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THE EFFECT OF THE IONOPHORES X-537A AND A23187 ON THE NORADRENALINE OUTPUT FROM PERIPHERAL ADRENERGIC NEURONES IN THE PRESENCE OF VARIOUS DIVALENT CATIONS

S. ITO, Y. NAKAZATO & A. OHGA

Department of Pharmacology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan

1 The effects of the ionophores, X-537A and A23187 on the noradrenaline output from peripheral adrenergic neurones of isolated vas deferens of the guinea-pig were investigated in the presence of various divalent cations.

2 X-537A (17 μM) caused an increase in the noradrenaline output in the presence of barium, calcium and strontium. The effectiveness of the cations was Ba > Ca ≥ Sr.

3 In the absence of calcium and in the presence of ethyleneglycol-bis(2-aminoethylether)-N,N,N',N',-tetraacetic acid (EGTA, 1 mM), the response was reduced by about 50% of that obtained in the presence of calcium.

4 Calcium was the most effective cation in stimulating noradrenaline output when reintroduced after pretreatment with A23187 (191 μM). The response increased in an almost linear fashion with the concentration of calcium 1 mM to 10 mM.

5 Excess magnesium (20 mM) reduced the response induced by X-537A in the presence of barium. However, it was without effect on the response produced by reintroduction of calcium after pretreatment with A23187.

6 The response induced by X-537A in the presence of barium increased with an increase in the concentration of external sodium from 25 mM to 143 mM.

7 It is suggested that X-537A may cause an increase in the noradrenaline output by depolarization as well as by transferring cations as an ionophore. On the other hand, A23187 may produce an increase in the noradrenaline output, transferring calcium across the membrane as a specific calcium ionophore.

Introduction

Calcium is critically involved in stimulus-secretion coupling in various secretory cells (Douglas, 1968; Rubin, 1970; Miledi, 1973). For example, electrical stimulation or depolarizing agents such as excess potassium require calcium to induce catecholamine release from adrenergic nerve terminals (Kirpekar & Misu, 1967; Bennett & Florin, 1975; Thoa, Wooten, Axelrod & Kopin, 1975; Nakazato, Toyosawa & Ohga, 1975).

Calcium ionophores, X-537A and A23187, have been extensively used to investigate the role of calcium in a variety of secretory systems (McLaughlin & Eisenberg, 1975) including transmitter release from the adrenergic (Thoa, Costa, Moss & Kopin, 1974) as well as cholinergic (Kita & Van der Kloot, 1976; Ito & Miledi, 1977) nerve terminals. However, these ionophores were found to form lipophilic complexes not only with calcium but also with various other cations such as barium and strontium and to transport them across lipid bilayer membranes (Pressman, 1973; Célis, Estrada-O. & Montal, 1974).

Kita & Van der Kloot (1976) found that X-537A caused transmitter release from frog motor nerve terminals in the presence of various divalent cations and suggested that the effect of X-537A was dependent on the ability to carry the divalent cations. However, it has been reported that X-537A had an ability to cause a substantial depolarization of striated muscle membranes (Cochrane & Douglas, 1975; Devore & Nastuk, 1975; Kita & Van der Kloot, 1976). If X-537A produces depolarization of the nerve terminal membranes, then the transmitter release could be evoked independently of the effects arising from its action as a divalent cation ionophore. We now have

another ionophore A23187 which is generally believed to be a more specific 'Ca-ionophore' (Pressman, 1973; Pfeiffer, Reed & Lardy, 1974).

The purpose of the present experiments was to compare the effects of X-537A and A23187 on the noradrenaline output from the peripheral adrenergic neurones of the isolated vas deferens of the guinea-pig in the presence of various divalent cations. The mechanisms of action of both ionophores are discussed.

Methods

Male guinea-pigs weighing between 450–650 g were stunned and bled to death. Vasa deferentia were isolated, fixed on the tip of fine metal wires and immersed in 1 ml of incubation medium. Media containing bicarbonate and phosphate buffers were continuously bubbled with 5% CO_2 in O_2 or 100% O_2 and the temperature was maintained at 37°C in a water bath. The pH was approximately 7.3.

The incubation was performed in the following way. The vasa deferentia were preincubated with Krebs or Ca-free Krebs solution for 40 min during which the solution was changed at 10 min intervals. This allowed the spontaneous release of noradrenaline to reach a low, steady level. The tissue was then transferred to the appropriate test solution and the medium changed at 20 min intervals throughout the incubation. The medium obtained after each 20 min incubation was acidified with concentrated perchloric acid and stored in the cold until centrifugation.

The principal incubation medium was Krebs solution of the following composition (mM); NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 10. For barium- or strontium-substituted Krebs solution, $CaCl_2$ was replaced with 2.5 mM of $BaCl_2$ or $SrCl_2$. In some experiments, $NaHCO_3$ and KH_2PO_4 were replaced with 2.5 mM Tris aminomethane buffer. The excess magnesium solution was prepared by increasing the concentration of $MgCl_2$ from 1.2 to 20 mM without adjusting the tonicity. For Ca-free solution, $CaCl_2$ was omitted and sometimes ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (1 mM) was added. Phenoxybenzamine (5×10^{-5} M) was added to all incubation media to inhibit the uptake of catecholamine.

Incubation media containing the ionophores X-537A or A23187 were prepared from stock solutions of the drugs in dimethylsulphoxide (DMSO) of which the final concentration was kept constant at 0.1%.

Following incubation, the acidified media were centrifuged at 25,000 *g* at 5°C for 10 minutes. The clear supernatants were then transferred to small test tubes and stored on ice until assay. The assay was performed by the fluorometric method of Anton & Sayre (1962).

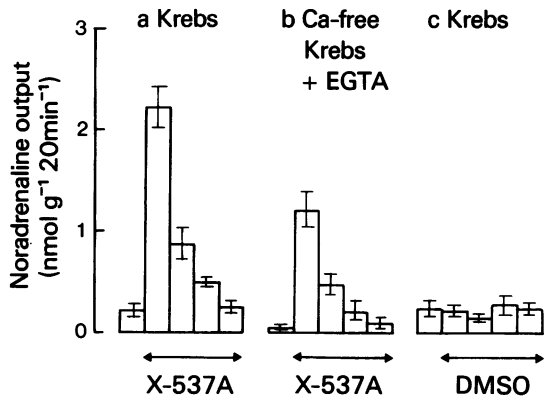

Figure 1 Stimulant effect of the ionophore X-537A on noradrenaline output from isolated vas deferens of guinea-pig in (a) the presence or (b) absence of calcium (2.5 mM). In (a) and (b) X-537A (17 μM) and in (c) the solvent, dimethylsulphoxide (0.1%) were present for the periods indicated by horizontal arrows. The incubation media are shown above the records. EGTA (1 mM) was added to Ca-free Krebs solution. Columns in this and following figures show mean noradrenaline output in successive 20 min incubation periods of five (a) and four (b and c) experiments, respectively. Vertical lines show s.e. mean.

Results

Effects of X-537A in the presence of calcium, barium and strontium

When vasa deferentia were exposed to X-537A (17 μM) in Krebs solution, the output of noradrenaline was increased to 2.23 nmol/gram. Noradrenaline output then gradually declined to the resting level, taking 40 to 60 min in spite of the presence of X-537A (Figure 1a). The effect of X-537A was decreased somewhat by exposure of the preparation to Ca-free Krebs solution for more than 1 h before the introduction of X-537A, but the difference in noradrenaline output from that obtained in the presence of calcium was insignificant. These results agree with the finding that noradrenaline output induced by X-537A remained unaltered when calcium was omitted from the media (Thoa *et al.*, 1974). However, when the preparation was exposed to Ca-free media containing EGTA (1 mM), the response induced by X-537A was decreased to 54% of that in the presence of calcium ($P < 0.005$) (Figure 1b). Addition of the solvent, DMSO (0.1%),

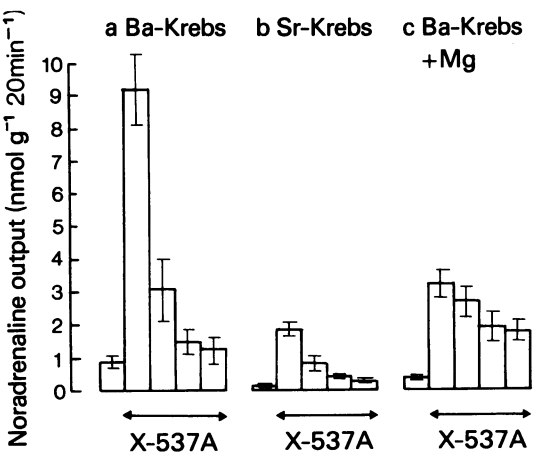

Figure 2 Stimulant effect of X-537A on noradrenaline output in the presence of (a) barium (2.5 mM) and (b) strontium (2.5 mM) and (c) the inhibitory effect of excess magnesium in Ba-Krebs solution. X-537A (17 μM) was present for the periods indicated by horizontal arrows. In (c), $MgCl_2$ (20 mM) was present. The incubation media are shown above the records. Columns are the mean of six (a and c) and four (b) experiments, respectively. Vertical lines show s.e. mean.

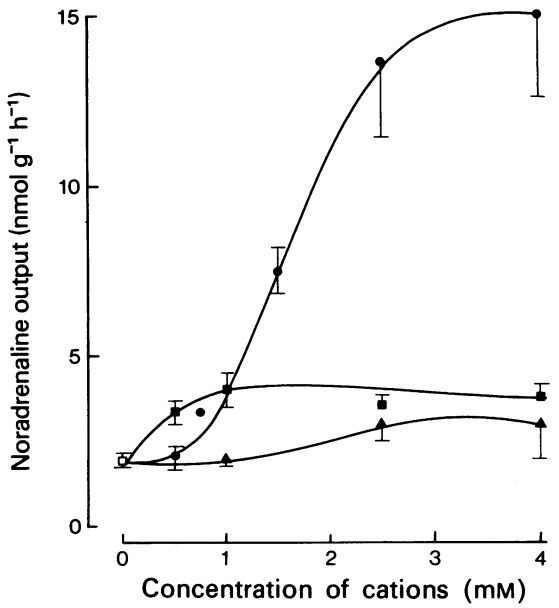

Figure 3 Relation between the effect of X-537A (17 μM) and the concentration of divalent cations, barium (●), calcium (■) and strontium (▲). Open squares (□) indicate the response induced by X-537A in the absence of calcium and the presence of EGTA (1 mM). Ordinate scale is the noradrenaline output per hour. Abscissa scale is the concentration of the cations. Symbols represent the sum of noradrenaline output (mean) of the first three 20 min incubation periods from three to five experiments; there were only two experiments with barium 0.75 mM. Vertical lines show s.e. mean.

alone had no effect on noradrenaline output as shown in Figure 1c. In Ba-Krebs or Sr-Krebs solutions, it was also without effect.

X-537A has much higher affinity for forming complexes with barium and strontium than with calcium (Pressman, 1973) and produced much larger increases in cholinergic transmitter release in the presence of barium and strontium than in the presence of calcium (Kita & Van der Kloot, 1976).

When calcium was replaced with either barium or strontium in the present experiments, X-537A also produced an increase in noradrenaline output. The response was greatest in the presence of barium, being about four times that obtained in the presence of calcium (Figure 2a). On the other hand, in the presence of strontium, the response was about 84% of that obtained in the presence of calcium and there was no statistical difference from that obtained in Ca-free Krebs solution containing EGTA (1 mM) (Figure 2b).

Relation between the effect of X-537A and the concentrations of the divalent cations

Dose-response relationships between the effect of X-537A and the concentration of divalent cations were obtained by plotting the amount of noradrenaline released during the first hour against the ion concentration (0.5 to 4 mM). The clearest relationship was observed in the presence of barium. As shown in Figure 3, the output of noradrenaline induced by X-537A increased with increasing concentrations of barium. On the other hand, in media containing either calcium or strontium, there was no comparable dose-response relationship (Figure 3). At the low range of the cation concentration, the responses were larger in the presence of calcium than in the presence of strontium, but they were fairly close at 2.5 mM and 4 mM.

Effects of magnesium and sodium on the noradrenaline output induced by X-537A in Ba-Krebs solution

X-537A was found to cause substantial depolarization of striated muscle fibre (Cochrane & Douglas, 1975; Devore & Nastuk, 1975; Kita & Van der Kloot, 1976). Thus the effect of X-537A on the noradrenaline output could result from a depolarization-induced influx of divalent cations as has been suggested in other

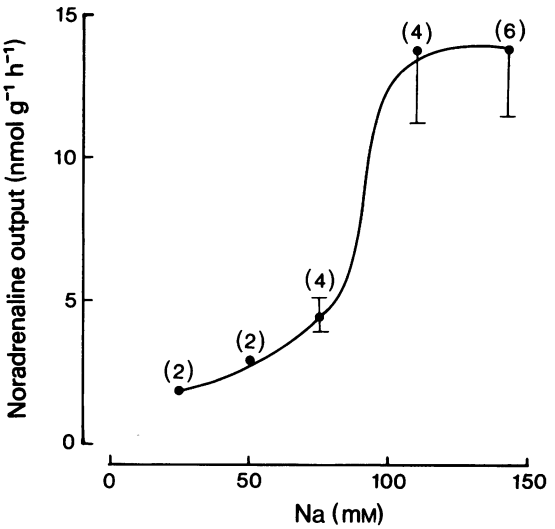

Figure 4 Relation between the effect of X-537A (17 μM) and the concentration of sodium in the Ba-Krebs solution. NaCl was replaced by sucrose. Ordinate scale is the noradrenaline output per hour. Abscissa scale is the concentration of sodium. Symbols represent the sum of noradrenaline output (mean) of the first three 20 min incubation periods. Numbers of experiments are shown beside each point.

systems (Nakazato & Douglas, 1974; Cochrane, Douglas, Mouri & Nakazato, 1975).

In many such systems excess magnesium has been found to compete with the potential-dependent influx of calcium and to inhibit the release of neurotransmitter substances and hormones (Rubin, 1974). It was therefore of interest to determine whether excess magnesium depressed the increase in the noradrenaline output induced by X-537A. $MgCl_2$ 20 mM was found to be effective in reducing the effect of X-537A only when added to Ba-Krebs solution. The inhibition rate was about 65% during the first 20 min incubation period (Figure 2c). No comparable inhibitory effects of excess magnesium were observed in Krebs, Sr-Krebs and Ca-free Krebs solutions.

Devore & Nastuk (1975) suggested that a depolarization of striated muscle membranes caused by X-537A may result from facilitation of sodium influx. If this occurs in the present adrenergic nerve terminal membranes, the noradrenaline output induced by X-537A should be affected by changing the concentration of external sodium. For this reason, the effect of X-537A in Ba-Krebs solution was observed in the presence of various concentrations of external sodium, tonicity being maintained with sucrose. As shown in Figure 4, the noradrenaline output caused by X-537A increased with increasing concentration of external sodium until it attained a maximum at 110 mM. No further increase in the noradrenaline output was obtained at 143 mM.

Effects of A23187 in the presence of calcium, barium and strontium

A23187 was a much less effective stimulus for catecholamine release from perfused adrenal medulla (Cochrane *et al.*, 1975) and from peripheral adrenergic neurones (Thoa *et al.*, 1974) than X-537A and was almost ineffective in releasing vasopressin from neurohypophyses (Nakazato & Douglas, 1974), although A23187 is the more selective calcium ionophore (Pressman, 1973).

On the other hand, if exposure to A23187 was performed in the absence of extracellular calcium, the subsequent reintroduction of calcium caused a substantial secretory response (Russell, Hansen & Thorn, 1974; Williams & Lee, 1974; Garcia, Kirpekar & Prat, 1975). It is assumed that once taken up by the tissue, the ionophore A23187, will not pass back easily into the aqueous medium because of its lipophilic nature (Cochrane *et al.*, 1975).

This prompted us to do the following experiments. Vasa deferentia were exposed to Ca-free Krebs solution containing A23187 in a concentration of 191 μM for 30 minutes. After this the ionophore was withdrawn, but the tissue was left in Ca-free solution for a further 20 min, during which time divalent cations were reintroduced. The reintroduction of calcium caused an increase in the noradrenaline output which reached a maximum during the first 20 min and then declined (Figure 5a). The reintroduction of barium caused less of an increase in noradrenaline output than that induced by calcium, although the response was variable from preparation to preparation (Figure 5b). Strontium was completely ineffective in producing release (Figure 5c). No comparable response was produced by reintroduction of calcium or barium after pretreatment with DMSO (0.1%) alone.

Effects of A23187 in the presence of various concentrations of calcium

Calcium seemed to be the only effective ion in producing a consistent increase in the noradrenaline output from adrenergic nerve terminals, when reintroduced after pretreatment with A23187. This result may reflect the property of the ionophore as a specific calcium carrier (Pressman, 1973). To confirm this we investigated whether the effect of A23187 on the calcium-induced response depends on the concentration of calcium (0.5 to 10 mM). Tris aminomethane buffer (2.5 mM) was used instead of the bicarbonate and phosphate buffers in order to avoid the precipitation of calcium. Under these conditions, calcium (2.5 mM)

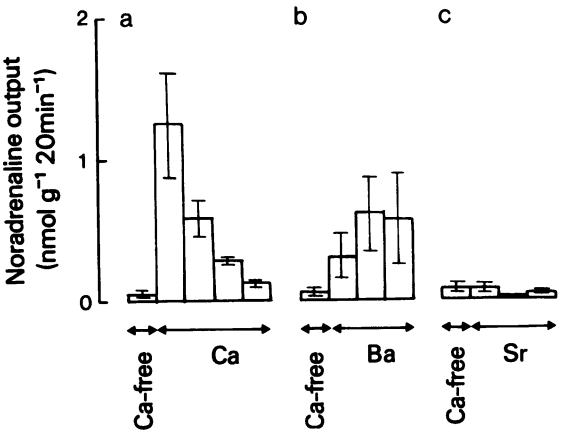

Figure 5 Effects of the reintroduction of (a) calcium, (b) barium and (c) strontium after pretreatment with the ionophore A23187 (191 μM) for 30 min in a Ca-free solution. After 20 min incubation in the absence of calcium, 2.5 mM of each cation was added. Columns are the mean of six (a and c) and seven (b) experiments. Vertical lines show s.e. mean.

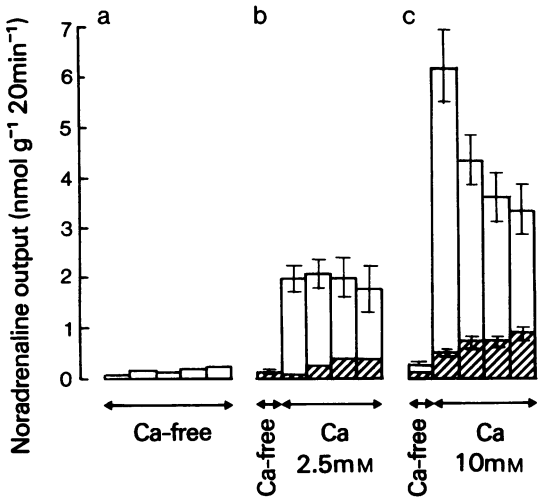

Figure 6 Effects of the reintroduction of calcium on the noradrenaline output after pretreatment with A23187. The records begin after exposure to A23187 (191 μM) for 30 min in a Ca-free solution. (a) The incubation was continued without reintroduction of calcium. After the 20 min incubation period in the absence of calcium, in (b) 2.5 mM and in (c) 10 mM calcium was added. The incubation medium contained Tris aminomethane buffer (2.5 mM) instead of the bicarbonate and phosphate buffers. The hatched areas show the basal output, when (b) 2.5 mM and (c) 10 mM calcium was reintroduced after pretreatment with dimethylsulphoxide (0.1%) alone. Columns are the mean of four (b and hatched columns in c) and seven (c) and the mean of two (a and hatched columns in b) experiments, respectively.

reintroduced after pretreatment with A23187, elicited a larger and more sustained output of noradrenaline than that occurring in the presence of bicarbonate and phosphate buffers (compare Figure 5a and Figure 6b). The response induced by the reintroduction of calcium increased with increasing concentration of calcium from 1 to 10 mM. In Figure 6c, the response induced by calcium 10 mM is shown. Without reintroduction of calcium, there was no response (Figure 6a). Pretreatment with DMSO alone again was not effective in producing a comparable response to that obtained after treatment with A23187 (hatched columns in Figure 6b and 6c). The amount of noradrenaline released during the first three 20 min incubation periods was algebraically added and plotted against the concentration of calcium (Figure 7).

Effects of magnesium on noradrenaline output induced by A23187

After pretreatment with A23187, the preparation was exposed to Ca-free medium (Tris aminomethane buffer) containing magnesium (20 mM) for 20 min and then 2.5 mM calcium was reintroduced. During the first 20 min following the reintroduction of calcium, the output of noradrenaline was decreased somewhat but recovered immediately after and eventually exceeded that of the control (compare Figure 6b and Figure 8a). In the presence of excess magnesium, even without reintroduction of calcium or with pretreatment with DMSO alone, there was a gradual but small increase in the resting noradrenaline output (Figure 8b and 8c).

Discussion

Kita & Van der Kloot (1976) found that X-537A was effective in stimulating transmitter release from frog motor nerve terminals in the presence of various divalent cations. They showed that the effectiveness of the cations was in the order Ba > Sr > Ca > Mg which was roughly parallel to the affinity sequence for X-537A (Pressman, 1973). Célis *et al.* (1974) reported that the ability of X-537A to move the cations through the lipid bilayer membrane was Ba > Ca > Sr > Mg. The effective sequence in the present experiments in X-537A-induced noradrenaline output (Ba > Ca ≥ Sr) seemed to be related to the

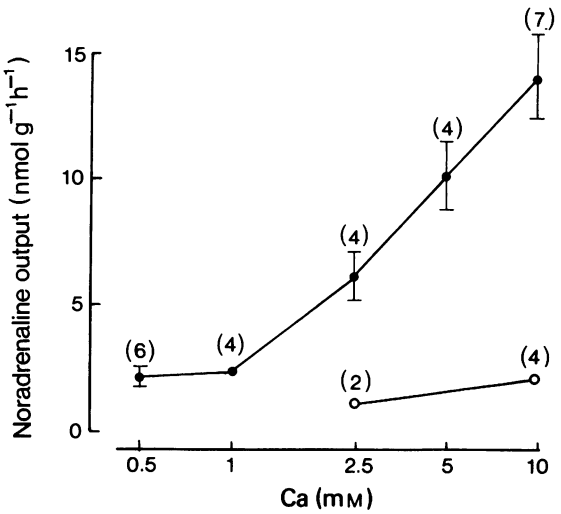

Figure 7 Concentration-dependent increase in the noradrenaline output induced by reintroduction of calcium after pretreatment with A23187 (191 μM; ●) and dimethylsulphoxide (0.1%; ○) for 30 min in a Ca-free solution. Ordinate scale is the noradrenaline output per hour. Abscissa scale is the concentration of calcium. Symbols represent the sum of noradrenaline output (mean) of the first three 20 min incubation periods. Vertical lines show s.e. mean. Numbers of experiments are shown beside each point.

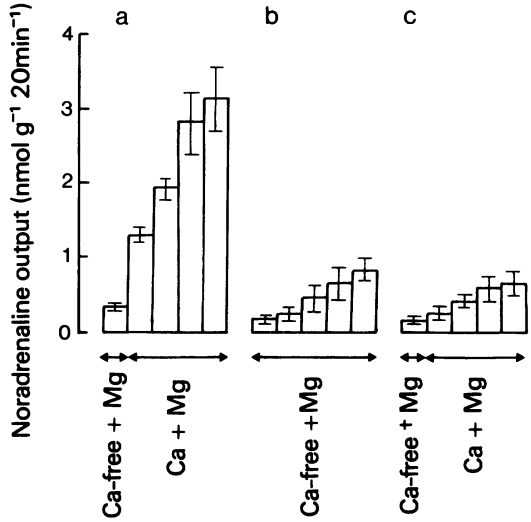

Figure 8 The effect of excess magnesium on the noradrenaline output induced by reintroduction of calcium after pretreatment with A23187. The record begins in the presence of $MgCl_2$ (20 mM) after exposure to (a and b) A23187 (191 μM) and (c) dimethylsulphoxide (0.1%) for 30 min in a Ca-free solution. After the first 20 min incubation period in Ca-free media, 2.5 mM of calcium was added in (a) and (c) but not in (b). Columns are the mean of five (a) and four (b and c) experiments. Vertical lines show s.e. mean.

ability to transfer ions through the lipid bilayer membrane. However, it should not be thought that this sequence simply reflected the ability of X-537A to move ions across the cell membrane, because the same sequence was also found when excess potassium was used to stimulate the release of noradrenaline (Nakazato *et al.*, 1975 and unpublished). Furthermore, Pressman (1973) found that the ability of X-537A to carry divalent cations across the lipid bilayer is exactly opposite to its ability to complex divalent cations. This is inconsistent with the findings of Célis *et al.* (1974).

Kita & Van der Kloot (1976) suggested that barium ions were transported most effectively by X-537A. If X-537A increases intracellular concentrations of barium by transporting the ions directly across the cell membrane, excess magnesium should not inhibit the noradrenaline output in the presence of barium. The affinity of barium for X-537A is about ten thousand times higher than that of magnesium (Pressman, 1973); it therefore seems unlikely that in Ba-Krebs solution, magnesium forms a complex with X-537A and thus inhibits the noradrenaline output. X-537A was found to produce a substantial depolarization of some striated muscles (Cochrane & Douglas, 1975; Devore & Nastuk, 1975; Kita & Van der Kloot, 1976). According to Devore & Nastuk (1975), the depolarization induced by X-537A may result from facilitation of sodium influx. If X-537A also produces depolarization of the adrenergic nerve terminals and results in the operation of voltage-dependent calcium channels, then the effect of X-537A could be inhibited by excess magnesium. In fact, it has been reported that the response induced by excess potassium was almost completely inhibited by excess magnesium in various adrenergic nerve terminals (Rubin, 1974). In the present experiments, the effect of X-537A in Ba-Krebs solution was inhibited by excess magnesium and furthermore, was dependent on the concentration of external sodium ions. These results suggest that the effect of X-537A could partly be due to depolarization-induced influx of barium. Because X-537A can transport monovalent cations in addition to divalent cations (Pressman, 1973), the large extracellular sodium concentration may allow an appreciable influx of sodium ion and depolarize the nerve terminal membranes in the presence of X-537A. In the presence of barium, X-537A may depolarize the nerve terminal membrane more easily than in the presence of calcium or strontium. This might explain why we could

obtain a clear dose-response relationship in the presence of barium. However, we do not intend to exclude completely the possibility that X-537A transports barium ions across the cell membrane, because even in the presence of excess magnesium X-537A caused a greater output of noradrenaline than that obtained in the absence of the divalent cations.

X-537A is known to complex catecholamines (Pressman, 1973) and to transfer them directly across the lipid bilayer membrane (Schadt & Haeusler, 1974), the chromaffin granule membranes (Johnson & Scarpa, 1974) and synaptosomal vesicular membranes (Holz, 1975). In the absence of external calcium and with EGTA, X-537A caused about 50% of the response obtained in the presence of calcium. This agrees with the observation of Thoa *et al.* (1974) who found that X-537A caused a non-exocytotic release of noradrenaline from the peripheral adrenergic neurones in the absence of external calcium. The effects of X-537A in the absence of external calcium may be due to the direct transport of noradrenaline across the cell membrane. Alternatively, they may be due to the release of intracellular stored calcium as assumed by Cochrane *et al.* (1975) and Nordmann & Currell (1975).

A23187, although not entirely specific, has been found to be a more selective carrier of calcium than X-537A (Pressman, 1973). Pfeiffer *et al.* (1974) reported that the relative complex stabilities as determined by an organic phase extraction technique were Ca > Mg > Sr > Ba. Our results obtained from the reintroduction of barium, strontium and calcium seemed to reflect the specificity of A23187 as a calcium ionophore. A similar selective effect of the reintroduction of calcium was reported by Williams & Lee (1974) on amylase release from mouse pancreatic fragments.

The reintroduction of calcium also caused an increase in the catecholamine output from perfused adrenal medulla after pretreatment with A23187 (Garcia *et al.*, 1975) and the secretory response of the exocrine pancreas in the presence of A23187 (Eimerl, Savion, Heichal & Selinger, 1974). Furthermore these effects were inhibited by excess magnesium. In disagreement with their results, excess magnesium failed to inhibit an increase in the noradrenaline output in the present experiments. The reason for the discrepancy in the effects of magnesium between ours and the previous reports is not known but may be due to the difference in tissues used.

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