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IN VIVO RELEASE OF ACETYLCHOLINE EVOKED BY BRACHIAL PLEXUS STIMULATION AND TITYUSTOXIN

ABDUL-SALAM ABDUL-GHANI, JOAQUIM COUTINHO-NETTO and HENRY F. BRADFORD
Biochemistry Department, Imperial College of Science and Technology, London SW7 2AZ, U.K.

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Abstract—The *in vivo* release of acetylcholine from the sensorimotor cortex evoked by sensory stimulation via the brachial plexus and by administration of tityustoxin has been studied using a superfusion technique. Electrical stimulation of the brachial plexus of the contralateral forelimb of anaesthetized animals evoked a significant increase in acetylcholine release from the sensorimotor cortex into the superfusion fluid. This was reversed on cessation of stimulation. Stimulation of the ipsilateral brachial plexus was without effect. Tityustoxin applied topically to the surface of the superfused area caused myoclonic jerks of the contralateral forelimb which was accompanied by an increase of 86 per cent in the rate of acetylcholine release. These effects of tityustoxin were entirely prevented by tetrodotoxin (0.1 μM) applied to the cortex surface via the superfusion cannula.

Changes in the rates of acetylcholine (ACh) release from the cerebral cortex *in vivo* have been reported to be caused by a wide range of drugs. Thus, an increase in release has been seen to follow treatment with Atropine and Scopolamine [1–8], Pentylenetetrazole [1, 3, 9–11] and picrotoxin [12]. Augmented acetylcholine release follows unilateral stimulation of the lateral geniculate body [6] and direct cortical stimulation [13]. In contrast, a decrease in acetylcholine release was observed to follow treatment with morphine [2, 14, 15], $^9\Delta$-tetrahydrocannabinol [16] and Pentobarbital [2, 17]. Most of these experiments were carried out using small cups placed on the exposed cortex to collect the released transmitter [1, 6].

Previous studies in our laboratory have shown that brachial plexus stimulation [18] and tityustoxin [19] caused a significant increase in the *in vivo* release of neurotransmitter amino acids to superfusion fluids washing the surface of the cerebral cortex [20].

This present study describes the effects of stimulation of the brachial plexus of the forelimb and the application of the depolarizing neurotoxin, tityustoxin, on the release of acetylcholine from the sensorimotor cortex *in vivo*. This toxin is a component of the venom of the yellow scorpion *Tityus serrulatus*. It is a polypeptide (molecular weight 6995 daltons) and appears to exert its effects by a depolarizing action [33].

MATERIALS AND METHODS

Superfusion method

Female hooded Rowett rats (220–250 g body wt) were used in all experiments. Anaesthesia was induced with avertine (250 mg/kg) and atropine (1.7 mg/kg) given intraperitoneally and repeated at smaller doses to maintain light anaesthesia as tested by positive corneal reflex during implantation of the cannulae. The animals were then cooled to approx. 21° (rectal temperature) in a cold room to reduce cerebral oedema during surgery. Body temperature was kept low during surgery due to anaesthetic action. After exposing the skull surface, a 4 mm diameter hole was cut in the bone immediately over the sensorimotor cortex, taking care to remove the underlying dura and arachnoid membranes without damaging the pia mater and the cortex surface. Then the sterilized cannula was fixed firmly into the hole using a dental powder–cyanoacrylic cement mixture.

The design, construction and method of implantation of the cannula (15 μl vol.; 4 mm diameter) has been described in full detail elsewhere [21]. The superfusion system is designed to prevent the delivery and collection leads from twisting whilst allowing the animal to move freely.

After implantation of the superfusion cannula the animal was returned to its cage and allowed at least 15 hr to recover before experiments were performed. All the surgery and subsequent experiments were performed in a temperature-stabilized room (24°) in which the lighting system was on a 12 hr 'lights on' and 12 hr 'lights off' cycle [20].

Superfusion fluid. This was sterilized saline (0.85% w/v) containing 1.3 mM $CaCl_2$.

Stimulation

In experiments involving sensory stimulation of the sensorimotor cortex, the nerve trunks of the brachial plexus of both forelimbs were exposed and gently clamped in small silver electrodes [18]. Electrical stimulation of the brachial plexus employed square wave pulses of 3–6 V amplitude, 1–3 mA current, 1 msec pulse duration and 2–3 Hz frequency. Seven-minute periods of stimulation were applied, and these always evoked clearly-visible muscular jerking in the ipsi-lateral forelimb. Clonic seizures were never seen to occur. Contralateral stimulation activated the cortex of the cannulated hemisphere, while ipsilateral stimulation should affect mainly the cortex of the uncannulated hemisphere.

In other experiments, tityustoxin, the purified scorpion venom toxin [22], was dissolved in sterile

saline (1.0 μM) and sterilized by filtration through a millipore filter pre-washed with albumin (1 mg/ml in sterile saline). The purified toxin was dissolved in saline and applied to the cortex via the superfusion cannula.

Tityustoxin. This is a basic polypeptide (mol. wt. 6995 daltons; pI, 8.25) which was purified from the venom of a Brazilian yellow scorpion (*Tityus serrulatus*) by the method of Coutinho-Netto and Diniz [22]. The procedure involved extraction and chromatographic separation on Sephadex G-50 and CM-Cellulose-52. The toxin was homogeneous as judged by electrophoresis in polyacrylamide gels with or without added sodium dodecyl sulphate. Only one N-terminal (L-lysine) was detected in the preparation. It showed an LD_{50} of 18 μg/kg in mice when administered intraperitoneally. The preparations were found to be relatively free of contamination by amino acids.

Determination of acetylcholine

The superfusate was continuously collected in 1-ml fractions in plastic tubes held in a fraction collector. These contained 20 μl of HCl (1.0 N), an amount sufficient to give a final pH of 3.5–4.0. They also contained 10 μl of eserine-sulphate (0.1 mg/ml) to give a final concentration of 0.01 mg/ml to prevent cholinesterase action. The fractions were each boiled at 100° for 10 min and stored in the freezer until analysis for acetylcholine which followed within 3 days after collection. The acetylcholine content was bioassayed using the guinea pig ileum preparation as described by Paton [23]. Acetylcholine perchlorate was employed as standard. A bracketing method of assay was employed in which the samples were alternated with standards selected to give a similar response. Spasmogenic substances present in the superfusate were inactivated by boiling for a few minutes in an alkaline medium. The responses were all antagonized by atropine (10^{-6} M final concentration) and destroyed by adding acetylcholine esterase to samples collected without added eserine. This characterized the signals as being due to acetylcholine and not to other substances which contract the ileum.

Tetrodotoxin. This was purchased from the Sigma Chemical Co., London.

RESULTS

Spontaneous release of acetylcholine. The rates of spontaneous release of acetylcholine from the sensorimotor cortex of both conscious and anaesthetized rats was measured. Relatively high values (7.0–7.5 ng/ml/min/cm^2) were found for conscious animals, whilst those anaesthetized, using an avertine–atropine mixture, showed a very much reduced rate of spontaneous acetylcholine release, i.e. 4.5–5.3 ng/ml/min/cm^2.

It seems that the pattern of spontaneous acetylcholine release is dependent on the state of activity of the cerebral cortex. In all subsequent described experiments animals were kept under light anaesthesia as monitored by the maintenance of a positive corneal reflex and a negative pain reflex.

Effect of brachial plexus stimulation. Stimulation of the brachial plexus contralateral to the cannulated cortex by application of square wave electrical pulses always evoked clear muscular jerking in the stimulated forelimb. This was accompanied by a significant increase (41 per cent; $P < 0.025$) in the rates of release of acetylcholine from the stimulated sensorimotor cortex of anaesthetized animals. These changes in acetylcholine release returned to control levels 10 min after cessation of the stimuli (Fig. 1). Stimulation of the brachial plexus of the forelimb ipsilateral to the hemisphere being superfused caused no change in acetylcholine release.

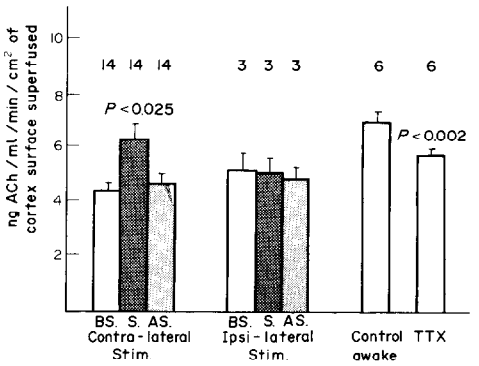

Fig. 1. *In vivo* release of acetylcholine from sensorimotor cortex at rest and during periods of sensory stimulation via the brachial plexus. Data are ACh levels in 10 min fractions. Histobars represent the mean ± S.E.M. for the number of observations indicated above them. These observations were collected from 5 animals for contralateral stimulation, 3 for ipsilateral stimulation, 3 for control awake and 3 for tetrodotoxin. Animals were lightly anaesthetized during brachial plexus stimulation. Code is as follows: BS = before stimulation; S = during stimulation; AS = after stimulation. In other experiments tetrodotoxin (TTX; 0.1 μM) was infused through the cannula using awake animals.

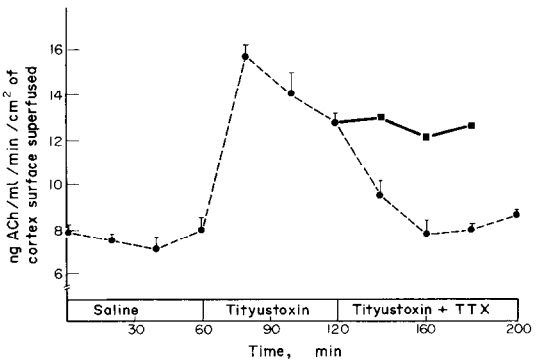

Fig. 2. *In vivo* release of acetylcholine from sensorimotor cortex at rest and during infusion of tityustoxin. Data are ACh levels in 10 min fractions. Points represent the mean ± S.E.M. for at least 9 observations from 3 animals. After an initial period of 60 min of superfusion with saline, tityustoxin was infused in saline (1.0 μM) for at least a further 60 min. A mixture of tityustoxin (1.0 μM) and tetrodotoxin (TTX; 0.1 μM) was then infused for at least a further 60 min. The solid line in the third period represents the level of ACh release when tityustoxin infusion alone was continued.

Effect of tityustoxin. Tityustoxin was introduced into the superfusion stream (1 nmole/ml superfusate) and delivered to the sensorimotor cortex of awake normally-behaved animals. Myoclonic jerks of the contralateral forelimb started 15 min later. The frequency of the limb jerks increased gradually and continued throughout the period of toxin infusion, but stopped entirely 20–30 min after washing the cortical tissue with toxin-free saline. When tetrodotoxin (0.1 μM in saline) was applied through the cannula together with tityustoxin, the myoclonic limb jerks were either decreased in frequency or entirely prevented.

The content of acetylcholine in the superfusate was sharply increased (2-fold) immediately after first applying the tityustoxin, but 20 min later during continuous infusion of the agent, the increase in acetylcholine release reduced and stabilized at 160 per cent of control levels. This additional acetylcholine release was reduced to zero when a mixture of tityustoxin (1 μM) and tetrodotoxin (0.1 μM) was introduced (Fig. 2). However, tetrodotoxin applied alone (0.1 μM in saline) reduced the locomotory activity of the conscious animals, and caused a relatively small but significant decrease of 20 per cent ($P < 0.02$) in the rates of spontaneous acetylcholine release (Fig. 1).

DISCUSSION

Spontaneous release of acetylcholine. The rates of release of acetylcholine from the sensorimotor cortex into the superfusate measured in awake animals was substantial (7.0–7.5 ng/ml/min/cm^2) and well maintained. Very similar rates of acetylcholine release were observed to occur from the visual cortex of conscious rabbits [17]. Since the two cortical areas were washed either continuously or periodically with salines containing anticholinesterase drugs, the acetylcholine released during normal resting activity could be expected to be efficiently collected since specific re-uptake mechanisms for acetylcholine are absent or ineffective [24, 37]. Thus diffusion from layers 5 and 6 of the cortex, where acetylcholine-releasing terminals are predominantly located [25, 26], should occur with little loss of acetylcholine, the flow of superfusate providing an additional trap. When cups were used to collect acetylcholine released from the cortical surface [1], it was found that after 15-min periods of collection an equilibrium was approached between the acetylcholine in the cup and the brain, due to a re-entry process. This suggests that acetylcholine leaves the cortex by simple diffusion and it would be transferred in the opposite direction if acetylcholine was added to the cup.

During anaesthesia induced by atropine–avertine (tribromoethanol), the rate of acetylcholine release was decreased by 40 per cent. This is essentially in agreement with previous reports on the actions of other kind of anaesthetics where the liberation of acetylcholine from the surface of the cortex was found to be dependent on the depth of anaesthesia [1, 7, 17, 27].

Acetylcholine release due to sensory stimulation. In view of the considerable cholinergic innervation of the cerebral cortex and its relatively high content of acetylcholine [1], the patterns of release evoked by its sensory stimulation via the nerves of the forelimb were entirely expected. Since only contralateral stimulation of these afferent fibres caused a significant increase in the release of acetylcholine (ipsilateral being without effect), a clear linkage to activation of nerve terminals in the appropriate cortical area is indicated. Any acetylcholine release due to changed blood-flow or other response to the stimulation must be small and was undetected by our collection and analysis system. Such responses would be expected to be bilateral. In addition, the cessation of the acetylcholine release-signal and the return to control levels when nerve stimulation was stopped indicates that well-controlled physiological processes had been activated.

In view of the absence of response to ipsilateral stimulation, it seems unlikely that the fibres of the corpus callosum, which connect the two homotropic cortical areas, employ acetylcholine as a neurotransmitter.

The same kind of sensory stimulation coupled with the same superfusing system caused a specific increase in the release of glutamate and GABA from rat sensorimotor cortex [18, 28]. Similar increases in acetylcholine release from the somatosensory cortex of cat have been evoked by contralateral stimulation of the forepaw or of the sciatic nerve [1]. The maximal effects were seen at lowest frequency, and ipsilateral stimulation was without effect. A small increase occurred when the sensorimotor cortex was activated by transcallosal stimulation.

Evoked release of acetylcholine from the visual cortex was detected following stimulation of the lateral geniculate body [6, 17, 29]. Using the method for spinal cord superfusion described by Morton [30], acetylcholine release was evoked by application of electrical stimulation to the cord surface [31, 32]. Newly synthesized (^{3}H)-acetylcholine has been preferentially released from the caudate nucleus and from the cerebral cortex, into the perfusate of a push–pull cannula following application of a wide range of different kinds of stimuli, i.e. electrical pulses, high-K^+ concentration and pentylenetetrazole [11], and Dudar and Szerb [7] have shown acetylcholine release due to application of 45 mM K^+ to the cortical surface.

The action of tityustoxin. Application of tityustoxin for 15 min directly onto the sensorimotor cortex produced contralateral myoclonic forelimb jerks. Similar responses were produced by other depolarizing agents, e.g. Veratrine and electrical pulses.

The release of acetylcholine from cerebral cortex *in vivo*, shown here, is complementary to *in vitro* studies where scorpion venom has been shown to increase its release from slices of rat cerebral cortex [34] and from segments of guinea-pig ileum [35].

As for sensory stimulation via the brachial plexus, tityustoxin also released GABA, glutamate and aspartate, other amino acids being unaffected [19]. Thus, though several neurotransmitter systems are activated by the two very different modes of stimulation, the responses do seem to be specific to neurotransmitters, other soluble compounds showing no tendency to efflux. These responses are not, there-

fore, likely to be due to non-specific increases in permeability of the cells of the CNS.

Since tetrodotoxin prevented both myoclonic jerks and release of acetylcholine in the present experiments, and the release of amino acid neurotransmitters in previous experiments *in vitro* [19] (this release being Ca^{2+}-dependent), activation of transmitter release mechanisms due to Ca^{2+}-entry is clearly indicated. Additional support for this interpretation comes from *in vitro* studies where depolarization evoked by both electrical stimuli and by administration of tityustoxin caused an increase in the release of acetylcholine from rat brain slices [34] and synaptosomes [36].

Our results also confirm that tityustoxin is likely to cause a nonspecific depolarization of neurones with subsequent specific release of neurotransmitters.

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