Br. J. Pharmac. (1984), **83**, 069–073

Biphasic effect of methylxanthines on acetylcholine release from electrically-stimulated brain slices

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1 The effect of caffeine and aminophylline on the release of acetylcholine (ACh) was investigated in slices of rat cortex perfused with Krebs solution at rest and during electrical stimulation at frequencies of 0.2, 1 and 5 Hz.

2 Both methylxanthines added to the superfusing Krebs solution at a concentration of 50 μM enhanced ACh release. Conversely, at a concentration of 0.5 mM both caffeine and aminophylline decreased ACh release.

3 Neither caffeine nor aminophylline affected the unstimulated ACh release.

3 Dipyridamole 10 μM potentiated the inhibitory effect of adenosine 30 μM on ACh release and antagonized both the stimulatory and inhibitory effects of caffeine on ACh release.

5 The inhibitory effect of caffeine was antagonized by cyclohexyladenosine (CHA) 0.5 μM and N-ethylcarboxamideadenosine (NECA) 5 μM.

6 The results indicate that methylxanthines exert both stimulatory and inhibitory effects on ACh release by acting on adenosine receptors. Methylxanthines may enhance the electrically-evoked ACh release by antagonizing the effect of endogenous adenosine on inhibitory adenosine receptors. On the other hand the mechanism through which methylxanthines decrease ACh release remains obscure.

Introduction

Theophylline and aminophylline antagonize the inhibitory effect of exogenous adenosine on acetylcholine (ACh) release from electrically stimulated guinea-pig myenteric plexus-longitudinal muscle (Vizi & Knoll, 1976) and rat cortical slices (Pedata *et al.*, 1983a). Theophylline also antagonizes the reduction in brain ACh turnover induced by 2-chloroadenosine in the rat (Murray *et al.*, 1982).

However, there is little information concerning the direct effect of methylxanthines on the brain cholinergic system. Phillis *et al.* (1980) observed that caffeine and theophylline increased the rate of ACh efflux from cerebral cortex in anaesthetized rats, and Murray *et al.* (1982) found that the intracerebroventricular administration of theophylline brought about a stimulation of ACh turnover in the hippocampus. Pedata *et al.* (1983b) found that caffeine was able to stimulate or inhibit ACh release from electrically stimulated brain slices, depending on the concentration and the stimulation frequency.

The aim of the present study was therefore to investigate the effects of caffeine and aminophylline on ACh release, and to ascertain whether their effects depend upon antagonism of adenosine.

It has been repeatedly suggested (Fredholm, 1980; Cardinali, 1980), that many of the central effects of methylxanthines may be due to their antagonism of the depressant action exerted by endogenous adenosine on brain neurones (Stone, 1981), presumably through a presynaptic inhibition of neurotransmitter release (Fredholm & Hedqvist, 1980).

A preliminary communication of part of these results was presented at a meeting of the British Pharmacological Society (Pedata *et al.*, 1983b).

Methods

Brain slices: preparation and electrical stimulation

The experiments were carried out on male Wistar rats of 150–200 g body weight. The animals were decapitated, the skull opened and the right and left parietal cortices were rapidly removed and plunged into cold Krebs solution with the following composition (mM): NaCl 118.5, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 10, $NaHCO_3$ 25, choline 0.02. The cortical slices were prepared and

stimulated according to the method of Beani *et al.* (1978). Briefly, the cortical samples, submerged in Krebs solution were cut into slices 400 μm thick by means of a microtome for fresh tissue. The slices were kept floating for 30 min in Krebs solution bubbled with 95% O_2 and 5% CO_2 and for another 20 min in the presence of physostigmine sulphate 3.8 μM. They were then transferred to Perspex superfusion chambers of 0.9 ml volume and perfused with gassed Krebs solution (containing physostigmine sulphate) at the rate of 0.5 ml min^{-1} at 37°C. After 20 min equilibration, the first sample was collected during a 5 min rest period. Then the slices were stimulated with rectangular pulses of alternating polarity, with current strength of 30 mA cm^{-2} and a pulse duration of 5 ms. Two or three cycles of stimulation were carried out for each preparation. The cycles were separated by 20 min intervals. With each cycle, 5 min stimulation periods at 0.2, 1 and 5 Hz stimulation frequency were followed by 10 min of rest in order to allow the washout of all ACh released by electrical stimulation. The actual net extra release caused by the electrical stimulation was estimated by subtracting the ACh release expected during 15 min of rest, calculated by multiplying by 3 the amount released during the 5 min rest preceding each stimulation cycle, from the whole amount found in the 15 min stimulation and washout.

Drugs were added to the perfusion fluid at the beginning of the 20 min interval between two stimulation cycles, taking into account the perfusion speed and dead space.

Acetylcholine assay

ACh content of the superfusate was quantified on the guinea-pig isolated ileum perfused with Tyrode solution containing cyproheptadine 3 nM and morphine 3 μM, preincubated for 60 min with tetrodotoxin 0.03 mM according to the procedure described by Beani *et al.* (1978).

The identity of the active substance in the samples with ACh was routinely checked by adding atropine or by alkaline hydrolysis of the samples. In order to prevent drugs added to the Krebs solution perfusing the slices from interfering with the bioassay, standard solutions of ACh containing the same concentration of drugs were used.

ACh release was expressed as ng g^{-1} of wet tissue min^{-1} of superfusion (mean ± s.e. mean).

Statistical analysis

Student's paired *t* test and two tailed *t* test were used in order to evaluate statistically significant differences. The regression lines were calculated by least squares analysis and tested for correlation coefficients (*r*).

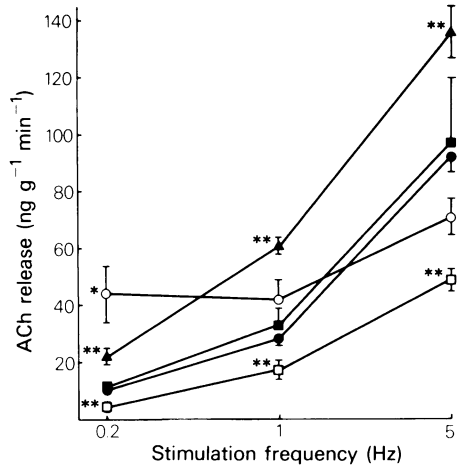

Figure 1 Effect of caffeine on acetylcholine (ACh) release from electrically stimulated cortical slices: (■) no drug, (●) 10 μM, (▲) 50 μM, (○) 100 μM, (□) 0.5 mM caffeine. Each point is the mean of at least 8–10 experiments; vertical bars: standard error of the mean. Statistically significant difference from no drug: $*P<0.05$ and $**P<0.01$ calculated by Student's two tailed *t* test.

Drugs

Freshly prepared solutions of the following drugs were used: acetylcholine, caffeine, physostigmine sulphate, α-methylparatyrosine (Sigma), aminophylline (Malesci), adenosine (Calbiochem), morphine sulphate (Carlo Erba), tetrodotoxin (Biochemia), cyproheptadine (Merck, Sharp & Dome), dipyridamole (Boeringer, Ingelheim), cyclohexyladenosine (CHA) (Boeringer, Mannheim). N-ethylcarboxamideadenosine (NECA) was a gift from Prof. H.C. Erbler of Byk Gulden, Konstanz.

Results

Comparison of the effects of caffeine and aminophylline on acetylcholine release

As shown in Figure 1, the increase in stimulation frequency was associated with a linear increase in ACh release ($r=0.82$, $P<0.01$). The addition of caffeine 10 μM to the superfusing Krebs solution was without effect. On the other hand, caffeine 50 μM significantly enhanced ACh release with a 112% increase at 0.2 Hz stimulation frequency and 85% and 47% increase respectively at 1 and 5 Hz. A linear relationship between stimulation frequency and ACh release could also be calculated with $r=0.93$, $P<0.01$. Caffeine at 100 μM concentration only enhanced ACh release at 0.2 Hz, while it had no effect at 1 and 5 Hz stimulation rates. On the other hand,

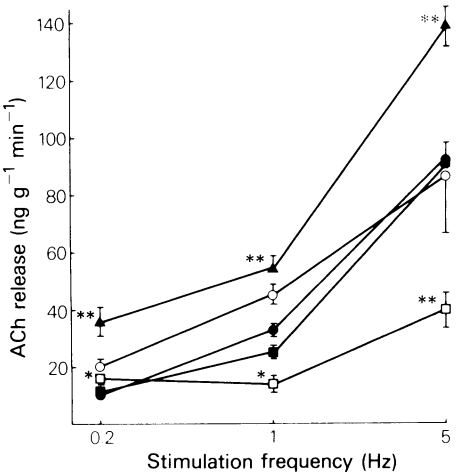

Figure 2 Effect of aminophylline on acetylcholine (ACh) release from electrically stimulated cortical slices: (■) no drug, (●) 10 μM, (▲) 50 μM, (○) 100 μM, (□) 0.5 mM aminophylline. Each point is the mean of at least 5–6 experiments; vertical bars: standard error of the mean. Statistically significant difference from no drug: $*P<0.05$ and $**P<0.01$ calculated by Student's two tailed t test.

caffeine at 0.5 mM concentration significantly decreased ACh release by approximately 50% at all frequencies tested.

Figure 2 shows that the effect of aminophylline on ACh release was qualitatively similar to that of caffeine: aminophylline had no effect when added at a concentration of 10 μM, it enhanced ACh release at 50 μM ($r= 0.80$, $P<0.01$), while at 100 μM it was without effect. On the other hand at 0.5 mM aminophylline significantly decreased ACh output at both 1 and 5 Hz stimulation frequencies.

Neither caffeine, as shown in Table 1, nor aminophylline (data not shown) affected the unstimulated ACh release.

Interaction between dipyridamole and caffeine

As shown in Table 1, a concentration of 30 μM of adenosine added to the perfusion fluid brought about a decrease in ACh release. The magnitude of the effect was proportional to the stimulation frequency. Dipyridamole at a concentration of 10 μM, which blocks adenosine uptake by cholinergic synaptosomes (Meunier & Morel, 1978), significantly potentiated the inhibitory effect of adenosine 30 μM at all stimulation frequencies used, though potentiation was particularly evident at 5 Hz. At this frequency dipyridamole and adenosine reduced ACh release to 13% of control values. On the other hand, dipyridamole 10 μM added alone to the superfusion fluid only slightly decreased ACh output at 5 Hz stimulation frequency.

Dipyridamole 10 μM completely antagonized both the stimulatory and inhibitory effects on ACh release brought about by caffeine 50 μM and 0.5 mM respectively (Table 1). At some of the stimulation frequencies tested, the effect of caffeine was even inverted.

Investigation of the inhibitory effect of methylxanthines

The inhibitory effect of caffeine 0.5 mM on ACh release from slices stimulated at 5 Hz was antagonized by two adenosine derivatives, CHA, added to the superfusion fluid at a concentration of 0.5 μM and NECA 5 μM as illustrated in Figure 3.

The inhibitory effect of caffeine was not affected by pretreating some of the rats ($n=4$), from which

Table 1 Interaction between dipyridamole, adenosine and caffeine on acetylcholine release (ng g^{-1} min^{-1} ± s.e. mean)

Drugs	*Concentration* (μM)	n	Stimulation frequency (Hz) 0	0.2	1	5
None	–	49	5.9±0.2	10.4±0.7	33.0±1.7	92.2±5.8
Adenosine	30	8	3.8±0.4	7.5±0.6*	11.8±1.4**	26.9±2.2**
Dipyridamole	10	3	5.1±0.5	8.3±2.8	34.3±2.5	73.3±2.2
Adenosine + dipyridamole	30 + 10	8	3.2±0.1	5.5±0.3°	7.8±0.4°°	11.3±0.8°°
Caffeine	50	10	6.5±0.4	22.1±1.9**	61.2±2.9**	136.4±9.1**
Caffeine + dipyridamole	50 + 10	8	7.2±0.6	4.6±0.3°	21.3±1.7°	79.2±3.6°°
Caffeine	500	11	5.8±0.4	4.2±0.4**	17.7±0.8**	49.1±4.2**
Caffeine + dipyridamole	500 + 10	4	5.0±0.3	11.5±1.0	34.7±3.5°°	98.2±7.3°

Statistically significant difference, calculated by Student's two tailed t test, from no drug $*P<0.05$ and $**P<0.01$; from adenosine or caffeine alone $°P<0.05$, $°°P<0.01$, n = number of experiments.

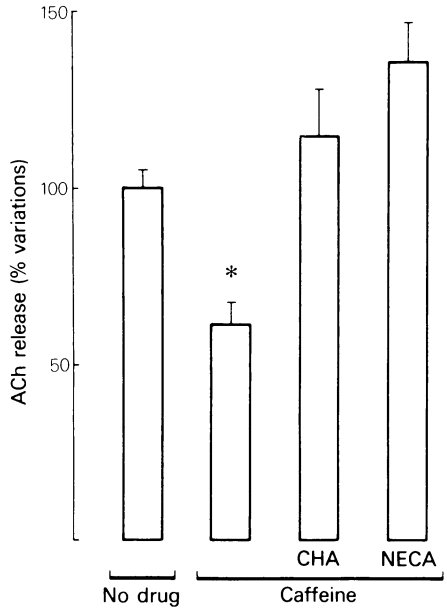

Figure 3 Antagonism by cyclohexyladenosine (CHA) 0.5 μM and N-ethylcarboxamideadenosine (NECA) 5 μM of the inhibitory effect of caffeine 0.5 mM on acetylcholine (ACh) release from brain slices stimulated at 5 Hz. Each column represents the mean of 4 experiments. Vertical bars: standard error of the mean. Statistically significant difference from control: $*P<0.05$, calculated by Student's paired t test.

the cortical slices were taken, with α-methyl-*p*-tyrosine (200 mg kg^{-1} i.p.) 24 h before they were killed.

Discussion

Our experiments demonstrate that caffeine and aminophylline exert a biphasic effect on ACh release from cortical slices. ACh release evoked by electrical stimulation was enhanced by the two methylxanthines at 50 μM and inhibited at 0.5 mM. The limited efficacy of 100 μM could be the result of the algebraic sum of the two opposite effects.

A biphasic effect of methylxanthines was described by Gustafsson *et al.* (1981) who showed that theophylline enhanced the neurogenic contractions of the electrically stimulated guinea-pig myenteric plexus-longitudinal muscle at concentrations up to 50 μM, while depressing them at high concentrations. It may be also mentioned that Phillis *et al.* (1980) observed that caffeine at 40 mg kg^{-1} was less effective than at 20 mg kg^{-1} in increasing ACh efflux from the cerebral cortex *in vivo*.

It is possible that methylxanthines enhance ACh output by antagonizing the depressant effect of endogenous adenosine released by electrical stimulation. A spontaneous release of adenosine from the brain was shown *in vivo* by Zetterstrom *et al.* (1982). Gustafsson *et al.* (1981) demonstrated that electrical stimulation increases the release of endogenous adenosine from isolated longitudinal muscle of guinea-pig ileum. In the electrically stimulated vas deferens preparation, the concentration of endogenous adenosine in the extracellular fluid was enhanced by the presence of dipyridamole (Fredholm *et al.*, 1982).

An indirect indication that adenosine might be released in a frequency-dependent manner also under our experimental conditions is given by the finding that the inhibitory effect of adenosine and adenosine plus dipyridamole on ACh release is more pronounced at a high than at a low stimulation frequency. It may be pointed out that the increase in ACh release from the cerebral cortex brought about by low concentrations of caffeine could contribute to the electroencephalographic and behavioural activation which follows its administration in man (Goldstein *et al.*, 1965).

The inhibitory effect of methylxanthines on ACh release also appears to be mediated through adenosine receptors since it is antagonized by the adenosine derivatives, CHA and NECA and by dipyridamole.

Two different types of adenosine receptors have been identified in various tissues, including the brain. The A_1-receptors are activated by nmolar concentrations of adenosine and result in inhibition of adenylate cyclase, while the A_2-receptors are activated by μmolar concentrations of adenosine resulting in stimulation of adenylate cyclase (Van Calker *et al.*, 1979). Spignoli *et al.* (1984) demonstrated that CHA, which shows a high affinity for A_1-receptors (Daly, 1982) decreased ACh output at nmolar concentrations. Conversely NECA, characterized by a relatively low affinity for both A_1- and A_2-receptors (Daly, 1982), stimulated ACh output at μmolar concentrations. Therefore, in attempting to explain the decrease in ACh release brought about by methylxanthines, the possibility of an inhibition of stimulatory A_2-receptors should be considered even if the antagonism by CHA, observed in our experiments, raises some doubt.

However in millimolar concentrations, methylxanthines are phosphodiesterase inhibitors and prevent cyclic AMP inactivation (Butcher & Sutherland, 1962). They also affect intracellular calcium mobilization (Isaacson & Sandow, 1967; Johnson & Inesi, 1969) and inhibit the release of adenosine from cortical slices (Stone *et al.*, 1981). Since the inhibitory effect of caffeine was not modified by pretreating

the rats with α-methyl-*p*-tyrosine in a dose which reduces brain catecholamine levels (Berkowitz *et al.*, 1970), the involvement of a noradrenergic mechanism can be excluded. Nevertheless, investigations are still needed to rule out mechanisms other than the blockade of adenosine receptors, in explaining the inhibitory action of methylxanthines on ACh release.

This work was supported by a grant from the University of Florence.

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(Received October 20, 1983. Revised February 27, 1984.)