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Effect of adenosine receptor agonists on spontaneous and K^+-evoked acetylcholine release from the in vivo rat cerebral cortex

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Repeated applications of elevated K^+ (100 mM) in artificial cerebrospinal fluid (CSF) were used to evoke an efflux of acetylcholine (ACh) from the in vivo rat cerebral cortex using a cortical cup technique. Elevated K^+ reproducibly increased the levels of ACh in cup superfusates by a factor of 3-5-fold above basal levels (27.2±9.7 nM). The adenosine A_1 receptor agonist N^6-cyclopentyl adenosine (CPA), at a concentration of 10^{-8} M, depressed basal, but not K^+-evoked ACh efflux. 10^{-6} M CPA increased basal, but did not alter K^+-evoked, ACh efflux. The A_2 selective agonist CGS 21680 did not alter either basal, or K^+-evoked, ACh efflux. The inhibitory effects of 10^{-8} M CPA on ACh efflux would be consistent with the presence of adenosine A_1 receptors on cholinergic nerve terminals in the cerebral cortex. At a higher concentration (10^{-6} M) CPA elevated basal release, possibly by activating low affinity A_2 receptors. The failure of CGS 21680 (10^{-8} M) to alter basal ACh release suggests an absence of high affinity A_2 receptors in these terminals. Whereas elevated K^+ in cup superfusates consistently enhanced ACh efflux from the cerebral cortex, this increase was not affected by either CPA or CGS 21680. High K^+-evoked release of cerebral cortical ACh may be an inappropriate model for the study of adenosine's actions on neurotransmitter release.

INTRODUCTION

Both A_1 and A_2 adenosine receptors have been demonstrated to regulate acetylcholine release from in vitro central cholinergic nerve terminals. A_1 receptor agonists, such as R-phenylisopropyl adenosine (R-PIA) and cyclohexyl adenosine (CHA), inhibited the veratridine-evoked release of acetylcholine (ACh) from purified rat striatal nerve terminals whereas the mixed A_1/A_2 agonist *N*-ethylcarboxamido adenosine (NECA) stimulated ACh release[1]. The involvement of A_2 receptors in the effect of NECA was established by showing that the NECA-evoked stimulation of release was enhanced after the application of the A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine. Similar results were obtained by Pedata et al.[15] using synaptosomes prepared from rat cerebral cortex and hippocampus. In these studies, CHA (0.01 μM) was a potent inhibitor of K^+-stimulated ACh release whereas NECA was inactive at concentrations of 0.1–1.0 μM.

Adenosine receptors also modulate ACh release from electrically stimulated rat cerebral cortical slices[20]. The A_1 agonist CHA (10 nM) potently depressed ACh release, although its effects were less pronounced at higher (100 μM) concentrations. NECA (100 μM) significantly enhanced ACh release. Addition of aminophylline to the superfusion fluid prevented both the inhibitory effects of CHA and the stimulating effect of NECA, confirming the involvement of A_1 and A_2 adenosine receptors. The present experiments were undertaken in order to compare these in vitro observations with an in vivo model of ACh release.

ACh release from the rat cerebral cortex into artificial CSF superfusates has been measured by high pressure liquid chromatography and electrochemical detection, using the newly available Immobilized Enzyme

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Reactor system from Chrompack, Inc. (New Jersey). The effects of adenosine receptor agonists on both basal and K^+-evoked ACh release were determined.

MATERIALS AND METHODS

Male Sprague–Dawley rats were anesthetized with halothane. After insertion of a tracheal cannula, anesthesia was sustained with methoxyflurane in air. Body temperature was controlled at 37°C with a rectal probe and heating pad. One femoral artery was cannulated for the measurement of blood pressure and withdrawal of arterial blood samples for pH and blood-gas measurement.

The dorsal surfaces of both cerebral hemispheres were exposed, and after reflection of the dura mater, oval cortical cups suspended in flexible mounting brackets were placed on both cortices[14,19]. The dorsal surface of the head around the cups was covered with a stabilized gel of 4% agar in artificial CSF. A monopolar EEG electrode was placed in both cups. ECoGs and arterial blood pressure were recorded on a Grass Polygraph. Artificial CSF, containing 2×10^{-5} M neostigmine bromide, pipetted into the cortical cups, was removed after a 30 min equilibration period and replaced with 200 μl of warmed (37°C) sterile artificial CSF, which had been bubbled with a gas mixture of 5% carbon dioxide in nitrogen. The same gas mixture was bubbled into the cortical cups. Cup fluid was maintained at 37°C with a heat lamp. Cup fluid was collected at 10 min intervals and replaced with fresh neostigmine-containing artificial CSF.

Depolarization of cortical tissues was elicited by the topical application of artificial CSF containing 100 mM K^+, prepared by replacing the appropriate amount of NaCl in the artificial CSF with an equivalent amount of KCl.

Seven rats (Series I) were used to determine the effects of topical applications of 100 mM K^+ on ACh release into the cortical cups. After a basal 10 min CSF (200 μl) collection, the cups were filled with 200 μl of high-K^+ CSF for 10 min, followed by two more normal CSF collections. After a 20 min break, this sequence was repeated twice, with a 10 min break between the sequences.

For studies (Series II) on the release of ACh during CPA and CGS 21680 application, the initial sequence of K^+ exposure (S_1), described above, was followed by a 20 min period during which CPA (10^{-8} M or 10^{-6} M) or CGS 21680 (10^{-8} M) were applied (the cup solution was replaced 3× during this period). The second and third K^+ depolarization sequences (S_2, S_3) were then repeated, with all solutions containing the appropriate concentration of CPA or CGS 21680. Five rats (10 cortices) were used to test for the effects of each concentration of CPA. CGS 21680 (10^{-8} M) was applied in the cup superfusates of 4 rats.

The collected cortical perfusates were ejected into chilled microvials, centrifuged at 1,200× g and stored at −20°C for later analysis by high-pressure liquid chromatography. The chromatographic system consisted of a Waters Associates' 510 pump, 717 Autosampler, 464 Electrochemical Detector equipped with a Ag/AgCl reference electrode and a platinum working electrode, 746 Data Module, and an Automated Gradient Controller. The column used was a Chromspher choline column (100×3.0 mm L×ID) coupled with a Chromsep IMER (IMmobilized Enzyme Reactor) type ACh and a guard column type RC (all Chrompack).

The chromatographic analysis was performed isocratically using a moving phase composition of: 0.2 M KH_2PO_4, 0.002 M tetramethylammonium hydroxide, and 0.005 M KCl, pH 7.4. This elution system was derived from the work of L. Gorman and D. Becker (Chrompack, Inc., New Jersey, Technical Bulletin).

The postcolumn effluent stream was monitored for chromatographic peaks at a platinum working electrode potential of 400 mV and a current range of ±10 nA. Peaks were identified and quantified by comparing retention times, peak heights, and areas with those of a known standard. The minimal detectable level of acetylcholine was 0.4 pmol which, with a 100 μl injection volume of perfusate, corresponded to a 4 nM ACh concentration.

Significant increases in ACh release into the cortical cups during K^+ application were determined by Student's t-test, comparing the concentration during K^+ exposure with that during the immediately preceding basal collection period. The effects of CPA and CGS 21680 on K^+-evoked ACh release are expressed as S_2/S_1 or S_3/S_1 ratios evoked during the three K^+ application periods. In each instance, stimulated efflux represents the increase in ACh concentrations above that in the immediately preceding basal collection period. The data were analyzed with the SPSS PC statistical program. A one-way analysis of variance (ANOVA) was performed and Student–Newman–Keuls ($P < 0.05$) or Sheffe's ($P < 0.01$) tests were utilized to determine statistical significance, with comparisons between control and each drug condition.

RESULTS

Physiological variables were monitored in all groups of animals by recordings of mean arterial blood pressure (MABP) and electrocorticogram (ECoG) and through the determination of arterial pH and gas tensions in samples obtained during the initial and final basal collection periods of each experiment. These data are presented in Table I. There were no significant changes in MABP as a result of the application of high K^+ CSF or CPA. Application of 100 mM K^+-containing CSF caused an initial marked depression of the ECoG, with some intermittent recovery (high voltage, low frequency potentials) becoming apparent during

TABLE I

Physiological variables in arterial blood at the start (Begin) and ending (End) of experiments (mean ± S.E.M.)

	MABP (mm Hg)		*pH*		*$PaCO_2$ (mm Hg)*		*PaO_2 (mm Hg)*	
	Begin	*End*	*Begin*	*End*	*Begin*	*End*	*Begin*	*End*
Series I								
100 mM K^+ ($n=7$)	112.4±3.1	114.7±5.7	7.40±0.01	7.40±0.01	36.4±1.5	34.7±1.1	85.3±3.0	81.8±2.8
Series II								
10^{-8} M CPA ($n=5$)	106.2±9.7	109.4±2.5	7.41±0.01	7.41±0.01	35.6±1.2	33.3±1.2	80.5±5.6	84.7±2.9
10^{-6} M CPA ($n=5$)	102.2±4.4	97.0±6.0	7.41±0.01	7.43±0.01	35.6±1.0	33.1±2.2	79.4±2.6	87.8±4.1
10^{-8} M CGS 21680 ($n=4$)	104.5±1.5	98.2±1.9	7.38±0.01	7.41±0.01	40.1±1.2	36.7±0.77	80.5±6.7	79.8±7.5

the latter phases of the 10 min application period. Electrical activity returned during the initial post-K^+ collection period, with the graded onset of high voltage, low frequency, potentials. Recovery to a pre-K^+ (low voltage, higher frequency) ECoG occurred slowly over a subsequent 10–20 min period.

Series I. Effects of high K^+ on ACh release

The initial basal level of ACh in the 10 min equilibrated cup superfusates was 27.2 ± 9.7 nM (mean ± S.E.M., $n = 14$). The effects of 3 successive high-K^+ applications on ACh efflux are presented in Fig. 1. The ACh concentrations in cup superfusates at the end of a 10 min period of K^+ application increased 3–5 fold over the immediately preceding basal levels; the increases reaching significance during the all three exposures to 100 mM K^+-CSF.

Series II. Effects of adenosine agonists on ACh efflux

Basal efflux. N^6-cyclopentyl adenosine (10^{-8} M) reduced spontaneous ACh release in the basal samples (B_2, B_3) preceding the S_2 and S_3 stimulated samples (B_1, 23.4 ± 9.4 nM; B_2, 8.7 ± 1.3 nM; B_3, 8.0 ± 0.8 nM, $n = 10$). The level in B_3 was significantly reduced ($P < 0.01$) in comparison to the equivalent basal superfusate sample in the control (Series I) animals (15.2 ± 2.1 nM, $n = 14$). Conversely, CPA (10^{-6} M) significantly ($P < 0.01$) elevated the level of ACh in B_3 (53.4 ± 17.5 nM, $n = 10$) in comparison to that in the equivalent sample in the control series. CGS 21680 (10^{-8} M) had no effect on the spontaneous efflux of ACh.

K^+-evoked efflux. Neither CPA (10^{-8} and 10^{-6} M) nor CGS 21680 (10^{-8} M) had any significant effect on K^+-evoked ACh release, although CPA (10^{-8} M) tended to increase the K^+-evoked release of ACh (Table II).

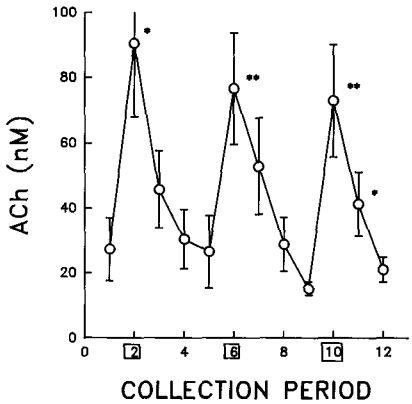

Fig. 1. Potassium-evoked release of acetylcholine into rat cerebral cortical cup superfusates. Superfusates were collected at 10 min intervals and analyzed by HPLC. Data are presented as the means (±S.E.M., $n = 14$) of superfusate ACh during three successive exposures to artificial CSF containing 100 mM K^+ applied during collection periods 2, 6 and 10 (□). There was a 20 min gap between collection periods 4 and 5 and a 10 min gap between collecting periods 8 and 9. See Materials and Methods for further details.

TABLE II

K^+-evoked efflux of ACh from the in vivo rat cerebral cortex (mean ± S.E.M.)

Drug present during S_2 & S_3	*K^+-evoked efflux (S_2/S_1)*	*K^+-evoked efflux (S_3/S_1)*
None ($n = 14$)	1.28 ± 0.38	1.47 ± 0.48
CPA 10^{-8} M ($n = 10$)	2.35 ± 0.39	2.55 ± 0.82
CPA 10^{-6} M ($n = 10$)	1.32 ± 0.12	0.99 ± 0.26
CGS 21680 10^{-8} M ($n = 8$)	1.42 ± 0.27	0.98 ± 0.18

DISCUSSION

The efflux of ACh from the in vivo cerebral cortex into artificial CSF superfusates in cortical cups has been extensively employed to study the effects of afferent pathway activation and of stimulant or depressant agents on cholinergic activity in the cerebral cortex[6,7,12,13,16,17]. To our knowledge, this is the first time a K^+-evoked release of ACh from the in vivo cerebral cortex has been described. However, the use of elevated K^+ in the perfusion medium of slices or synaptosomes is recognized as an accepted technique for evoking ACh-release[5,15].

200 μl of 100 mM K^+ artificial CSF proved to be an effective, reproducible, stimulus for eliciting ACh efflux from the rat cerebral cortex. When applied for a 10 min period, the applications of high-K^+ CSF provoked a 3–5-fold increase in ACh levels in cup superfusates. Peak ACh release occurred during the period of application of high K^+ CSF and levels returned to normal within 10–20 min of the cessation of its application. During the period of high-K^+ superfusion, the amplitude of the ECoG was initially markedly diminished, and in some instances became virtually isoelectric. Presumably as the K^+-level in the superfusates diminished with its diffusion into the underlying cortex, the ECoG started to recover with the intermittent appearance of large, low frequency waves. Electrical activity recovered slowly over a 10–20 min period following the removal of high-K^+ CSF. In those instances in which ECoG activity disappeared, it is likely that spreading depression had been initiated[10]. The ACh that appears in superfusates of the cerebral cortex is assumed to be released from the nerve terminals of both extrinsic and intrinsic cholinergic neurons[7,8,16].

Adenosine receptor agonists are known to affect ACh release from synaptosomal and slice preparations. Spignoli et al.[20] demonstrated that the adenosine receptor A_1 agonist cyclohexyl adenosine (CHA) at concentrations of 10 nM and 100 μM significantly depressed ACh release from electrically stimulated rat cerebral cortical slices, although the 10 nM concentration had the most pronounced effect. Conversely, a mixed A_1/A_2 agonist, *N*-ethylcarboxidamido adenosine (NECA), which was inactive at 10 μM, significantly increased ACh release at 100 μM. CHA (0.01 μM) and adenosine (30 μM) decreased K^+-evoked [^{3}H]ACh release from rat cerebral cortical synaptosomes, whereas on this preparation NECA was inactive at 0.1 and 1.0 μM[15]. However, Corrieri et al.[2] were unable to demonstrate an inhibitory effect of adenosine (10 μM or 10 mM) on the K^+-evoked release of ACh from guinea-pig cortical synaptosomes. Pedata et al.[15] have made the interesting point that the K^+-evoked release of ACh from cortical synaptosomes is much less sensitive to the inhibitory effects of adenosine and CHA than is the release of ACh from electrically stimulated rat cortical slices.

The A_1 adenosine receptor agonist CPA, at a concentration of 10^{-8} M, depressed spontaneous ACh release from the in vivo rat cerebral cortex. If the assumption is made that this ACh is released as a consequence of ongoing activity in cholinergic pathways in the cerebral cortex, our findings support the conclusion of Spignoli et al.[20] and Pedata et al.[15], namely that there are inhibitory A_1 receptors on cholinergic nerve terminals. In contrast, at a concentration of 10^{-6} M, CPA significantly enhanced spontaneous ACh release from the in vivo cerebral cortex. These results with CPA can be compared to those observed in experiments on ischemia-evoked release of the excitant amino acid neurotransmitters, glutamate and aspartate, from the rat cerebral cortex in which whilst CPA at 10^{-10} M depressed release, it had no apparent effect at 10^{-6} M. On the basis of these results, together with further experiments using selective A_1 and A_2 receptor antagonists, Simpson, et al.[19] suggested that ischemia-evoked excitant amino acid release was depressed by A_1 receptor activation and that co-activation of low affinity A_{2b} receptors by high concentrations of CPA reversed or blocked the A_1 mediated response. A similar response pattern appears to characterize the effects of CPA on ACh release. The high affinity A_2 receptor agonist CGS 21680 (10^{-8} M) did not affect spontaneous ACh release into cortical superfusates. We have previously shown that at this concentration which would activate A_{2a} receptors, CGS 21680 can depress γ-aminobutyric acid (GABA) release from the rat cerebral cortex[14]. Neither CPA (10^{-8} M; 10^{-6} M) nor CGS 21680 (10^{-8} M) had any effect on K^+-evoked ACh release. This result is comparable to the lack of effect of these agents on the K^+-evoked release of glutamate, aspartate, and GABA from the rat cerebral cortex (unpublished observations).

The absence of a reduction in K^+-evoked release may be explicable by the frequently observed failure of adenosine agonists to reduce Ca^{2+} entry into K^+-depolarized synaptosomes[3,9,11]. In contrast, Ca^{2+} entry into synaptosomes evoked by electrical stimulation is abolished by adenosine[4,18]. Shinozuka et al.[18] have suggested that K^+-induced depolarization may stimulate a mechanism of Ca^{2+} entry that is adenosine insensitive and different from the mechanism of Ca^{2+} entry occurring during electrical stimulation. The involvement of different Ca^{2+} channels could also explain the observation[15] that adenosine and CHA are less effective at inhibiting ACh release from K^+-depolarized synaptosomes than from electrically stimulated brain slices as well as the failure of adenosine (10 μM and 10 mM) to depress K^+-evoked release of ACh from guinea-pig cortical synaptosomes[3].

In conclusion, the present results demonstrate that applications of 100 mM K^+-containing CSF elicit a release of endogenous ACh from the rat in vivo cerebral cortex. The A_1 adenosine receptor agonist, CPA (10^{-8} M) inhibits the spontaneous, but not the K^+-evoked efflux of ACh. At a 10^{-6} M concentration, it enhances spontaneous release, but fails to alter K^+-evoked release. The A_2 receptor agonist CGS 21680 (10^{-8} M) was without effect on either spontaneous or K^+-evoked ACh release. The results suggest that K^+-evoked transmitter release may constitute an inappropriate model with which to study drug effects on the in vivo release of acetylcholine.

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