Br. J. Pharmac. (1986), **88**, 671–676

Chronic caffeine treatment reduces caffeine but not adenosine effects on cortical acetylcholine release

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1 The effects of both adenosine and caffeine on the release of acetylcholine (ACh) were investigated in slices of cerebral cortex taken from rats pretreated for 30 days with caffeine (100 mg kg^{-1} daily, dissolved in their drinking water) at rest and during electrical stimulation at frequencies of 0.2, 1 and 5 Hz. The effect of this treatment on adenosine binding sites was also investigated in cortical membranes using N-cyclohexyl-(^{3}H]-adenosine ([^{3}H]-CHA) as a ligand.

2 The chronic caffeine treatment did not change animal growth patterns. Spontaneous exploratory activity appeared to be increased at the 3rd day but was unchanged at the 30th day when compared with controls.

3 Caffeine-treatment increased the number of high affinity binding sites for [^{3}H]-CHA by 64% over the control values. Low affinity binding site density and affinity constants were unaffected.

4 Adenosine 30 μM added to the superfusion fluid decreased electrically stimulated ACh release both in rats drinking tap water and rats drinking caffeine.

5 In rats drinking tap water, caffeine added to the superfusion fluid at a concentration of 50 μM enhanced ACh release, while at 0.5 mM it decreased ACh output from the slices. Both effects were abolished by pretreatment with caffeine *in vivo*.

6 The results indicate that prolonged consumption of high doses of caffeine causes changes in the responsiveness of cholinergic neurones to caffeine. The change is not shared by adenosine, through whose recognition sites caffeine is believed to act. It is therefore possible that the adaptive changes following repeated caffeine administration involve either only the coupler-transducer mechanism activated by the antagonist, or effects unrelated to receptors.

Introduction

Caffeine, consumed in the form of coffee, tea or cola, is the psychotropic agent most widely used in the world by either sex and in practically all age groups (Barone & Roberts, 1984). Tolerance develops rapidly to most of the behavioural and peripheral effects of caffeine in man (Wedemeyer, 1920; Goldstein *et al.*, 1969; Robertson *et al.*, 1981) and in rodents (Butcher *et al.*, 1984; Chou *et al.*, 1985). A clinically relevant dependence has not yet been described (see Eichler, 1976).

Little is known of the neurochemical changes occurring during the development of caffeine tolerance. Caffeine is chemically related to adenosine and evidence indicates that most of its actions are brought about by the blockade of adenosine receptors (Smellie *et al.*, 1979; Phillis & Wu, 1981; Fredholm, 1982). In rodents repeated administration of caffeine increases the number of adenosine binding sites in the brain (Murray, 1982; Fredholm, 1982; Boulenger *et al.*, 1983; Chou *et al.*, 1985). Adenosine added to the superfusing solution brings about a dose-dependent decrease in ACh released from electrically stimulated cortical slices (Pedata *et al.*, 1983a). Caffeine, added to the superfusing solution at the concentration of 50 μM enhances acetylcholine (ACh) release. Conversely, at a concentration of 0.5 mM caffeine decreases ACh release (Pedata *et al.*, 1984).

In the present study we investigated whether chronic caffeine treatment modifies the action of adenosine and of caffeine on ACh release from the cerebral cortex. The effects on the number of adenosine binding sites in cortical membranes and on exploratory activity were also studied.

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A preliminary account of some of these results was given at a meeting of the British Pharmacological Society (Bartolini *et al.*, 1985).

Methods

Animals and treatment

Male Wistar rats, with an initial body weight of 180 g, received caffeine 100 mg kg^{-1} dissolved in the drinking water for 30 days. Three separate sets of experiments were carried out on a total of 42 controls receiving tap water and 44 treated rats. Animals were housed, two per cage, in a climatized (24°C) constant daylight cycle (12 h) room, with free access to water and food. Water intake and weight were measured daily. Treatment was discontinued 48 h before the animals were killed to avoid interference of dietary caffeine on release and binding experiments. Membranes for binding assay were obtained from each batch of treated animals and controls.

Brain slice preparation and electrical stimulation

The animals were decapitated, the skull opened and the right and left parietal cortices were rapidly removed and plunged into cold Krebs solution of 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 oxygenated 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% 0_2 and 5% CO_2, and for an additional 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 superfused with gassed Krebs solution (containing physostigmine sulphate) at the rate of 0.5 ml min^{-1} at 37°C. Following 20 min equilibration, the first sample was collected during a 5 min rest period. Slices were then stimulated with rectangular pulses of alternating polarity, with a current strength of 30 mA cm^{-2} and a pulse duration of 5 ms. Two cycles of stimulation, separated by 20 min interval, were carried out. Within each cycle, 5 min stimulation periods at 0.2, 1 and 5 Hz stimulation frequency were followed by 10 min rest. Samples from each stimulation period and the following rest period were collected in order to allow the washout of all the ACh released by the electrical stimulation. The extra release caused by electrical stimulation was estimated by subtracting the ACh release expected during 15 min 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 perfusate sample. Drugs were added to the superfusion fluid at the beginning of the 20 min interval between two stimulation cycles, taking into account the perfusion speed and dead space.

Acetylcholine assay

The ACh content of the superfusate samples was quantified on the guinea-pig isolated ileum perfused with a Tyrode solution containing cyproheptadine (3 nM), morphine (3 μM) and preincubated for 60 min with tetrodotoxin (30 μM) according to the procedure described by Beani *et al.* (1978).

The identity with ACh of the active substance in the samples was routinely checked by adding atropine or by alkaline hydrolysis of the samples. In order to prevent the drugs which were added to the Krebs solution superfusing the slices, from introducing an uncalculated bias in the estimation of the ACh released, the ACh standards contained the same concentration of the drugs as did the samples.

Membrane preparation and binding assay

Adenosine binding sites have been characterized using N-cyclohexyl-[^{3}H]-adenosine ([^{3}H]-CHA), as previously described (Corradetti *et al.*, 1984). In brief, after decapitation the skull was opened and the cerebral cortex was rapidly dissected out and homogenized in 15 vol ice cold sucrose (0.32 M) in Teflon glass homogenizers (Thomas Elvejner-Potter). Following 1000 *g* (10 min at 0°C) centrifugation, the supernatant was further centrifuged at 30,000 *g* for 30 min at 0°C and resuspended in 25 ml Tris-HCl buffer at pH 7.4. This procedure was repeated twice in order to remove all the endogenous adenosine from membranes. After resuspension in 10 vol of Tris-HCl buffer and incubation for 30 min with adenosine deaminase (2 units ml^{-1}) at room temperature, the homogenates were centrifuged (30,000 *g*, 30 min, 0°C), resuspended in 20 vol Tris-HCl buffer and stored at −70°C for not more than one month. Saturation curves were carried out on critical membranes prepared from caffeine-treated and control rats by adding increasing concentrations (0.2–60 nM) of [^{3}H]-CHA (13.5 Ci $mmol^{-1}$; New England Nuclear) to a constant amount of membranes (about 200 mg protein in 0.6 ml final volume). All experiments were performed in triplicate. Non-specific binding was quantified by adding 10 μM of unlabelled CHA. Samples, filtered through GF/B filters (Whatman) were washed three times with 3 ml ice cold Tris-HCl buffer. Bound radioactivity was counted in 10 ml Instagel (Packard) scintillation fluid by means of a Tricarb 460C counter with 40% efficiency. Displacement curves were carried

out by adding increasing concentrations of cold CHA in the presence of 10 nM [^{3}H]-CHA. Binding parameters were calculated according to the criteria indicated by Munson & Rodbard (1980) and Klotz (1982). Protein content was measured according to the method described by Lowry *et al.* (1951).

Exploratory activity

Spontaneous activity was investigated in naive rats by means of a symmetrical Y shaped runway (Mulas *et al.*, 1970). The number of complete entries with all four feet into the arms of the runway were counted over a period of 3 min. With this procedure both spontaneous alternation and exploratory activity were assessed.

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*).

Drugs

Freshly prepared solutions of the following drugs were used: acetylcholine, caffeine, physostigmine sulphate (Sigma), adenosine (Calbiochem), morphine sulphate (Carlo Erba), tetrodotoxin (Biochemia), cyproheptadine (Merck, Sharp & Dome), calf adenosine deaminase, cyclohexyladenosine (Boehringer Mannheim), N-cyclohexyl-[^{3}H]-adenosine (specific activity 13.5 Ci mmol^{-1}, New England Nuclear).

Results

Effect of chronic caffeine treatment on mortality, body weight and exploratory activity

No differences between the treated and control rats were found in mortality and body weight. Among the 42 controls, 3 died and the final body weight of the others was 366 ± 1 g. Of the 44 treated rats, 4 died and body weight at the end of treatment was 367 ± 21 g. An increase in exploratory activity of treated rats with no change in alternation was observed on day 3. The number of entries in the Y maze arms was 4.20 ± 0.35 in 15 treated animals and 3.00 ± 0.25 in 12 control rats ($P < 0.05$). No difference between the two groups was found on day 30.

Effect of chronic caffeine treatment on N-cyclohexyl-[^{3}H]-adenosine binding sites

Figure 1 shows the Scatchard analysis of N-cyclohexyl-[^{3}H]-adenosine ([^{3}H]-CHA) binding to cortical membranes from rats drinking tap water or caffeine solution. Two distinct binding sites can be recognized in both groups: a high affinity binding site with an apparent K_D of 1.3 ± 0.1 nM and a low affinity binding site with a K_D of 93 ± 1 nM. In the controls the B_{max} were 112 ± 4 and 417 ± 4, respectively. The Hill plot was linear ($r = 0.99$) and had a slope of 0.51 ± 0.02. The semi-logarithmic plot confirmed, reliably, the total B_{max} obtained by extrapolation from the Scatchard plot. In the caffeine-treated rats the B_{max} of the high affinity sites was 184 ± 16 fmol mg^{-1} protein with a statistically significant 64% increase over the controls ($P < 0.01$, two tailed *t* test). The B_{max} of the low affinity binding sites was unchanged (395 ± 70 fmol mg^{-1} protein). The *n* values obtained from the Hill plot were 0.59 ± 0.06.

Displacement curves carried out on membranes obtained from the same groups of animals used for the saturation curves gave IC_{50} values of 38 and 41 nM for control and caffeine-treated rats, respectively, as calculated by Hill plots. The Hill plots showed a significant correlation coefficient ($r = 0.99$, $P < 0.01$) with *n* values of 0.50 in both groups.

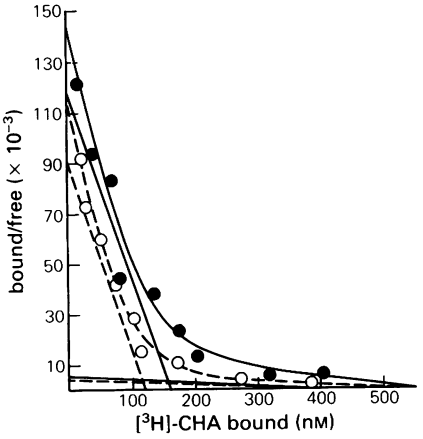

Figure 1 Scatchard analysis of saturation isotherms of specific N-cyclohexyl-[^{3}H]-adenosine ([^{3}H]-CHA) binding to cortical membranes from control (○) and caffeine-treated rats (●). Each point represents the mean of 4 determinations in triplicate. Binding was measured over a radioligand concentration range from 0.2 to 60 nM, non specific binding was determined in the presence of 10 μM unlabelled cyclohexyladenosine. Fitting of the curves (Munson & Rodbard, 1980) gave two components for both controls (dashed lines) and caffeine-treated rats (solid lines). Note that membranes from caffeine-treated rats show an increase in number of high affinity sites in comparison with controls (184 ± 16 *vs* 112 ± 4 fmol-mg^{-1} protein, $P < 0.01$).

Effect of adenosine on acetylcholine release

As shown in Figure 2 the increase in stimulation frequency brought about the same linear increase in ACh release in the control and caffeine-treated rats ($r = 0.99$, $P < 0.02$ and $r = 0.99$, $P < 0.02$ respectively). The addition of adenosine 30 μM caused a statistically significant decrease in ACh release at all frequencies tested in both groups of rats. In control rats, adenosine exerted its maximum inhibitory effect at stimulation frequencies of 1 and 5 Hz (61% and 66% decrease) while a 37% decrease occurred at 0.2 Hz. In caffeine-treated rats, adenosine produced a decrease of about 48% at all the stimulation frequencies. No statistically significant differences were found between the decrease induced by adenosine in control and caffeine-treated rats at the same stimulation frequency. The basal release at rest was 5.41 ± 0.51 in control rats and 6.48 ± 1.13 in caffeine-treated rats: adenosine induced a decrease of about 30% in both groups of rats.

Effect of caffeine on acetylcholine release

Figure 3 shows that with the addition of caffeine 50 μM to the superfusing Krebs solution, a statistically significant increase in ACh release from the stimulated cortical slices taken from the rats drinking tap water was seen. In caffeine-treated rats, caffeine exerted no statistically significant effect. In rats drinking tap water, caffeine increased ACh release at rest (+ 42%) and brought about a 124% increase at 0.2 Hz, a 43% at 1 Hz and a 31% at 5 Hz stimulation frequency. Conversely in caffeine-treated rats, caffeine caused no significant increase of ACh release both at rest and at 0.2 Hz (+ 3%) and caused a decrease of − 13% and − 10% at 1 and 5 Hz stimulation frequency, respectively.

Figure 4 shows the effect of caffeine 0.5 mM on ACh release in the rats drinking tap water or caffeine. In control rats, caffeine brought about a significant decrease in ACh release at all frequencies tested, ranging from − 59% at 0.2 to − 40% at 5 Hz. No effect was seen at rest. In the caffeine-treated rats, caffeine added to the superfusing Krebs solution did not affect ACh release. The small increase observed, + 13% at 1 and + 17% at 5 Hz stimulation frequency was not statistically significant.

Discussion

The daily dose of 100 mg g^{-1} of caffeine in the present experiment is apparently rather large for rats.

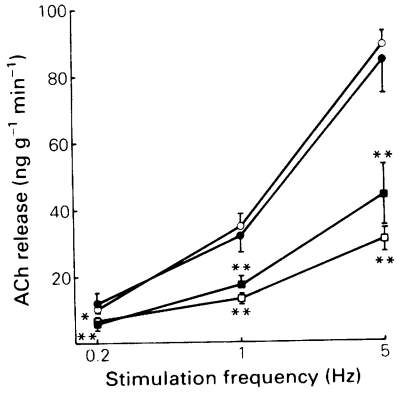

Figure 2 Effect of adenosine on acetylcholine (ACh) release from electrically stimulated cortical slices prepared from rats drinking tap water or a caffeine solution: (○) rats drinking tap water, no drug in the superfusing Krebs; (●) caffeine-treated rat (100 mg kg^{-1} orally for 30 days), no drug in the superfusing Krebs; (□) rats drinking tap water, adenosine (30 μM) in the superfusing Krebs; (■) caffeine-treated rats, adenosine in the superfusing Krebs. Each point is the mean of 4–6 experiments; vertical lines show standard error of the mean. Statistically significant difference from no drug: $*P < 0.05$; $P < 0.02$; calculated by student's two tailed t test.

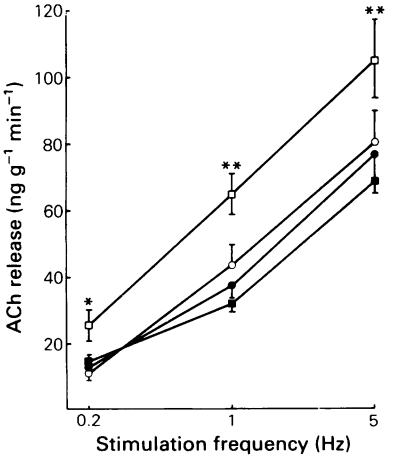

Figure 3 Effect of caffeine on acetylcholine (ACh) release from electrically stimulated cortical slices prepared from rats drinking tap water or a caffeine solution: (○) rats drinking tap water, no drug in the superfusing Krebs; (●) caffeine-treated rats (100 mg kg^{-1} orally for 30 days), no drug in the superfusing Krebs; (□) rats drinking tap water, caffeine (50 μM) in the superfusing Krebs; (■) caffeine-treated rats, caffeine in the superfusing Krebs. Each point is the mean of 4–6 experiments; vertical lines show standard error of the mean. Statistically significant difference from no drug: $*P < 0.05$; $**P < 0.01$, calculated by Student's two tailed t test.

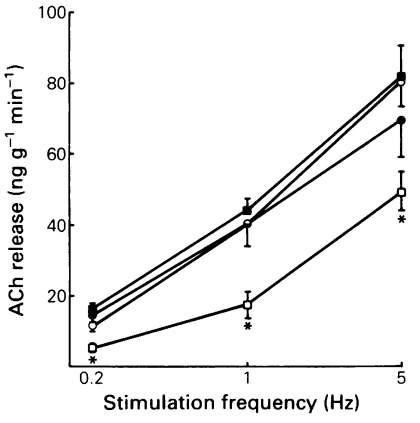

Figure 4 Effect of caffeine on acetylcholine (ACh) release from electrically stimulated cortical slices prepared from rats drinking tap water or a caffeine solution: (○) rats drinking tap water, no drug in the superfusing Krebs; (●) caffeine-treated rats (100 mg kg^{-1} orally for 30 days) no drug in the superfusing Krebs; (□) rats drinking tap water, caffeine (0.5 mM) in the superfusing Krebs; (■) caffeine-treated rats, caffeine in the superfusing Krebs. Each point is the mean of 4–6 experiments; vertical lines show standard error of the mean. Statistically significant difference from no drug: $*P<0.01$ calculated by Student's two tailed t test.

However, after a month of treatment, mortality, body weight and gross behaviour in treated rats were not different from controls. This finding excludes gross caffeine toxicity. If correction is made for surface area, this dose would be equivalent to the daily consumption of 10 cups of coffee containing 100 mg of caffeine each in man (Cardinali, 1980; Boulenger *et al.*, 1982).

A moderate increase in exploratory activity was observed on the third day of caffeine treatment but it was not present on the 30th. This indicates the development of tolerance. Similarly, Chou *et al.* (1985) observed the development of tolerance to a challenge dose of caffeine following a 2 week treatment with low doses of caffeine.

At least two different types of adenosine receptors have been identified in various tissues, including the brain. They can be classified as A_1 and A_2 receptors in terms of order of potency of agonists (Stone, 1984; Hamprecht & Van Calker, 1985). Both receptors are inhibited by methylxanthines, including caffeine (Daly, 1982). The ligands commonly used for receptor binding studies only bind selectively to the A_1 type.

Following chronic caffeine administration an increase of A_1 adenosine binding sites was found in rat cortical membranes by Fredholm (1982) and Murray (1982) and in reticular formation membranes by Chou *et al.* (1985). The increase in the number of binding sites ranged between 25 and 30%. In our experiments we found a larger increase of + 64% in the number of cortical binding sites. However, by extending the concentrations of ligand used, Patel *et al.* (1982) and Corradetti *et al.* (1984) demonstrated two sub-types of A_1 binding sites with a large difference in affinity in the cortex. In the present experiments it was demonstrated that the increase involved only the high affinity binding sites. However, in spite of the increase in the number of high affinity A_1 binding sites, the effect of adenosine on ACh release was not modified in the cortical slices taken from caffeine-treated rats and the effect of caffeine at both 50 and 500 μM concentration was abolished. Thus no relationship appears to exist between the changes following chronic caffeine treatment in the number of high affinity binding sites and the purinergic modulation of ACh release. It should be mentioned that according to Fredholm (1982) the increase of adenosine receptors did not affect cyclic AMP accumulation and inhibition of lipolysis induced by adenosine analogues.

The depressant effect of adenosine on ACh release is strongly reduced (Pedata *et al.*, 1983b) in 24 month old rats in which low affinity A_1 receptors have disappeared (Corradetti *et al.*, 1984). Therefore, the low affinity subtype of the A_1 receptors seems to be involved in the depressant effect of adenosine on cortical ACh release. Since chronic caffeine treatment induces no increase of the low affinity receptors, no change in adenosine action should be expected.

On the other hand, both stimulatory and inhibitory effects of caffeine on ACh release are abolished in the caffeine-treated rats. Both effects appear to involve purine receptors (Pedata *et al.*, 1984). It is therefore difficult to account for their disappearance, the unchanged effect of adenosine and the increase in the A_1 receptor high affinity subtype.

This apparent contradiction could indicate that agonists and antagonists bind to the same purine recognition sites but activate different coupler-transducer mechanisms of which only that associated with antagonist actions undergoes adaptive changes following repeated caffeine administration. However, direct affects of chronic caffeine administration unrelated to adenosine receptors should also be taken into consideration.

Caffeine has been shown to exert direct effects on calcium storage and translocation (Johnson & Inesi, 1969). Changes in calcium concentration could therefore affect caffeine modulation of the calcium-dependent electrically-evoked ACh release.

This work was supported by a grant from the Italian Ministry of Education.

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(Received December 12, 1985.
Revised February 19, 1986.
Accepted March 12, 1986.)