for DMPP (1 μM) was observed in both stimulation conditions, this difference could reflect competitive antagonism between TC and the increased levels of ACh released by longer pulses. TC (0.1 μM), in a concentration virtually devoid of effect on [^{3}H]-ACh release, applied in both S_1 and S_2, blocked the excitatory effect of DMPP (1 μM, applied 3 min before S_2) and prevented the desensitization by DMPP (10 μM, applied 15 min before S_2) in relation to its ability to increase evoked tritium outflow.

TC (0.1 μM), applied in both S_1 and S_2 (S_1 average: 35.0 ± 3.1 × 10^3 d.p.m. g^{-1}) did not significantly modify the excitatory effect of CGRP used in a supramaximal concentration (400 nM, 1 ms pulse duration, $n = 4$) (see Figure 3). The effect of CGRP (200 nM, 40 μs pulse duration) on evoked [^{3}H]-ACh release, when applied in S_2 in the presence of DMPP (1 μM, added to the bathing solution 3 min before both S_1 and S_2) remained about the same (see Figure 4); DMPP (1 μM, 40 μs pulse duration) alone increased [^{3}H]-ACh release (S_1 average: 42.6 ± 3.7 × 10^3 d.p.m. g^{-1}, $n = 4$). In contrast, when the intensity of stimulation was increased by increasing pulse duration to 1 ms (S_1 average: 37.8 ± 3.5 × 10^3 d.p.m. g^{-1}, $n = 21$), the excitatory effect of CGRP (200 nM) on [^{3}H]-ACh release was greatly enhanced (Figure 4).

Discussion

The results show that the facilitatory effect of CGRP on evoked [^{3}H]-ACh release from the phrenic motor nerve endings depends on the presence of endogenous adenosine, which is tonically activating A_{2a}-adenosine receptors. Presynaptic activation of nicotinic receptors did not contribute to the excitatory effect of CGRP on ACh release.

CGRP markedly facilitated electrically evoked [^{3}H]-ACh release from rat phrenic nerve endings. It has been shown that CGRP receptor-mediated effects are positively coupled to the adenylate cyclase adenosine 3':5'-cyclic monophosphate (cyclic AMP) transducing system (Takami *et al.*, 1986; Kobayashi *et al.*, 1987), and additionally, it was demonstrated that substances that increase intracellular cyclic AMP levels also increase evoked [^{3}H]-ACh release (Correia-de-Sá *et al.*, 1992). Cyclic AMP is involved in regulating metabolic activity associated with synthesis, mobilization and storage of ACh (Wilson, 1974). It appears unlikely that the effect of CGRP is primarily mediated by an action on the synthesis of the transmitter, but rather by other mechanisms involved in transmitter release (i.e. increase release probability, mobilization, recruitment of active release zones), because after the labelling with [^{3}H]-choline and its washing out, hemicholinium-3 was added to prevent further re-uptake of [^{3}H]-choline, particularly that originating from hydrolysis of released [^{3}H]-ACh. In this condition, the synthesis of [^{3}H]-ACh was prevented (Takagi *et al.*, 1970).

CGRP can increase the frequency of miniature endplate potentials (Jinnai *et al.*, 1989). This effect is thought to reflect presynaptic events facilitating spontaneous ACh release from motor nerve endings. The increase in spontaneous tritium outflow, observed in a few experiments, immediately after exogenously applied CGRP is consistent with the above studies, and suggests that CGRP is also able to enhance spontaneous ACh release.

In the present experimental conditions, where using electrical stimulation with pulses of 1 ms duration, but not 40 μs duration, it is likely that local depolarization (TTX-insensitive) of the motor nerve endings occurs, causing increase in the probability of quantal release (see e.g. Katz, 1969; Dudel, 1989). This could explain why CGRP did not increase [^{3}H]-ACh release with pulses of 40 μs duration, but did increase the release of [^{3}H]-ACh evoked by pulses of 1 ms duration. Thus, the effect of CGRP may depend (a) on the amount of ACh, itself, being released and regulating its own release through the presynaptic nicotonic receptors (Wessler *et al.*, 1986), or (b) on the release of a factor that occurs concomitantly with the release of ACh. Substances released together with ACh upon electrical stimulation from motor nerve terminals are adenine nucleotides (Silinsky, 1975; Ribeiro & Sebastião, 1987; Smith, 1991; Cunha & Sebastião, 1993) and adenosine (Ribeiro & Sebastião, 1987; Smith, 1991; Cunha & Sebastião, 1993).

The increase in release of [^{3}H]-ACh evoked by nerve stimulation (5 Hz, 750 pulses) is, in part, regulated through activation of nicotinic receptors. The nicotinic agonist, DMPP, enhanced, and the antagonist, TC, decreased electrically evoked [^{3}H]-ACh release. The inhibitory effect of TC and its dependency on the duration of the stimulation pulse, taken together with the fact that no change was found in the sensitivity of the nicotinic receptors to agonists, namely to DMPP, when comparing the effect of DMPP in both (40 μs and 1 ms pulse duration) stimulation conditions, indicate that these nicotinic autoreceptors mediate a positive feedback and, therefore, ACh may enhance its own release during repetitive nerve stimulation. The facilitatory action of DMPP declined when the incubation time was increased from 3 min to 15 min before stimulation. Moreover, a concentration of 10 μM DMPP was less effective than a concentration of 1 μM, suggesting that the presynaptic nicotinic receptor like other nicotinic receptors present in muscle, ganglia, sympathetic neurones and central neurones can be desensitized by increasing the concentration of the agonist, or by prolonging incubation time with the agonist (for a review see Wessler, 1989). Pretreatment with the nicotinic antagonist, TC, prevented the desensitization induced by a high concentration of DMPP (10 μM) incubated for 15 min.

The increase in the evoked [^{3}H]-ACh release caused by ADA is in agreement with previous findings (Ribeiro & Sebastião, 1987), suggesting that endogenous adenosine is tonically inhibiting ACh release. However, when the width of the stimulation pulse was increased from 40 μs to 1 ms, ADA (0.5 u ml^{-1}) had virtually no effect on [^{3}H]-ACh release, whereas when the amount of ADA (2.5 u ml^{-1}) was increased a consistent excitatory effect was obtained. This probably results from greater increase in extracellular adenosine accumulation, by release as such and/or formed from ATP degradation (Cunha & Sebastião, 1993), requiring higher amounts of ADA to be inactivated. In both stimulation conditions (40 μs and 1 ms pulse duration), no significant change was observed in the dose-response curves obtained for the excitatory effect of the A_{2a} stable adenosine agonist, CGS 21680C. This suggests that the sensitivity for the A_{2a}-adenosine receptor agonist was not markedly modified by increasing stimulus intensity and by the increase in endogenous adenosine levels.

It was demonstrated in the present work that the excitatory effect of CGRP on evoked [^{3}H]-ACh release depends on

the presence of endogenous adenosine, since ADA, an enzyme that inactivates adenosine to the inactive compound inosine (Arch & Newsholme, 1978), prevented this effect. Furthermore, we concluded that facilitation by CGRP cannot be evident without A_{2a} receptor activation from the findings that CGRP maximal excitation was blocked by pretreatment with the A_{2a}-adenosine antagonist, PD 115,199, and, that the ADA prevention of the CGRP effect on [^{3}H]-ACh release was almost completely reversed by applying the non-hydrolysable A_{2a}-adenosine agonist, CGS 21680C, together with ADA. The potentiating effect of CGS 21680C on the excitatory action of CGRP was indeed mediated through A_{2a} receptors, since this effect was completely antagonized by PD 115,199. The facilitation of evoked [^{3}H]-ACh release by CGRP was greatly potentiated when the A_1-inhibitory adenosine receptors were antagonized by the A_1 receptor antagonist, DPCPX, which indicates that tonic A_1-adenosine receptor activation is in part counteracting the A_{2a}-adenosine receptor-mediated potentiation. In the experimental conditions used in the present work, no interaction was found between CGRP and nicotinic receptor activation, because pretreatment with the nicotinic antagonist, TC, applied in a concentration sufficient to block the excitatory effect of DMPP, did not significantly modify the facilitatory action of CGRP, contrasting with the effects caused by ADA, CGS 21680C, PD 115,199 and DPCPX when applied in the same conditions.

The A_{2a} receptor agonist, CGS 21680C, but not the nicotinic receptor agonist, DMPP, (1) potentiated the effect of CGRP, when applied in conditions where no facilitation by CGRP on evoked [^{3}H]-ACh release was observed (5 Hz frequency, 40 μs), and (2) reversed the blockade of the effect of CGRP induced by ADA. The peptide facilitatory action on neurotransmission, observed when ACh release was induced by pulses of 1 ms duration, was reproduced in preparations stimulated with 40 μs pulses pretreated with CGS 21680C. These results suggest that the increase in extracellular endogenous adenosine, rather than the increase in ACh release, plays a critical role in the CGRP facilitation of ACh release depending on the presynaptic A_{2a}-adenosine receptor activation. The fact that both the receptor for CGRP (Takami *et al.*, 1986; Kobayashi *et al.*, 1987) and the A_{2a}-adenosine receptor (Correia-de-Sá *et al.*, 1992), are positively coupled to the adenylate cyclase/cyclic AMP transducing system, could indicate that cooperation between CGRP and CGS 21680C occurs at the level of the second messenger transducing system.

We recently showed (Correia-de-Sá & Riberior, 1993), that the excitatory effect of the catalytic subunit adenylate cyclase activator, forskolin, on evoked [^{3}H]-ACh release from rat phrenic nerve endings depends on tonic A_{2a}-adenosine receptor activation. The increase in evoked [^{3}H]-ACh release from rat phrenic motor nerve endings in consequence of adenylate cyclase stimulation via receptor activation, by the A_{2a}-adenosine agonist (CGS 21680C), or by CGRP, is about twice as effective as direct activation induced by forskolin. Since it has been reported that forskolin preferentially binds and activates adenylate cyclase when this enzyme is bound to the stimulatory G-protein G_s (Battaglia *et al.*, 1986), and, consequently, interacts synergistically with agents that activate adenylate cyclase through G_s-proteins (Seamon & Daly, 1983), our observations might suggest that signal transduction amplification occurs at the receptor/G_s-protein level. Whether the activation of A_{2a}-adenosine receptors, facilitate interaction of forskolin or CGRP with adenylate cyclase by recruiting G_s proteins and increasing the availability of the catalytic subunit (Alousi *et al.*, 1991), remains to be investigated. Besides suggestions that CGRP increases ACh release via a cyclic AMP-dependent pathway (Mullholland & Jaffer, 1990), this neuropeptide can also, in part, increase ACh release through activation of neuronal calcium channels (Wiley *et al.*, 1992).

In conclusion, the present results support a physiological role for the A_{2a}-adenosine receptors present at the motor nerve terminals. It appears that substances that facilitate neurotransmitter release through activation of adenylate cyclase increasing cyclic AMP accumulation, need the presence of endogenous adenosine tonically activating A_{2a}-adenosine receptors.

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References

ALOUSI, A.A., JASPER, J.R., INSEL, P.A. & MOTULSKY, H.J. (1991). Stoichiometry of receptor-G_s-adenylate cyclase interactions. *FASEB J.*, **5**, 2300–2303.

ARCH, J.R.S. & NEWSHOLME, E.A. (1978). The control of metabolism and the hormonal role of adenosine. In *Essays in Biochemistry*, Vol. 14, ed. Campbell, P.N. & Aldridge, W.N. pp. 82–123. New York: Academic Press.

BATTAGLIA, G., NORMAN, A.B., HESS, E.J. & CREESE, I. (1986). Forskolin potentiates the stimulation of striatal adenylate cyclase mediated by D-1 dopamine receptors, guanine nucleotides and sodium fluoride. *J. Neurochem.*, **46**, 1180–1185.

CORREIA-DE-SÁ, P. & RIBEIRO, J.A. (1993). Facilitation of [^{3}H]-ACh release by forskolin depends on A_2-adenosine receptor activation. *Neurosci. Lett.*, **151**, 21–24.

CORREIA-DE-SÁ, P., SEBASTIÃO, A.M. & RIBEIRO, J.A. (1991). Inhibitory and excitatory effects of adenosine receptor agonists on evoked transmitter release from phrenic nerve endings of the rat. *Br. J. Pharmacol.*, **103**, 1614–1620.

CORREIA-DE-SÁ, P., TIMÓTEO, M.A. & RIBEIRO, J.A. (1992). Presynaptic A_2 adenosine receptors of the motor nerve endings of the rat are coupled to adenylate cyclase/cyclic AMP transducing system. *Br. J. Pharmacol.*, **105** (Suppl.), 312P.

CUNHA, R.A. & SEBASTIÃO, A.M. (1993). Adenosine and adenine nucleotides are independently released from both the nerve terminals and the muscle fibers upon electrical stimulation of the innervated skeletal muscle of the frog. *Pflügers Arch.*, **424**, 503–510.

DUDEL, J. (1989). Nerve terminal depolarization, calcium inflow and transmitter release. In *Neuromuscular Junction*, ed. Sellin, L.C., Libelius, R. & Thesleff, S. pp. 149–159. Amsterdam: Elsevier.

JINNAI, K., CHIHARA, K., KANDA, F., TADA, K. & FUJITA, T. (1989). Calcitonin gene-related peptide enhances spontaneous release from the rat motor nerve terminal. *Neurosci. Lett.*, **103**, 64–68.

KATZ, B. (1969). *The Release of Neural Transmitter Substances.* pp. 31–39. Liverpool: Liverpool University Press.

KOBAYASHI, H., HASHIMOTO, K., UCHIDA, S., SAKUMA, J., TAKAMI, K., TOHYAMA, M., IZUMI, F. & YOSHIDA, H. (1987). Calcitonin gene-related peptide stimulates adenylate cyclase activity in rat striated muscle. *Experientia*, **43**, 314–316.

MULLHOLLAND, M.W. & JAFFER, S. (1990). Stimulation of acetylcholine release in myenteric plexus by calcitonin gene-related peptide. *Am. J. Physiol.*, **259**, G934–G939.

OHHASHI, T. & JACOBOWITZ, D.M. (1988). Effects of calcitonin gene-related peptide on neuromuscular transmission in the isolated rat diaphragm. *Peptides*, **9**, 613–617.

RIBEIRO, J.A. & SEBASTIÃO, A.M. (1987). On the role, inactivation and origin of endogenous adenosine at the frog neuromuscular junction. *J. Physiol.*, **384**, 571–585.

RODRIGO, J., POLAK, J.M., FERNANDEZ, L. GHATEI, M.A., MULDERRY, P. & BLOOM, S.R. (1985). Calcitonin gene-related peptide immunoreactive sensory and motor nerves of the rat, cat, and monkey esophagus. *Gastroenterology*, **88**, 129–135.

SEAMON, K.B. & DALY, J.W. (1983). Forskolin, cyclic AMP and cellular physiology. *Trends Pharmacol. Sci.*, **9**, 120–123.

SILINSKY, E.M. (1975). On the association between transmitter secretion and the release of adenine nucleotides from mammalian motor nerve terminals. *J. Physiol.*, **247**, 145–162.

SMITH, D.O. (1991). Sources of adenosine released during neuromuscular transmission in the rat. *J. Physiol.*, **432**, 343–354.

TAKAGI, H., KOJIMA, M., NAGATA, M. & KUROMI, H. (1970). On the site of action of hemicholinium-3 at the rat phrenic nerve-diaphragm preparation with special references to its multiple presynaptic actions. *Neuropharmacology*, **9**, 359–367.

TAKAMI, K., HASHIMOTO, K., UCHIDA, S., TOHYAMA, M. & YOSHIDA, H. (1986). Effect of calcitonin gene-related peptide on the cyclic AMP level of isolated mouse diaphragm. *Jpn. J. Pharmacol.*, **42**, 345–350.

TAKAMI, K., KAWAI, Y., UCHIDA, S., TOHYAMA, M., SHIOTANI, Y., YOSHIDA, H., EMSON, P.C., GIRGIS, S., HILLYARD, C.J. & MACINTYRE, I. (1985). Effect of calcitonin gene-related peptide on contraction of striated muscle in the mouse. *Neurosci. Lett.*, **60**, 227–230.

WESSLER, I. (1989). Control of transmitter release from the rat motor nerve by presynaptic nicotinic and muscarinic autoreceptors. *Trends Pharmacol. Sci.*, **10**, 110–114.

WESSLER, I., HALANK, M., RASBACH, J. & KILBINGER, H. (1986). Presynaptic nicotinic receptors mediating a positive feed-back on transmitter release from the rat phrenic nerve. *Naunyn-Schmied. Arch. Pharmacol.*, **334**, 365–372.

WESSLER, I. & KILBINGER, H. (1986). Release of [^{3}H]-acetylcholine from a modified rat phrenic nerve-hemidiaphragm preparation. *Naunyn-Schmied. Arch. Pharmacol.*, **334**, 357–364.

WILEY, J.W., GROSS, R.A. & MACDONALD, R.L. (1992). The peptide CGRP increases a high-threshold Ca^{2+} current in rat nodose neurons via a pertussis toxin-sensitive pathway. *J. Physiol.*, **455**, 367–381.

WILSON, D.F. (1974). The effects of dibutyryl cyclic adenosine 3',5'-monophosphate, theophylline and aminophylline on neuromuscular transmission in the rat. *J. Pharmacol. Exp. Ther.*, **188**, 447–452.

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