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Acetylcholine output from the ischemic rat cerebral cortex: effects of adenosine agonists

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The efflux of acetylcholine (ACh) from the ischemic rat cerebral cortex was examined using the cortical cup technique and an HPLC with electrochemical detection assay. Four vessel occlusion of the cerebral circulation caused a rapid increase in ACh efflux into the cortical superfusates, which was then sustained during the 20 min period of occlusion. Reperfusion was associated with a rapid return of ACh efflux to basal levels. The A_1 and A_2 selective adenosine receptor agonists, N^6-cyclopentyladenosine (10^{-8} and 10^{-10} M) and CGS 21680 (10^{-8}), failed to significantly alter ischemia-evoked release of ACh. Because ACh is known to enhance NMDA receptor mediated neuronal depolarization and intracellular Ca^{2+} levels, and to potentiate L-glutamate-induced neural degeneration, the present findings suggest that ACh could contribute to ischemic brain injury.

Previous studies in this laboratory have demonstrated that during a 20 min period of four vessel occlusion-induced cerebral ischemia, there is a pronounced increase in the efflux of neurotransmitter amino acids (glutamate, aspartate, γ-aminobutyric acid) into cerebral cortical superfusates[5,6]. The question, therefore, arises as to whether the efflux of other neurotransmitters, such as acetylcholine (ACh), from the cerebral cortex would be similarly affected by ischemia. An enhanced release of ACh from the ischemic hippocampus has been reported[1].

In a separate study, a focal ischemic lesion (middle cerebral artery occlusion) caused a decrease in ACh efflux from the ischemic region of rat cerebral cortices[9]. ACh efflux continued to be depressed below basal levels for 30 min following middle cerebral artery occlusion. This result appeared to be inconsistent with the observation that tissue ACh levels in the rat cortex decline during ischemia[1]; a likely consequence both of reduced synthesis and a release of ACh into the extracellular space.

An understanding of the changes in interstitial fluid ACh during cerebral ischemia has assumed new significance with recent observations that ACh potentiates responses to L-glutamate and the NMDA receptor agonist *N*-methyl-D-aspartate (NMDA) and, in particular, that ACh enhances NMDA-evoked rises in intracellular calcium[2,10]. Enhancement of excitotoxic amino acid (EAA)-evoked increases in intracellular calcium may account for the ability of ACh to potentiate glutamate-induced neurodegeneration in cultured hippocampal neurons[3].

A further evaluation of the effects of cerebral ischemia on ACh levels in the cerebral cortex, therefore, appeared to be essential for any understanding of the role that ACh might play in ischemia-evoked neuronal death. In the light of the actions of ACh on glutamate receptor-evoked neural toxicity we have evaluated the effects of an A_1 and an A_2 selective adenosine agonist on ACh release.

Male Sprague–Dawley rats (350–450 g, Charles River) were anesthetized with halothane. After insertion of a tracheal cannula, anesthesia was sustained with methoxyflurane. One femoral artery was cannulated for blood gas and pH analysis. The right and left common carotid arteries were isolated and separated

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from their accompanying nerve trunks. A length of dental floss was looped around each artery and exteriorized through the neck incision. The ends of each carotid snare were held together with adhesive tape. Following placement of the animal's head in a Narashige SH-8 non-traumatic head holder, a continuous midline incision was made along the top of the skull and extended along the dorsum of the neck. After removal of the overlying muscles the vertebral arteries were coagulated with a monopolar electrode inserted into the alar foramina of the first cervical vertebra. The neck incision was then closed with Michel wound clips. The dorsal and dorso-lateral surfaces of both cerebral hemispheres were exposed by removal of the overlying frontal and parietal bones, with a thin strip of bone left intact along the midline to protect the dorsal sagittal venous sinus. The dura mater–arachnoid complex overlying both hemispheres was reflected and oval cortical cups suspended in flexible mounting brackets were lowered onto both hemispheres, so that the frontal, parietal and occipital cortical surfaces were exposed within the cups. Each cup was filled with an artificial cerebrospinal (CSF) solution to ensure that there was no leakage of fluid from the cups. The dorsal surface of the head was then covered with 4% agar in artificial CSF to protect the exposed surfaces of the skull and stabilize the cups. A monopolar EEG lead was placed in both cups with the tip of the electrode adjacent to the cortical surface. EEGs and arterial blood pressure were recorded on a Grass Polygraph.

Artificial CSF, containing 2×10^{-5} M neostigmine bromide (a concentration which enabled us to reliably detect resting basal ACh release), 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.

Three groups of animals were involved in this study. The first group was used to study the release of ACh during ischemia. The second and third groups were used to evaluate the effects of a selective A_1 adenosine receptor agonist (N^6-cyclopentyl adenosine, CPA; 10^{-8} M) and a selective A_2 agonist (CGS 21680; 10^{-8} M) on ischemia-evoked ACh release.

The experimental protocol was as follows: two consecutive basal 10-min collection periods were succeeded by administration of drug; 20 min was then allowed for equilibration and two 10 min post-drug basal collections were obtained. Two 10 min collections were obtained during the period of ischemia, followed by four reperfusion samples after the carotid snares had been released. The onset of ischemia was evidenced by a flattening of the cortical encephalograms recorded from both hemispheres. The collected superfusate samples were immediately ejected into chilled microvials, centrifuged and stored at −20°C. High pressure liquid chromatography (HPLC) analyses of perfusate ACh content were conducted within a few hours using a Chromspher choline column and Chromsep IMER (immobilized enzyme reactor; ACh type)[7]. In this system, the ACh was reacted with the enzymes acetylcholinesterase and choline oxidase in the IMER. Acetylcholinesterase hydrolyzed the ACh to choline and acetate. The resultant choline was then oxidized to hydrogen peroxide and betaine by the choline oxidase. Hydrogen peroxide was detected with a Waters Model 464 electrochemical detector, using a platinum electrode set at a potential of +400 mV. The hydrogen peroxide corresponding to ACh was quantified by integrating the area under the peak and comparing this to a standard curve covering the range of ACh values. Statistical differences between the control group and experimental groups were determined by a one-way analysis of variance and Student–Newman–Keul's or Sheffe's test (SPSSPC statistical program). A probability < 0.05 was accepted as denoting a significant difference.

Physiological variables were monitored in all groups of animals by recording of mean arterial blood pressure (MABP) and EEG and through the determination of arterial pH and gas tensions in a basal sample

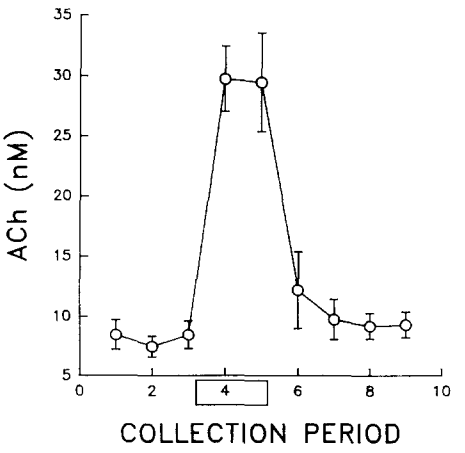

Fig. 1. Ischemia-evoked release of acetylcholine into rat cerebral cortical cup superfusates. Superfusates were collected at 10 min intervals and analyzed by HPLC. Data are presented as the means (±S.E.M., $n = 12$) of superfusate ACh. 3×10 min basal collection periods are shown, after which cerebral ischemia of 20 min duration was induced by occlusion of the carotid arteries (the vertebral arteries having previously been permanently occluded). The carotid snares were then released and four more 10 min CSF samples were collected.

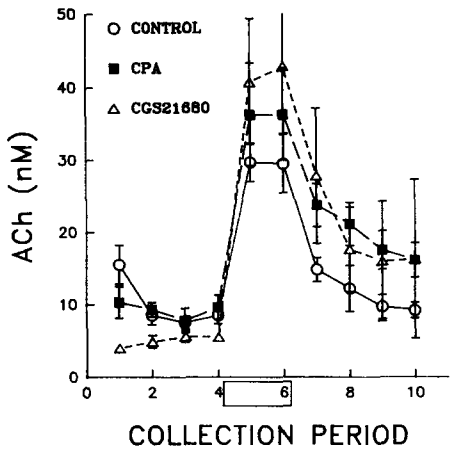

Fig. 2. CPA (10^{-8} M) and CGS 21680 (10^{-8} M) fail to alter ischemia-evoked increases in ACh release from the rat cerebral cortex.

obtained prior to the ischemic period and in a second sample obtained at the end of the reperfusion period (collection 10). No significant differences in pH, P_aCO_2, or P_aO_2 were noted between these samples in the control or treatment groups, indicating that the animals readily tolerated the period of cerebral ischemia. Occlusion of the carotid arteries consistently resulted in an isoelectric EEG during the entire ischemic period, with partial recovery occurring prior to the end of the experiment, signifying that release of the carotid ties had resulted in reperfusion.

ACh concentrations in the pre-ischemia, basal, cortical superfusates were stable at levels of 7.8 ± 1.1 nM (mean $\pm$ S.E.M.; $n = 11$). ACh output increased dramatically during the initial 10 min period of cerebral ischemia (Fig. 1), rising to 29.7 ± 2.7 nM ($P < 0.001$), and remained at this level during the second ischemic collection. Following removal of the carotid snares ACh levels in cortical superfusates declined rapidly and had returned to basal levels within 20 min of the onset of reperfusion.

As is evident in Fig. 2, administration of the adenosine A_1 receptor agonist CPA (10^{-8} M; $n = 10$) in the cortical cup superfusate failed to affect either the basal or ischemia-evoked release of ACh. CPA (10^{-10} M), tested in 8 rats, was also without significant effect on basal or ischemia-evoked release of ACh (data not presented). Whilst the A_2 selective adenosine receptor agonist, CGS 21680 (10^{-8} M; $n = 8$), tended to increase ischemia-evoked release, this effect was not significant.

A marked ($\times 4$) increase in the ACh content of rat cerebral cortical superfusates occurred during cerebral ischemia. This increase was sustained for the duration of the ischemic episodes (20 min) and ACh levels rapidly reverted to control levels after reperfusion commenced. The increase in ACh efflux is in contrast to the results of Scremin and Jenden[9], who observed a reduction in the ACh content of cerebral cortical superfusates following middle cerebral artery occlusion in the rat, but is comparable to the increase in extracellular levels observed in the ischemic hippocampus[1]. The reasons for this discrepancy may relate to the relative degrees of impairment of cortical blood flow achieved in the two series of experiments. Scremin and Jenden[9] recorded a 60% decrease in regional cortical blood flow to 47 ± 6 ml/100 g/min, a value which should have been adequate to sustain cortical viability. Unfortunately no cortical electroencephalographic recordings were taken in their experiments and it is therefore impossible to know how severely cortical functioning was affected. The four vessel occlusion method causes a severe decrease in cerebral blood flow and a confirmatory loss of cortical EEG activity was observed in each of the present experiments.

The failure of the A_1 adenosine receptor agonist (CPA) to influence ischemia-evoked ACh release was somewhat unexpected in the light of previous evidence indicating the presence of inhibitory A_1 receptors on central cholinergic nerve terminals[4,11]. However, we have previously reported an inability of CPA (10^{-8} M) to inhibit K^+-evoked release of ACh from the rat cerebral cortex[7]; a situation which may be comparable to that pertaining to ischemia-evoked release, in which extracellular K^+ levels are elevated. The A_2 selective agonist CGS 21680 (10^{-8} M) also failed to modify ischemia-evoked ACh release. Again this result is consistent with its inability to affect K^+-evoked ACh release[7]. It appears that the mechanisms underlying ACh release from the cerebral cortex during ischemia are resistant to inhibition by adenosine agonists.

In that ACh potentiates NMDA receptor-evoked neuronal depolarization[2] and NMDA receptor elicited increases in intracellular calcium[10], it is not surprising that ACh, acting on muscarinic receptors, potentiates glutamate-evoked neuronal degeneration[3]. The increases in extracellular ACh levels observed in our experiments suggest that it may enhance excitotoxic amino acid-induced neuronal destruction associated with cerebral ischemia. Blockade of muscarinic receptors may provide one approach to reducing cerebral injuries resulting from ischemic episodes.

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