J. Physiol. (1961), **156**, *pp.* 515–522
With 3 text-figures
Printed in Great Britain

THE STIMULANT EFFECT OF BARIUM ON THE RELEASE OF ACETYLCHOLINE FROM THE SUPERIOR CERVICAL GANGLION

BY W. W. DOUGLAS, D. W. LYWOOD* AND R. W. STRAUB†
From the Department of Pharmacology, Albert Einstein College of Medicine, Yeshiva University, New York 61, *N.Y., U.S.A.*

(*Received* 21 *November* 1960)

Calcium and magnesium are known to influence the quantity of acetylcholine (ACh) that is released by impulses in cholinergic nerves. Thus the output of acetylcholine from the superior cervical ganglion during stimulation of the preganglionic trunk is reduced when the perfusion medium is deficient in calcium (Harvey & MacIntosh, 1940) or when it contains excess magnesium, and is increased when the medium contains calcium in excess of the usual amount (Hutter & Kostial, 1954); and there is electrophysiological evidence indicating that these two ions have similar antagonistic effects on the release of acetylcholine from motor nerve endings in skeletal muscle (del Castillo & Katz, 1956).

The present paper offers evidence that barium, a divalent ion closely related to calcium and magnesium, also influences acetylcholine release from cholinergic nerves. A preliminary account of the findings was presented at the meeting of the Physiological Society in Lund last July (Douglas, Lywood & Straub, 1960).

METHODS

Preparation. Cats were anaesthetized with chloralose, 80 mg/kg, I.V., following induction with ethylchloride and ether. One or other superior cervical ganglion was prepared for perfusion after the method of Kibjakow (1933) with the modifications described by Perry (1953). The whole post-ganglionic trunk was included in the ligature embracing the nervous structures cranial to the ganglion, i.e. nerves IX, X, XI, and XII, to prevent leakage of perfusion fluid. The reservoirs containing the perfusion fluids were kept in a water-bath at 38° C. When one fluid was substituted for another, the dead space of the perfusion system was rapidly flushed through a side arm in the inflow cannula in the common carotid artery, and the common carotid artery itself flushed through a second cannula inserted into the lingual artery.

Stimulation. The preganglionic trunk was separated from the vagus and stimulated through bare platinum electrodes with pulses of 0·5 msec duration delivered through a stimulus

* Present address: National Institute for Medical Research, Mill Hill, London, N.W.7.
† Present address: Physiological Institute, Robert-Koch Strasse, Cologne, Germany.

isolation unit. Biphasic pulses were used to avoid polarization (action potential records showed that such stimuli did not set up repetitive responses). A liquid paraffin pool bathed the preparation and the nerve was not moved on the electrodes throughout the experiment. The frequency of stimulation was 2/sec except where otherwise noted. Occasionally continuous stimulation was employed, but since ACh output falls off rapidly with such stimulation (see MacIntosh, 1959) short periods of stimulation were usually alternated with similar periods of rest. This latter procedure, which was previously adopted by Hutter & Kostial (1954), led to the release of approximately equal amounts of ACh in successive periods of stimulation and allowed experimentally induced variations in ACh output to be more easily examined. The usual procedure was to stimulate for 5 min and to collect all the effluent during this period and the next 2 min. In this way a 'stimulation sample' was obtained containing all the ACh released by the stimulus without any being carried over into the next sample as a consequence of dead space in the collecting system. Then followed a period of 5 min during which a 'resting sample' of perfusate was collected in the absence of stimulation. This cycle was repeated as often as necessary.

Solutions. Four different perfusion fluids were used. (1) Locke's solution contained (mM): NaCl 154, KCl 5·6, $CaCl_2$ 2·2, Na_2HPO_4 2·15, NaH_2PO_4 0·85, glucose 10·0. (2) Locke's solution with barium was as (1) except that 6 mM $BaCl_2$ was added. (3) Barium-substituted Locke's solution was as (1) except that $CaCl_2$ was replaced by an equimolar amount of $BaCl_2$ (i.e. 2·2 mM). (4) Calcium-free Locke's solution was as (1) except that $CaCl_2$ was omitted. All perfusions contained 10^{-5} g eserine sulphate/ml. and were equilibrated with pure O_2. Their pH was close to 7·0.

Precipitation of barium. Before testing the ACh content of the barium-containing perfusates, barium was precipitated by the addition of Na_2SO_4 in amounts sufficient to leave an excess of sulphate of about 10 mM; the $BaSO_4$ thus precipitated was removed by filtration through a sintered glass filter. Separate measurements showed that ACh added to solutions which were treated in this way could be quantitatively recovered, and that any remaining traces of barium or the excess sulphate did not cause any detectable effect on the cat's blood pressure in the amounts injected during the assays.

Assay. ACh was assayed by its depressor effect on the systemic blood pressure of the eviscerated cat under chloralose (MacIntosh & Perry, 1950). Blood-pressure records were made with a pressure transducer and ink-writing oscillograph. Sample tests showed that the depressor effects were abolished by atropine sulphate 0·5 mg/kg I.V., and that no depressor activity appeared in the samples when eserine sulphate was omitted from the perfusion fluid.

RESULTS

Locke's solution with barium

The addition of 6 mM of barium to Locke's solution caused a striking increase in the amount of ACh liberated by preganglionic stimulation in each of four experiments. All these were performed in the manner illustrated in Fig. 1; thus, perfusion was begun with Locke's solution and four control samples of effluent were collected. The first and third of these were obtained in the absence of nerve stimulation; the second and fourth during preganglionic stimulation at 2 shocks/sec. Immediately after the second period of stimulation (i.e. the fourth sample) perfusion with Locke's solution was suspended and perfusion began with Locke's solution with barium. Again four samples were collected, samples 5 and 7 in the absence of stimulation and 6 and 8 during stimulation. Finally, perfusion was

resumed with Locke's solution and two more resting samples (9 and 11) and two more stimulation samples (10 and 12) were obtained.

In all these experiments the ACh content of the samples collected during stimulation in the presence of barium (samples 6 and 8) exceeded that obtained in Locke's solution alone. In three of the four experiments the effect was much more pronounced in the first of these periods of stimulation in the presence of barium (as in Fig. 1); but in the fourth experiment

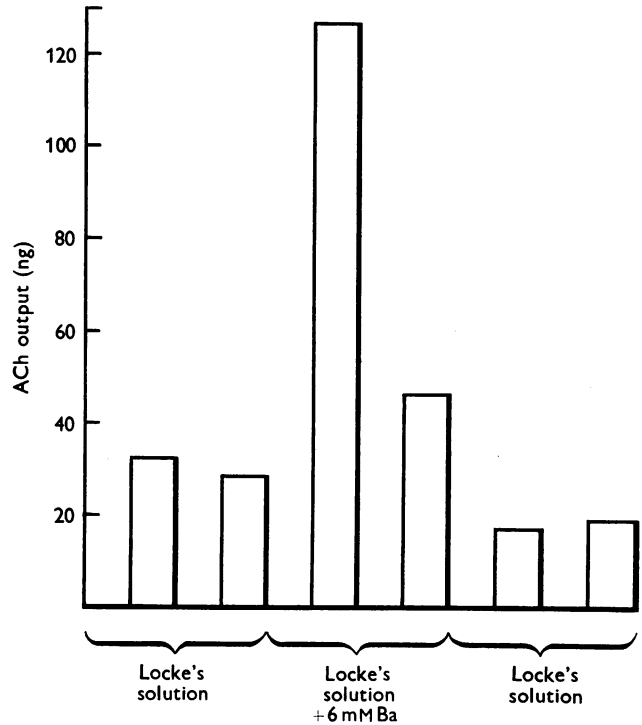

Fig. 1. Increased ACh output caused by adding 6 mM of barium to Locke's solution. Each block represents ACh output from the perfused superior cervical ganglion for a 5 min period of preganglionic stimulation at 2/sec.

the two samples obtained during stimulation in barium were equally active. Barium approximately doubled ACh output in three of these experiments and caused about a fourfold increase in another (Fig. 1).

The samples obtained in the intervals between periods of stimulation in Locke's solution usually contained no ACh detectable by the assay method used. Occasionally, when the assay cat was particularly sensitive, a few nanograms of ACh were detected in these 'resting samples'. No obvious increase in this 'resting output' of ACh occurred during the first period after switching from Locke's solution to Locke's solution with barium

(sample 5) but in one experiment the samples collected following stimulation in the presence of barium contained more ACh than the control resting samples collected in Locke's solution: the amount was too small to be assayed accurately. It was clear, however, from the assays of these 'resting samples' that any effect of barium on ACh output in the absence of stimulation could not account for the large output which accompanied preganglionic stimulation.

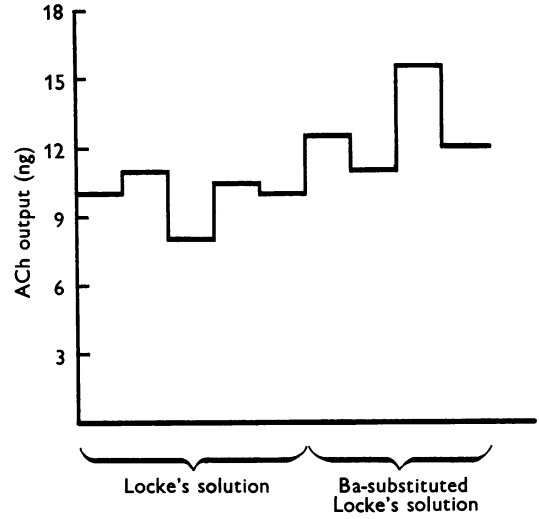

Fig. 2. Maintenance of ACh output on substituting barium for calcium. The values plotted represent the ACh output in successive 5 min periods from the perfused superior cervical ganglion during continuous preganglionic stimulation at 1/sec.

Barium-substituted Locke's solution

When the preganglionic trunk was stimulated continuously at 1/sec while the ganglion was perfused with Locke's solution, the output of ACh was low but well sustained. If the Locke's solution were changed for another, identical in all respects except that calcium was replaced by an equimolar amount of barium (barium-substituted Locke's solution), ACh output was maintained (Fig. 2). Calcium-free solutions do not support ACh output in this way (Harvey & MacIntosh, 1940; and below) and it therefore appeared that barium could be substituted for calcium in these conditions. This possibility was more rigorously tested in experiments in which calcium deprivation (and a fall in ACh output) was established before testing the effect of barium. In such experiments the ganglion was first perfused with Locke's solution. Then this perfusion was changed to calcium-free Locke's solution and ACh output was shown to fall. Thereafter perfusion was continued with barium-substituted Locke's

solution, and finally again with Locke's solution. In each of these experiments ACh output fell to very low values with calcium-free Locke's solution. But soon after beginning with barium-substituted Locke's solution ACh output recovered to levels similar to those found in Locke's solution (Fig. 3). No ACh was detected in the samples obtained during perfusion with barium-substituted Locke's solution in the absence of stimulation.

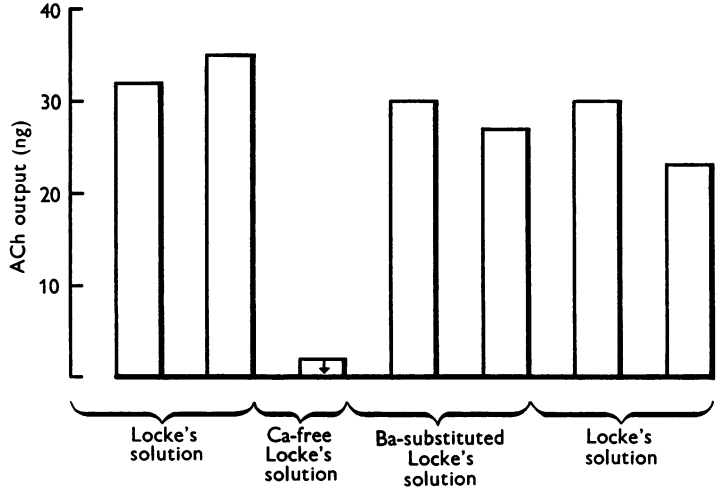

Fig. 3. The restorative effect of barium on ACh output depressed by calcium deprivation. Each block represents ACh output from the perfused superior cervical ganglion for a 5 min period of preganglionic stimulation at 2/sec. Note: no output of ACh in the absence of calcium was detected; the value plotted gives an upper estimate based on the sensitivity of the assay.

DISCUSSION

The effect of barium at the superior cervical ganglion resembles that of calcium, in that when added to Locke's solution it augments ACh output and when added to calcium-free Locke's solution it restores ACh output. Although calcium has an obviously important role in controlling ACh release its mode of action is obscure. Hodgkin & Keynes (1957) have demonstrated that there is an increased entry of calcium ions into squid nerve during activity, and that this increases with increasing extracellular calcium-ion concentration, as does the efflux of ACh from the ganglion. They have also found that this influx of calcium is depressed by magnesium ions, as is the efflux of ACh from the ganglion. Both they, and Birks & MacIntosh (1957) have postulated that calcium ions release ACh by entering the nerve terminals during the impulse and disrupting the ACh-retaining structures. It seems probable that barium acts in the same way. Barium

is not only closely related to calcium in its chemical properties, but also appears to penetrate axons during the impulse; thus Greengard & Straub (1959) have observed action potentials in mammalian B and C fibres maintained in isotonic barium chloride, and have concluded that the inward current is carried by barium ions.

The hypothesis that barium acts in much the same way as calcium on what we might loosely refer to as the 'calcium receptor for ACh release' readily explains the two principal observations in our experiments; the stimulant effect of barium on ACh release when added to Locke's solution, and its restorative effect on ACh output when added to calcium-free Locke's solution. The hypothesis, however, does not adequately account for such large increases in ACh output as shown in Fig. 1, for in Hutter & Kostials' (1954) experiments the addition of 6–14 mM of calcium no more than doubled ACh output. This would suggest that barium has some additional action. It has been known for some time that barium can lead to repetitive responses from nerve terminals (Dun & Feng, 1940) and axons (Lorente de Nó & Feng, 1946) and this might obviously increase ACh output. Alternatively, barium may influence ACh output by altering the shape of preganglionic action potentials, for barium is known to prolong the falling phase (negative after-potential) of the action potential in muscles (Fatt & Ginsborg, 1958) and in nerves (Lorente de Nó & Feng, 1946), including mammalian B and C fibres (Greengard & Straub, 1959); and there is electrophysiological evidence indicating that when the action potential is thus increased (as for example by passing hyperpolarizing currents through the nerve terminals) the output of 'transmitter' is increased (del Castillo & Katz, 1956).

Whatever the explanation, it is clear that barium increases the amount of ACh released by preganglionic stimulation, and it is of interest to relate this to some of its known pharmacological actions. Although the best known action of barium is its direct spasmogenic effect on smooth muscle, Ambache (1946) and Feldberg (1951) obtained evidence that it could also excite ganglion cells in the intestine; and Ambache (1949) showed that barium excited ganglion cells when injected into the superior cervical ganglion. These effects of barium in the intestine or superior cervical ganglion were depressed by hexamethonium or nicotine. It is possible that some of the ganglion-stimulating effect of barium is due to its increasing ACh release from active presynaptic terminals. This would certainly account for the inhibitory effects of nicotine and hexamethonium. It must be mentioned, however, that Ambache (1949) was able to obtain stimulant effects of barium on the superior cervical ganglion after chronic degeneration of preganglionic endings, so that barium seems to be able to stimulate ganglion cells directly. Barium is also known to have an

eserine-like effect at the neuromuscular junction (Feng, 1937), and it will be of interest to see whether this too is attributable to barium increasing ACh output.

SUMMARY

1. Experiments have been made on the effect of barium on the release of ACh from the perfused superior cervical ganglion of the cat.

2. The addition of 6 mM of barium to the Locke's solution used to perfuse the ganglion increased the amount of ACh released by preganglionic stimulation about twofold or more.

3. When calcium was absent from the perfusion fluid, ACh output could be sustained or restored by the addition of 2 mM barium.

4. It is suggested that barium may act in part by participating in the calcium-receptive mechanisms for ACh release, and in part by altering the action potentials of preganglionic nerves.

This investigation was supported by research grants (B-1093 and B-1319) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service.

REFERENCES

AMBACHE, N. (1946). Interaction of drugs and the effect of cooling on the isolated mammalian intestine. *J. Physiol.* **104**, 266–287.

AMBACHE, N. (1949). The nicotinic action of substances supposed to be purely smooth-muscle stimulating. (B) Effect of $BaCl_2$ and pilocarpine on the superior cervical ganglion. *J. Physiol.* **110**, 164–172.

BIRKS, R. I. & MACINTOSH, F. C. (1957). Acetylcholine metabolism at nerve endings. *Brit. Med. Bull.* **13**, 157–161.

DEL CASTILLO, J. & KATZ, B. (1956). Biophysical aspects of neuro-muscular transmission. *Progr. Biophys.* **6**, 122–170.

DOUGLAS, W. W., LYWOOD, D. W. & STRAUB, R. W. (1960). The effect of barium on the release of acetylcholine from the cat's superior cervical ganglion. *J. Physiol.* **154**, 39–40 *P*.

DUN, F. T. & FENG, T. P. (1940). Studies on the neuro-muscular junction. XX. The site of origin of the junctional after discharge in muscles treated with guanidine, barium or eserine. *Chin. J. Physiol.* **15**, 433–444.

FATT, P. & GINSBORG, B. L. (1958). The ionic requirements for production of action potentials in crustacean muscle fibres. *J. Physiol.* **142**, 516–543.

FELDBERG, W. (1951). Effects of ganglion blocking substances on the small intestine. *J. Physiol.* **112**, 177–196.

FENG, T. P. (1937). Studies on the neuromuscular junction. VII. The eserine-like effects of barium on motor nerve endings. *Chin. J. Physiol.* **12**, 177–195.

GREENGARD, P. & STRAUB, R. W. (1959). Restoration by barium of action potentials in sodium-deprived mammalian B and C fibres. *J. Physiol.* **145**, 562–569.

HARVEY, A. M. & MACINTOSH, F. C. (1940). Calcium and synaptic transmission in a sympathetic ganglion. *J. Physiol.* **97**, 408–416.

HODGKIN, A. L. & KEYNES, R. D. (1957). Movements of labelled calcium in squid giant axons. *J. Physiol.* **138**, 253–281.

HUTTER, O. F. & KOSTIAL, K. (1954). Effect of magnesium and calcium ions on the release of acetylcholine. *J. Physiol.* **124**, 234–241.

KIBJAKOW, A. W. (1933). Über humorale Übertragung der Erregung von einem Neuron auf das Andere. *Pflüg. Arch. ges. Physiol.* **232**, 432–443.

LORENTE DE NÓ, R. & FENG, T. P. (1946). Analysis of effect of barium upon nerve with particular reference to rhythmic activity. *J. cell. comp. Physiol.* **28**, 397–464.

MACINTOSH, F. C. (1959). Formation, storage and release of acetylcholine at nerve endings. *Canad. J. Biochem. Physiol.* **37**, 343–356.

MACINTOSH, F. C. & PERRY, W. L. M. (1950). Biological estimation of acetylcholine. *Methods med. Res.* **3**, 78–92.

PERRY, W. L. M. (1953). Acetylcholine release in the cat's superior cervical ganglion. *J. Physiol.* **119**, 439–454.