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STIMULATION OF ACETYLCHOLINE OUTPUT FROM BRAIN SLICES CAUSED BY THE IONOPHORES BrX-537A AND A 23187

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1 The effect of two ionophores, BrX-537A (Bromolasolacid) and A 23187, on acetylcholine (ACh) output from brain slices was studied.

2 The slices were prepared from rat cerebral cortex, incubated in Krebs solution containing physostigmine and ACh output determined by bioassay.

3 Both ionophores enhanced ACh output. BrX-537A exerted its maximal effect, a six fold increase, at a concentration of 1.8 μM, while A 23187 caused a three fold increase at a concentration of 58 μM.

4 When the slices were incubated in a Ca-free medium, the effect of A 23187 on ACh output was abolished while that of BrX-537A was only reduced. BrX-537A was also active when disodium edetate (EDTA) was added to the Ca-free medium.

5 The activity of BrX-537A was not affected by the presence of tetrodotoxin in the incubation medium.

6 The stimulation of ACh output elicited by KCl (25 mM) was increased further by hyoscine, but not by BrX-537A. Hyoscine however had no effect when ACh output was stimulated by BrX-537A.

7 The effect of BrX-537A on ACh output was potentiated by the addition of Mg^{2+} (9.3 mM) to the incubation medium and was reduced in a Mg-free medium.

8 It is concluded that A 23187 stimulates ACh output by transporting extracellular Ca^{2+} into cholinergic nerve endings. The effect of BrX-537A does not depend only on Ca^{2+} but also on other mechanisms.

Introduction

According to Katz & Miledi (1965) the penetration of Ca^{2+} into the prejunctional nerve terminals triggers acetylcholine (ACh) release. Ionophoric antibiotics are able to transport cations across lipid membranes and are therefore useful tools for investigation of the role of Ca^{2+} and other ions in neurotransmitter release.

In this paper the effects of two ionophores, BrX-537A (Bromolasolacid) and A 23187 on ACh release from brain slices were investigated. The two ionophores differ in many ways. A 23187 transports divalent cations but not alkali-metal cations across biological membranes (Case, Vanderkooi & Scarpa, 1974). BrX-537A shows higher affinity than the parent compound X-537A for divalent cations but is similar to it in also complexing both alkali ions and amines (Pressman, 1973).

A 23187 and X-537A stimulate the release of dopamine, noradrenaline and γ-aminobutyric acid (GABA) from synaptosomes (Holz, 1975; Colburn, Thoa & Kopin, 1976; Levi, Roberts & Raiteri, 1976), and the release of glutamate, taurine and GABA from the rat cerebral cortex *in vivo* (Collins, 1977). Kita & Van der Kloot (1976) demonstrated that X-537A increases the spontaneous and phasic quantal release of ACh at the neuromuscular juntion and Richter (1977) showed in brain slices that X-537A brings about a transient increase in ACh output, a slight stimulation of choline acetyltransferase and an inhibition of choline uptake. Electrophysiological studies demonstrated that A 23187 stimulates ACh output at the neuromuscular junction (Cull-Candy, Lundh & Thesleff, 1976; Kao, Drachman & Price, 1976). However, no information is available on the effect of A 23187

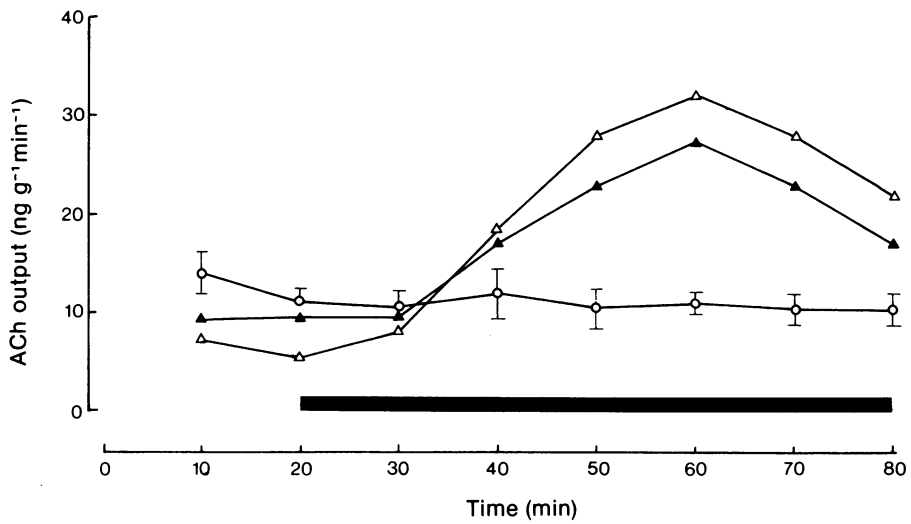

Figure 1 Effect of dimethylsulphoxide (DMSO, 25 mM, ○) $n = 10$, BrX-537A (1.8 μM, △) $n = 2$, and A 23187 (58 μM, ▲) $n = 2$ on acetylcholine (ACh) output from brain slices. Symbols represent means and vertical bars show s.e. means. The horizontal bar indicates the presence of DMSO alone or of the ionophores dissolved in DMSO.

on ACh release from brain tissue and the mechanism of action of the two ionophores on ACh release has not yet been fully clarified.

Methods

ACh output from brain slices was investigated by the method of Vizi (1972) with minor modifications. Slices were prepared from the cerebral cortex of adult male Wistar rats killed by decapitation. The slices weighing 60–100 mg and less than 0.8 mm thick were incubated in 3 ml of Krebs solution gassed with 5% CO_2 in O_2 at 37°C. The normal Krebs solution had the following composition (mM): NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.5. In some experiments modified Krebs solutions were used: KCl concentration was increased to 25 mM by replacement of a corresponding amount of NaCl; Ca-free and Mg-free solutions were obtained by omission of $CaCl_2$ and $MgSO_4$ respectively; Mg^{2+} was raised to 9.3 mM by addition of $MgCl_2$. Physostigmine sulphate 0.053 mM was added to the incubation medium.

Following a 60 min preincubation period, fresh medium was substituted every 10 min and its ACh content was determined by bioassay on the guinea-pig ileum as described by Paton & Vizi (1969). When hyoscine was added to the incubation medium the bioassay was carried out on the leech muscle (Bartolini & Pepeu, 1967). Concentrated solutions (1–10 mg/ml) of BrX-537A or A 23187 were made in dimethylsulphoxide (DMSO) and were diluted 500 to 2000 fold as necessary. The highest concentration of DMSO attained in the incubation medium was 25 mM and while this had no effect on ACh output, it was nevertheless added to all controls. BrX-537A was obtained from Roche and A 23187 from Eli Lilly Co. Tetrodotoxin was purchased from Biochemia.

Results

The mean spontaneous release of ACh from 60 cortical slices incubated in normal Krebs solution was 7.8 ± 0.4 ng g^{-1} min^{-1}. In preliminary experiments BrX-537A (42 μM) was added to the incubation medium for 10 minutes. At the end of this period no change in the ACh content of the medium was found. However, a marked but irregular increase, up to 10 times the basal output, was detected in the first and second collection period after the wash-out of the ionophore. In order to obtain reproducible results, smaller concentrations of the ionophores were therefore added to the incubation medium and their effects were studied for six collection periods. In Figure 1 the effects of BrX-537A (1.8 μM) and A 23187 (58 μM) on ACh output are shown and compared with the output in the presence of DMSO (25 mM) only. In the experiments with DMSO only the results are

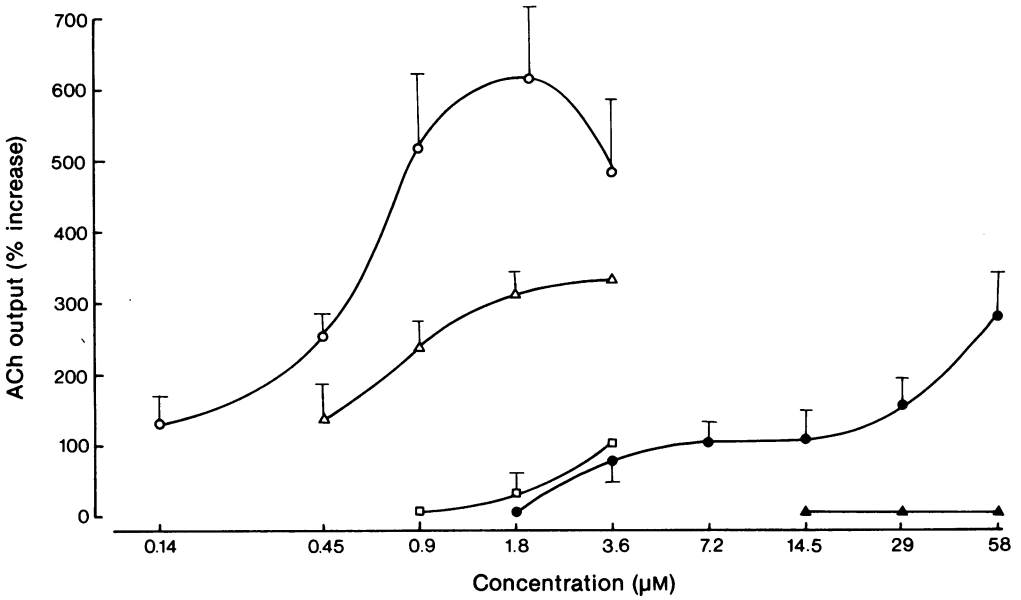

Figure 2 The relationship between concentration of ionophores in the incubation medium and peak increase in acetylcholine (ACh) output expressed as % increase over the basal output. Each symbol represents the mean of 4 to 6 observations; vertical bars indicate s.e. means. Symbols without vertical bars represent the mean of 2 observations. (○) BrX-537A in normal Krebs solution; (△) BrX-537A in Ca-free Krebs solution; (□) BrX-537A in Ca-free + EDTA (1 μM) Krebs solution; (●) A 23187 in normal Krebs solution; (▲) A 23187 in Ca-free solution.

the mean of 10 experiments while the average of 2 experiments is shown for the results with each one of the two ionophores. The experiments were chosen because the peak effect occurred in the same collection period. In 3 other experiments for each ionophore the maximum increase was observed in the second or fourth collection period after the addition of the ionophore.

The increase in ACh output elicited by the two ionophores was dose-dependent as shown in Figure 2, in which the peak increase is plotted against the log dose. Each point is the average of 4 to 6 experiments. It can be seen that BrX-537A was considerably more active than A 23187. BrX-537A 1.8 μM brought about a six fold increase of ACh output; at this concentration A 23187 had no effect and a three fold increase was obtained at 58 μM. Higher concentrations could not be used since the solution became cloudy. Figure 2 also shows that when the brain slices were incubated in Ca-free medium from the beginning of the experiment, the effect of BrX-537A on ACh was reduced; a further marked reduction was observed when disodium edetate (EDTA, 1 μM) was also added to the medium. The largest concentration of BrX-537A still exerted a small stimulatory effect on ACh output. On the other hand, A 23187 had no effect in Ca-free Krebs solution even without the addition of EDTA. The spontaneous ACh output in Ca-free Krebs solution was 8.4 ± 1.1 ng g^{-1} min^{-1} ($n = 12$).

Incubation of brain slices in the presence of tetrodotoxin (0.3 μM) did not change the increase in ACh output elicited by BrX-537A which at the concentration of 1.8 μM brought about a maximum increase of $698 \pm 180\%$ over the basal level.

Figure 3 shows that when ACh output was already stimulated by the addition of KCl 25 mM to the incubation medium, BrX-537A elicited no further increase. In contrast the large ACh output occurring in a medium with KCl 25 mM was further increased by the addition of hyoscine 0.26 μM. However, hyoscine did not enhance the ACh output already stimulated by the presence of BrX-537A.

Variations in the Mg^{2+} concentration of the incubation medium affected the spontaneous ACh output which was 5.5 ± 0.5 ng g^{-1} min^{-1} ($n = 4$) in the pres-presence of $MgSO_4$ 9.3 mM and 13.8 ± 1.3 ($n = 7$) in Mg-free medium. The difference between this value and the output in normal Krebs is statistically significant ($P < 0.01$). Figure 4 shows that the effect of BrX-537A on ACh output was greatly influenced by changes in the Mg^{2+} concentration of the incubation

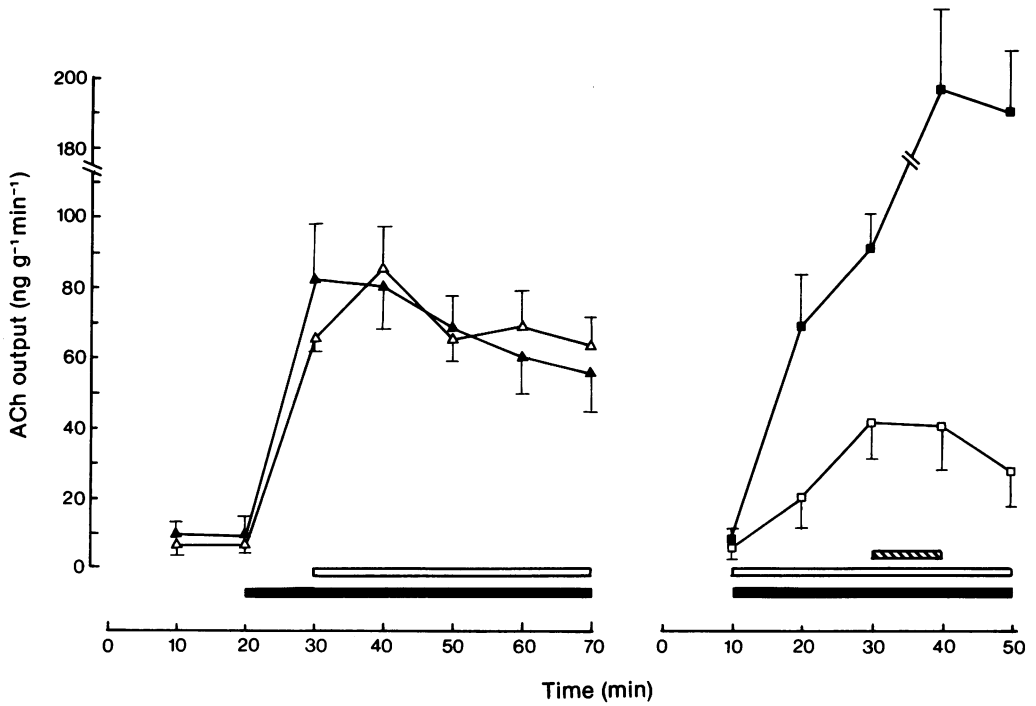

Figure 3 Acetylcholine (ACh) output from cerebral slices incubated in Krebs solutions containing KCl 25 mM (▲), KCl 25 mM + BrX-537A 0.9 mM (△), KCl 25 mM + hyoscine 0.26 μM (■), BrX-537A 1.8 mM + hyoscine 0.26 μM. (□). Symbols represent means and vertical bars show s.e. mean. The black horizontal bar indicates incubation with KCl, the open horizontal bar that with BrX-537A, the hatched bar the presence of hyoscine. Note that BrX-537A has no effect in the presence of high K^+ and hyoscine is inactive in the presence of BrX-537A.

medium. The increase in ACh output elicited by BrX-537A 1.8 μM was significantly ($P < 0.01$) larger in the presence of Mg^{2+} 9.3 mM and significantly ($P < 0.01$) smaller in the Mg-free medium than in normal Krebs solution.

Discussion

The values for spontaneous ACh output obtained in our experiments are similar to those reported by Bowers (1967) and Vizi (1972). High K^+ and hyoscine increased ACh output, which demonstrated the viability of the slices. ACh output was also substantially increased when the two ionophores BrX-537A and A 23187 were added to the incubation medium. However, the two ionophores showed some differences. First, BrX-537A was considerably more active than A 23187. A six fold increase was obtained with a concentration of BrX-537A 30 times smaller than the concentration of A 23187 required to induce a 3 fold increase. The effective doses of BrX-537A in our experiments were slightly smaller than those of the parent compound X-537A required to enhance ACh release from the neuromuscular junction (Kita & Van der Kloot, 1976) or from brain slices (Richter, 1977). The ionophore X-537A was more active than A 23187 in stimulating the output of catecholamines from the adrenal medulla (Cochrane, Douglas, Mouri & Nakazato, 1975), or of labelled noradrenaline from synaptosomes (Fairhurst, Julien & Whittaker, 1976; Colburn *et al.*, 1976). Conversely A 23187 was much more potent than X-537A when Ca transport was measured directly (Pressman, 1973; Kafka & Holz, 1976; Fairhurst *et al.*, 1976).

A second difference between the two ionophores was shown by omitting Ca^{2+} from the incubating medium. In a Ca-free medium A 23187 had no effect on ACh output. This finding confirms the previous observations that a Ca-free medium abolishes the action of A 23187 on the release of catecholamines from the adrenal medulla (Cochrane *et al.*, 1975),

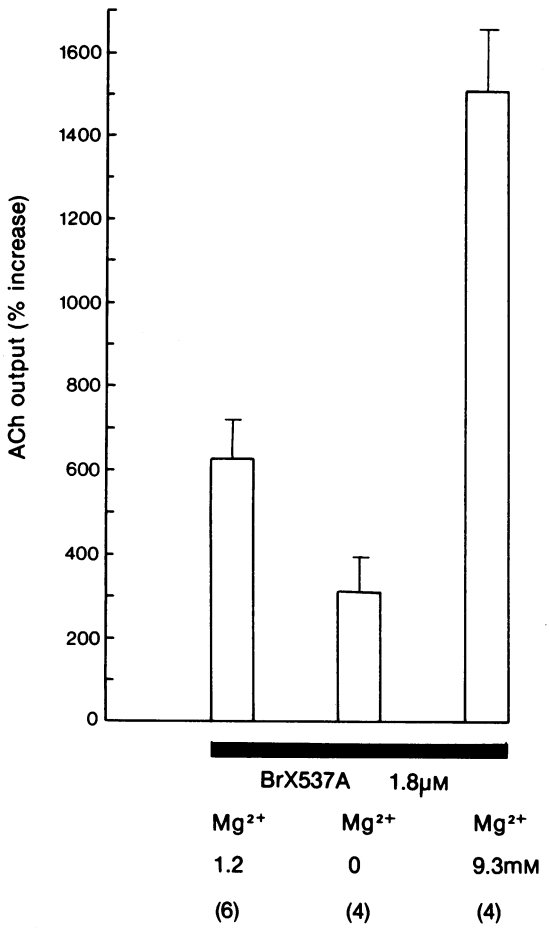

Figure 4 Effect of BrX-537A (1.8 μM) on acetylcholine (ACh) output from brain slices incubated in media with different Mg^{2+} concentrations. Each column represents the mean of the peak increases, expressed as a percentage of the respective basal output. Number of observations in parentheses. The differences between the columns are statistically significant with $P < 0.01$.

dopamine from striatal synaptosomes (Holz, 1975) and noradrenaline from the vas deferens (Thoa, Costa, Moss & Kopin, 1974). It appears therefore that the action of A 23187 on neurotransmitter release depends strictly upon the presence of extracellular Ca^{2+}.

On the other hand in a Ca-free medium the stimulatory effect of BrX-537A on ACh output was smaller than in normal Krebs solution and a further marked reduction was observed when EDTA was also added to the Ca-free medium although the stimulatory effect on ACh output was not completely abolished. Cochrane & Douglas (1974) and Kita & Van der Kloot (1975) observed that the effect of X-537A on histamine extrusion from mast cells and ACh release at a neuromuscular junction were abolished by the addition of a Ca-chelator to the Ca-free medium. Under the same conditions the stimulation of catecholamine release from perfused adrenal glands by X-537A, although strongly reduced, was not totally abolished (Cochrane *et al.*, 1975). However, according to Holz (1975) and Richter (1977), output stimulation of dopamine from synaptosomes and of ACh from brain slices by X-537A is not affected by the lack of Ca^{2+} and the presence of a Ca-chelator in the incubation medium. The dependence on Ca^{2+} of the action of BrX-537A and its parent compound on neurotransmitter release appears to change with both the experimental conditions and the dose. They apparently stimulate the release by two mechanisms, one of which does not require Ca^{2+} and the possibility of a partial depolarization should be considered (Devore & Nastuck, 1975).

Gomez, Dai & Dinitz (1973) found that tityustoxin, a scorpion venom, stimulated ACh output from brain slices and that this effect was blocked by the addition of tetrodotoxin, a well known inhibitor of the inward fast Na^+ channels (Narahashi, Moore & Scott, 1964). We found that the addition of tetrodotoxin to the incubation medium did not modify the increase in ACh output caused by BrX-537A. This finding confirms the observation of Devore & Nastuk (1975) that on the frog skeletal muscle, X-537A does not interact with the voltage-dependent Na^+ channels which are inhibited by tetrodotoxin.

Hyoscine and other anti-acetylcholine drugs enhance ACh output from brain slices only when they are depolarized by incubation in high K^+ (Bertels-Meeuws & Polak, 1969). Since hyoscine had no effect in the presence of BrX-537A, a depolarization of the brain slices by the ionophore seems unlikely, a conclusion also supported by the tetrodotoxin experiments. BrX-537A on the other hand did not enhance K^+-stimulated ACh output. According to Gerhards, Röttcher & Straub (1964a) the K^+-stimulated ACh release is also Ca-dependent. However, in the isolated intestine of the guinea-pig the maximum effect was observed with a Ca^{2+} concentration of 2.2mM and since in our experiments the Ca^{2+} concentration was 2.5 mM the stimulation was therefore already maximal.

Gerhards, Röttcher & Straub (1964b) showed that in the guinea-pig isolated ileum the addition of Mg^{2+} causes a progressive increase in spontaneous ACh liberation, accompanied by an increased synthesis. A similar observation was made by Dettbarn & Rosenberg (1966) in lobster isolated nerves. However, Mg^{2+} depresses the ACh release and synthesis stimulated by high K^+ concentration in the isolated ileum (Ger-

hards *et al.*, 1964a) and in brain slices (Molenaar & Polak, 1970). In our experiments an increase in Mg^{2+} concentration in the incubation medium had little effect on spontaneous ACh output, but potentiated the increase in ACh release elicited by BrX-537A. Conversely in a Mg-free medium the spontaneous output rose and the effect of BrX-537A was significantly smaller. It appears therefore that the effects of BrX-537A and K^+ on ACh output have different ion specificities. Whether the changes in Mg^{2+} concentration influence the releasing effect of BrX-537A because Mg^{2+} is transported inside the nerve endings or because they modify the ACh stores acted upon by the ionophore is matter for future investigation.

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