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EFFECTS OF METHOXYVERAPAMIL ON THE STIMULATION BY Ca^{2+}, Sr^{2+} AND Ba^{2+} AND ON THE INHIBITION BY Mg^{2+} OF CATECHOLAMINE RELEASE FROM THE ADRENAL MEDULLA

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1 Bovine adrenal glands were perfused with Ca^{2+}-free Locke solution and catecholamine release was induced either by the introduction of Ca^{2+}, Sr^{2+} or Ba^{2+} into the perfusion fluid or by the substitution of Na^+ by an osmotically equivalent amount of sucrose.

2 Methoxyverapamil (D600) at a concentration of 3×10^{-4} M blocked the release of catecholamines in response to Ca^{2+}, Sr^{2+} or Ba^{2+} stimulation but failed to block the release evoked by the omission of Na^+.

3 Mg^{2+} (10 to 20 mM) blocked the release induced by Na^+-deprivation; however, this inhibitory effect of Mg^{2+} was not modified by D600.

4 D600 blocked the increase in the efflux of ^{45}Ca from the perfused gland induced by the introduction of Ca^{2+} into the perfusion fluid and blocked the uptake of ^{45}Ca into adrenal medullary slices induced by K^+ depolarization.

5 The results suggest that Ca^{2+}, Sr^{2+} and Ba^{2+} may enter the chromaffin cell through the same channel and that this channel is blocked by D600. Mg^{2+} may enter the cell through the same Ca^{2+} channel but with a high rate of permeation or it may enter through a channel which is resistant to D600. Alternatively, Mg^{2+} may exert its inhibitory effect at an extracellular site.

Introduction

It has been demonstrated that Ca^{2+} is a necessary requirement for secretion to occur from those tissues which share the feature of storing their secretory products in subcellular granules (Douglas, 1974; 1975; Trifaró, 1977). Among these tissues is the adrenal medulla for which the requirement for Ca^{2+} was early established (Douglas & Rubin, 1961). Furthermore, it has been shown that replacement of extracellular Ca^{2+} by either Sr^{2+} or Ba^{2+} is effective in maintaining the stimulation-induced release of catecholamines (Douglas & Rubin, 1964a); moreover, Ba^{2+} itself is a potent secretagogue, even in the absence of extracellular Ca^{2+} (Douglas & Rubin, 1964b).

It seems, therefore, that an increase in intracellular Ca^{2+} is a necessary event in order to trigger release. Increase in cytoplasmic Ca^{2+} may be produced by conditions which interfere either with the sequestration of Ca^{2+} by subcellular organelles or with the efflux of Ca^{2+} from the cell (Lastowecka & Trifaró, 1974; Aguirre, Pinto & Trifaró, 1977). However, it is more likely that under physiological conditions an increase in Ca^{2+} entry, through a specific Ca^{2+} channel, would account for this increment in intracellular Ca^{2+}. Experiments published previously suggested that Ca^{2+} movements in the adrenal medulla may involve both Na-Ca and Ca-Ca exchange mechanisms (Baker & Rink, 1975; Aguirre *et al.*, 1977). These experiments also suggest that the late Ca^{2+} movement may be involved in stimulus-secretion coupling (Baker & Rink, 1975; Aguirre *et al.*, 1977). The entry of Ca^{2+} through a channel of similar characteristics to the 'late Ca^{2+} channel' of the squid axon seems to be involved in the exocytotic release of catecholamines from the adrenal medulla in response to acetylcholine and high K^+ stimulation (Pinto & Trifaró, 1976). However, under other experimental conditions, as for example during the exocytotic release produced by Na^+-deprivation, it is the intracellular and not the extracellular Ca^{2+} which seems to be involved (Lastowecka & Trifaró, 1974; Aguirre *et al.*, 1977). Although the catecholamine release induced by Na^+ omission is not dependent on extracellular Ca^{2+}, an increase in the concentration of extracellular Mg^{2+} blocks the hormone release (Lastowecka & Trifaró, 1974).

The present experiments were carried out to study further the characteristics of this specific channel, which seems to be involved in stimulus-secretion coupling, and to see if Sr^{2+}, Ba^{2+} and Mg^{2+} enter

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the chromaffin cell through the same channel. Methoxyverapamil (D600) a drug which blocks both Ca^{2+} influx in excitable tissues (Fleckenstein, 1971; Fleckenstein, Grun, Tritthart & Byron, 1971; Baker, Meves & Ridgway, 1973) and hormone release from the neurohypophysis (Dreifuss, Grau & Nordmann, 1973; Russell & Thorn, 1974) and the adrenal medulla (Pinto & Trifaró, 1976) was used in these experiments.

Methods

Bovine adrenal glands obtained from a slaughterhouse were perfused *in vitro* as described by Trifaró, Poisner & Douglas (1967).

The composition of the perfusion and incubation fluids was (mM): (a) standard Locke solution: NaCl 154, $CaCl_2$ 2.2, $MgCl_2$ 1.2, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85 and dextrose 10; (b) Na^+-free Locke solution was of the same composition as the standard Locke solution except that the NaCl was replaced by an osmotically equivalent concentration of sucrose; (c) Ca^{2+}-free Locke and Ca^{2+}-free, Na^+-free Locke solutions were similar to the solutions mentioned in (a) and (b) respectively, except that $CaCl_2$ was omitted from the media; (d) high potassium Locke solution contained 56 mM K^+ of which 53 mM was as KCl and 3 mM as K_2HPO_4 and KH_2PO_4. In this solution NaCl was reduced by an equivalent amount (50.4 mM); (e) Sr^{2+}-Locke and Ba^{2+}-Locke solutions were of the same composition as the standard Locke solution, except that $CaCl_2$ was replaced by either 2.2 mM $SrCl_2$ or 5 mM $BaCl_2$. In the experiments in which D600 was tested, the incubation or the perfusion fluids contained 0.06% ethanol which did not affect either the spontaneous or evoked catecholamine and ^{45}Ca effluxes. All solutions were equilibrated with 5% CO_2 in O_2 and the final pH of the solutions was 7.2.

The glands were perfused at room temperature (25°C) by means of a multichannel peristaltic pump (Buchler) at a constant rate (10 ml/min). In the experiments in which the ^{45}Ca efflux was studied, cortex-free adrenals were perfused for 20 min with normal Locke solution (equilibration period) and, in order to wash out the extracellular Ca^{2+}, perfusion was changed to Ca^{2+}-free (disodium edetate (EDTA), 2 mM) Locke for another period of 25 min. Perfusion was then continued for 5 min with the same Ca^{2+}-free solution but without EDTA. This was followed by a 4 min period (labelling phase) in which the glands were exposed to ^{45}Ca, 5 μCi/ml (specific activity: 2.27 μCi/nmol). After this labelling phase, perfusion was continued with Ca^{2+}-free Locke medium for 50 to 80 min to wash out extracellular ^{45}Ca. In some experiments the time required to wash out the extracellular space was determined after exposing the glands

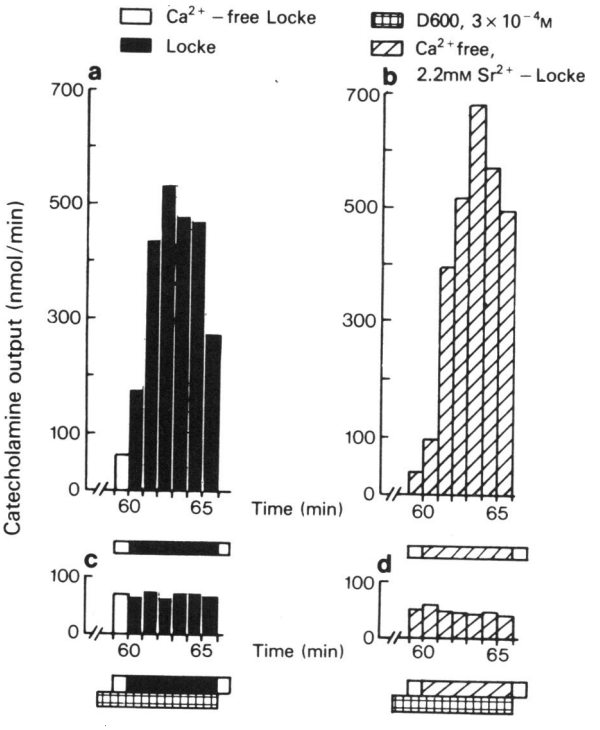

Figure 1 Effects of methoxyverapamil (D600) on the release of catecholamines induced by reintroduction of Ca^{2+} or introduction of Sr^{2+} into the perfusion medium. Four bovine adrenal glands were perfused with Ca^{2+}-free Locke solution (□) for 60 min. Thereafter the perfusion fluid was changed to (a) standard Locke (■) or to (b) Ca^{2+}-free, 2.2 mM Sr^{2+}-Locke solution (■) for 6 min. In (c) and (d) the effect of D600 (3×10^{-4} M) on two of these glands is illustrated. Similar results were obtained with 29 other glands. The adrenals were perfused at room temperature (25°C) with a flow rate of 10 ml/min. The perfusion solutions were gassed with a mixture of 5% CO_2 in O_2. Samples were collected from the perfusates at 1 min intervals; these were assayed for catecholamine content as indicated in the Methods.

for 4 min to [^{14}C]-sorbitol, 1 μCi/ml (specific activity: 200 μCi/μmol). After the radioactive pulse, samples of the perfusates were collected by a fraction collector for a period of 1 min at 1 or 2 min intervals. Aliquots (0.5 ml) of each sample were transferred to vials containing 15 ml Aquasol (New England Nuclear, Boston, Mass.), and the radioactivity was measured in a liquid scintillation spectrometer (SL 40 Intertechnique). The ^{45}Ca remaining in the tissue was determined as previously described (Aguirre *et al.*, 1977). The desaturation curves, the rate coefficients and the relative rate coefficients were calculated as described elsewhere (Aguirre *et al.*, 1977).

For the ^{45}Ca uptake studies, adrenal medullary slices were prepared. Bovine adrenal medullae were separated from their cortices. Four to six slices (200 to 300 μm thick) were obtained from each medulla by means of a Stadie-Riggs slicer. The average weight of the slices was 95 ± 1 mg (n = 173). The slices were divided into groups of 12 to 15 and they were incubated at 37°C for 60 min in standard Locke solution. Then the slices were incubated for 30 min in Ca^{2+}-free Locke solution with or without D600 (3×10^{-4} M). This was followed by a period of incubation (20 min; loading phase) with Locke solution containing low Ca^{2+} (0.1 mM) and 2.5 μCi ^{45}Ca/ml. During the loading phase, groups of slices were incubated with Locke solution containing either 5.6 mM or 56 mM KCl in the presence or in the absence of D600. After the loading phase, each slice was incubated separately in six changes in 10 ml standard Locke over a 60 min period. During this last period, the slices that were loaded with ^{45}Ca in the presence of D600 were also incubated in the presence of this Ca^{2+} antagonist. At the end of this period, the slices were placed in scintillation vials and digested with 1 ml of concentrated HNO_3. The acid was evaporated to dryness and the residue was decolourized with H_2O_2 and evaporated again to dryness. Distilled water (0.2 ml) and 10 ml Aquasol were added to the vials and the radioactivity measured.

In those experiments where the catecholamine output was measured, samples from the perfusates were added to tubes (0°C) containing 10 μl of 1.9 N HCl per ml of perfusate. The catecholamine content of these samples was assayed by the trihydroxyindole fluorimetric method described by Anton & Sayre (1962).

Chemicals

The chemicals were obtained from the following sources: ^{45}Ca (50 mCi/mmol) and [^{14}C]-sorbitol (200 mCi/mmol) New England Nuclear, Boston, Mass. Methoxyverapamil (D600) was a generous gift from Drs Oberdorf and Sharma, Knoll, A.C., Ludwigshafen, Germany.

Results

Effects of methoxyverapamil (D600) on the release of catecholamines induced by Ca^{2+}, Sr^{2+} and Ba^{2+}

Adrenal glands were perfused for 60 min with Ca^{2+}-free Locke solution. Then the perfusion was continued with standard Locke solution for a period of 6 min (Figure 1a). Each time that 2.2 mM Ca^{2+} was re-introduced into the perfusion fluid, a significant increase in the catecholamine output was observed

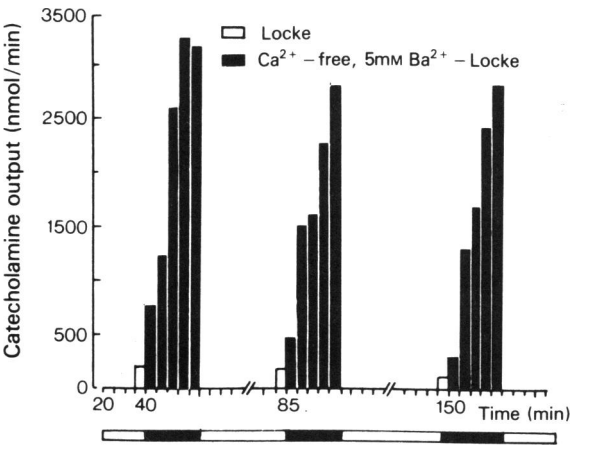

Figure 2 Effect of Ba^{2+} on the output of catecholamines. A bovine adrenal gland was perfused for three successive periods of 5 min each with Ca^{2+}-free, 5 mM Ba^{2+}-Locke solution (■). Each of these 5 min stimulation periods was separated from the next by 40 and 60 min of perfusion with Locke solution (□). Similar results were obtained in 3 other experiments. Other conditions were as described in Figure 1.

(Δ = 217 to 598%, n = 10). However, when D600 at a concentration of 3×10^{-4} M was present in the perfusion medium, the Ca^{2+} re-introduction response was blocked (Figure 1c). In the presence of D600, the inhibition of the Ca^{2+}-evoked catecholamine release was 88.9 + 3.8% (n = 6). This concentration of D600 was used because we have demonstrated previously that it produced 86% and 85% inhibition in the output of catecholamines in response to acetylcholine and high potassium, respectively (Pinto & Trifaró, 1976).

A similar set of experiments using 2.2 mM Sr^{2+} were carried out. As with the re-introduction of Ca^{2+} into the perfusion fluid, the introduction of Sr^{2+} evoked a significant increase in the output of catecholamines (Δ = 190 to 620%, n = 8). One such experiment is shown in Figure 1b. D600 (3×10^{-4} M) also blocked the response to Sr^{2+} introduction (Figure 1d). The Sr^{2+}-evoked release of catecholamines was decreased by 95.1 ± 1.3% (n = 9) in the presence of D600.

It has been demonstrated that Ba^{2+}, in contrast to Ca^{2+} and Sr^{2+}, is itself capable of inducing catecholamine release (Douglas & Rubin, 1964b). When Ca^{2+} is replaced by Ba^{2+} in the perfusion fluid, an increase in the catecholamine output is observed (Douglas & Rubin, 1964b). Therefore, the effect of D600 on the release of catecholamine induced by this ion substitution was studied. Four adrenal glands were perfused for three successive periods of 5 min

each with Ca^{2+}-free, 5 mM Ba^{2+}-Locke solution. Each of these 5 min stimulation periods was separated from the next by 40 and 60 min of perfusion with standard Locke solution respectively (Figure 2). There was a marked increase in the catecholamine output each time Ba^{2+} was introduced into the perfusion medium (Figure 2). When compared to the first stimulation period, the catecholamine outputs during the second and third stimulation periods decrease by 10 and 20% respectively. This decrease in the adrenal medullary response to successive exposures to secretagogues has been described previously (Douglas & Rubin, 1961; Lastowecka & Trifaró, 1974). Four other adrenal glands were also perfused and stimulated by Ba^{2+} as indicated above, except that during the second period of stimulation, D600 (3×10^{-4} M) was present in the perfusion medium. D600 was introduced into the medium 20 min before the second stimulation and it was withdrawn from the perfusion fluid together with Ba^{2+}. The response to Ba^{2+} stimulation was significantly blocked by D600 (Figure 3). Table 1 shows the mean values obtained in these experiments. It should also be noticed that 60 min after the removal of D600 from the perfusion medium the catecholamine output in response to the third Ba^{2+} stimulation had completely recovered (Figure 3).

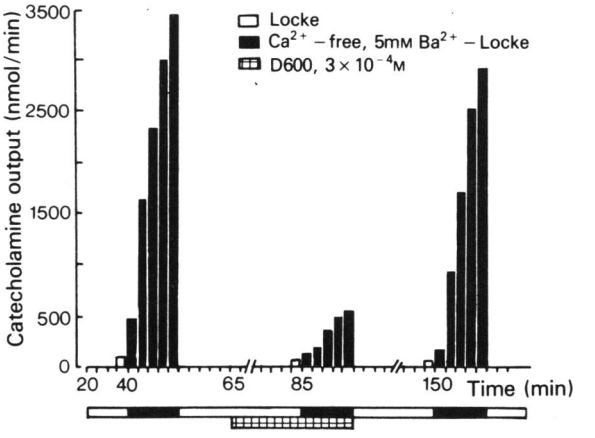

Figure 3 Effect of methoxyverapamil (D600) on the Ba^{2+}-evoked release of catecholamines. A bovine adrenal gland was perfused alternately with Locke solution (□) and Ca^{2+}-free, 5 mM Ba^{2+}-Locke solution (■) as described in Figure 2. During the second stimulation period, D600 (3×10^{-4} M) was present in the perfusion fluid. Similar results were obtained in 3 other experiments. Other conditions were as described in Figure 1.

Effect of methoxyverapamil on the ^{45}Ca efflux in response to Ca^{2+} re-introduction

We have demonstrated previously that the efflux of ^{45}Ca from adrenals perfused with solutions containing Ca^{2+} is reduced by D600 (Aguirre *et al.*, 1977). We have now studied the effect of D600 under the present experimental conditions and obtained similar results.

Cortex-free adrenal glands were perfused and loaded with ^{45}Ca as described in the Methods. The glands were perfused for 60 min with Ca^{2+}-free Locke

Table 1 Effect of methoxyverapamil (D600) on the release of catecholamines induced by Ba^{2+}.

Expt. No.	*Increased catecholamine release (nmol/min) 1st stimulation*	*2nd stimulation*	*Ratio of 2nd to 1st stimulation*
1	+2725	+2312	85%
2	+3807	+5051	133%
3	+540	+346	64%
4	+512	+330	64%
	Mean ± s.e. mean 86 ± 16%		
5	+3090	+415	13%
6	+2750	+425	15%
7	+522	−22	0%
8	+532	−4	0%
	Mean ± s.e. 7 ± 4%		

In experiments 1 to 4, adrenal glands were perfused first with standard Locke solution and during 1st and 2nd stimulation periods they were perfused with Ca^{2+}-free, 5 mM Ba^{2+} Locke solution. In experiments 5 to 8, during 2nd stimulation period, D600 (3×10^{-4} M) was present in the perfusion fluid. The discrepancies in the absolute values among stimulations are due to differences in the weight of the bovine adrenal glands used.

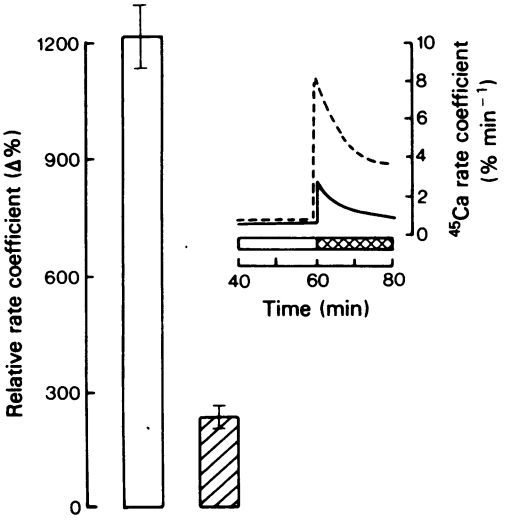

Figure 4 Effect of methoxyverapamil (D600) on the ^{45}Ca efflux in response to Ca^{2+} reintroduction. The insert shows the rate coefficients obtained with two cortex-free bovine adrenal glands that were perfused and labelled with ^{45}Ca as described in the Methods. After 60 min of perfusion with Ca^{2+}-free Locke solution (□), the perfusion was continued for 10 min with standard Locke solution (▨). Forty min after the experiment was started, D600 (3×10^{-4} M) was added to the perfusion fluid of one of the glands (continuous line). This gland was perfused with a solution containing D600 during the rest of the experiment. Samples of the effluents were collected and the ^{45}Ca radioactivity was determined as indicated in Methods. The columns represent the relative rate coefficients calculated from data obtained in similar experiments in the absence (open columns) or in the presence of D600 (hatched columns). The rate coefficient and the relative rate coefficient were calculated as described in the Methods.

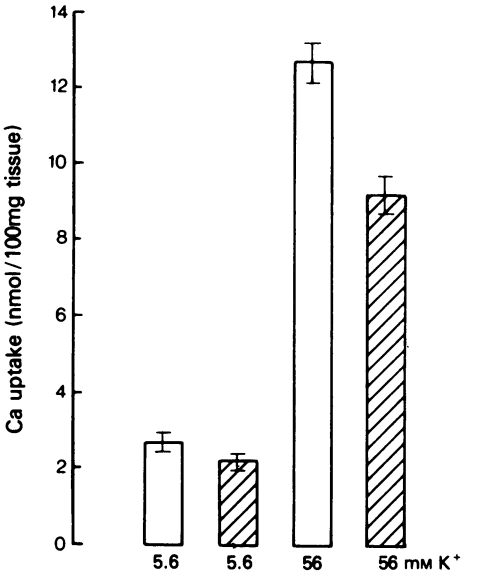

Figure 5 Effects of K^+ depolarization and methoxyverapamil (D600) on the ^{45}Ca uptake into adrenal medullary slices. The slices were prepared, incubated and the ^{45}Ca radioactivity measured as described in Methods. D600 (3×10^{-4} M) was present in the experiments indicated by hatched columns. The K^+ concentration is shown below each column. Each column represents the mean results from 12 medullary slices; vertical bars show s.e. mean.

solution. Then the perfusion fluid was changed to standard Locke solution. Previously, we have shown that the efflux of ^{45}Ca from cortex-free adrenals can be resolved into three different components by graphical analysis and that the third component is linear from 35 to 100 min of perfusion (Aguirre *et al.*, 1977). From these results, it is assumed that all radioactivity present in the perfusates after 40 min is the unidirectional flux of ^{45}Ca representing the washout of intracellular Ca^{2+}.

The insert in Figure 4 shows that there was a significant increase in the ^{45}Ca rate coefficient upon Ca^{2+} re-introduction and that this increase was significantly reduced by D600. Furthermore, the same figure shows that when the results were expressed as relative rate coefficients, the presence of D600 produced 80% inhibition of the ^{45}Ca efflux. This again is a suggestion that Ca-Ca exchange is the main mechanism responsible for the enhanced ^{45}Ca efflux observed during Ca^{2+} re-introduction.

Effect of methoxyverapamil on the uptake of ^{45}Ca into adrenal medullary slices

In order to obtain more direct evidence for the inactivation by D600 of Ca^{2+} entry into the adrenal medulla, experiments on ^{45}Ca uptake into adrenal medullary slices were performed. Slices were used in this case, because the results obtained are more homogeneous than those with perfused glands where there is a great deal of variation in the data, mainly due to individual differences in the ^{45}Ca uptake and in the rate of the washout of the extracellular space. Figure 5 shows that the exposure of adrenal slices to 56 mM K^+ produced an increase of 4.7 times in the uptake of ^{45}Ca. This effect of a depolarizing concentration of K^+ is in agreement with previous observations in the adrenal medulla (Douglas & Poisner, 1962; Baker & Rink, 1975).

In the presence of D600 (3×10^{-4} M), the uptake of ^{45}Ca into slices under resting conditions was not

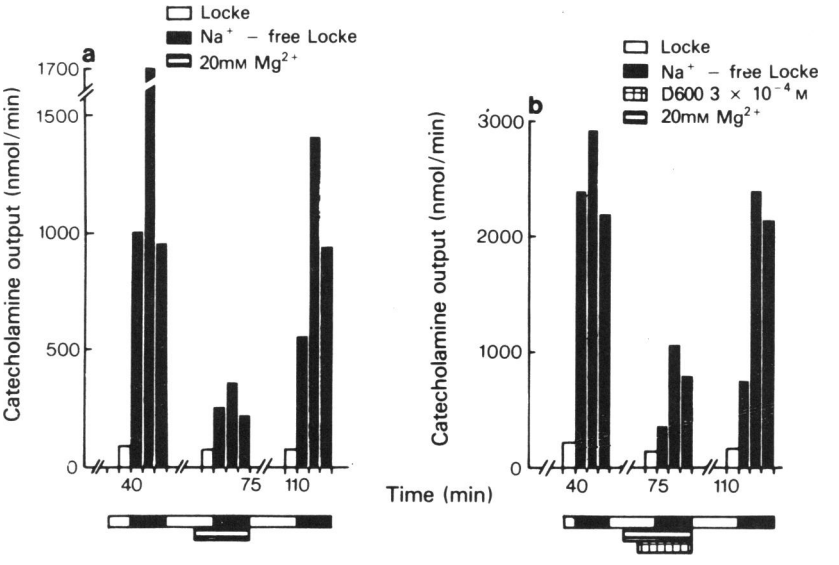

Figure 6 Effect of Mg^{2+} and methoxyverapamil on the output of catecholamines evoked by Na^+ omission. Two bovine adrenal glands (a) and (b) were perfused alternately with Locke solution (□) and Na^+-free Locke solution (■). During the second perfusion period with Na^+-free Locke solution, the solution contained 20 mM Mg^{2+} (▭) in (a) and Mg^{2+} plus 3×10^{-4} M D600 (▭) in (b). Similar results were obtained in 8 other experiments. Other conditions were as described in Figure 1.

affected (Figure 5). However, the ^{45}Ca uptake in response to 56 mM K^+ decreased significantly ($P < 0.001$). The inhibition in the presence of D600 was 27% (Figure 5), a value which agrees with the figure of 33% which can be calculated from data published by Baker & Rink (1975). The decrease in the ^{45}Ca uptake observed by these authors was interpreted as due to the inactivation of the late Ca^{2+} channel by prolonged depolarization induced by 56 mM K^+.

Effect of methoxyverapamil on the inhibitory action of Mg^{2+} upon catecholamine release induced by Na^+-deprivation

D600 has no effect on the release of catecholamines induced by Na^+-deprivation (Pinto & Trifaró, 1976). The increased release of catecholamines produced by Na^+ omission is practically or totally blocked by the presence of 10 or 20 mM Mg^{2+} in the perfusion fluid, either in the presence or in the absence of extracellular Ca^{2+} (Lastowecka & Trifaró, 1974). These findings have suggested that perhaps the site of action of Mg^{2+} during the blockade of the release reaction was intracellular (Lastowecka & Trifaró, 1974). For all the above reasons, the effect of D600 on the inhibitory action of Mg^{2+} was studied.

Adrenal glands were perfused with standard Locke solution and stimulated three times by Na^+-deprivation. During the second stimulation period, 10 or 20 mM Mg^{2+} with or without D600 was present in the perfusion fluid (Figures 6 and 7). D600 was present in the perfusion fluid 20 min before the second stimulation period and it was removed from the medium at the end of this stimulation period. D600, in concentrations that blocked the effects of Ca^{2+}, Sr^{2+} and Ba^{2+} on catecholamine release, did not modify the inhibitory effect of Mg^{2+} on the catecholamine release induced by Na^+-deprivation (Figures 6 and 7). The lack of effect of D600 was observed either in the presence or in the absence of extracellular Ca^{2+} (Figures 6 and 7). Table 2 summarizes the results obtained in experiments done in the presence of either 10 or 20 mM Mg^{2+}.

Discussion

It is well established that extracellular Ca^{2+} is a necessary requirement for hormone and transmitter release to occur in several secretory tissues, including the adrenal medulla (Douglas, 1974; 1975; Trifaró, 1977). However, the exact role that Ca^{2+} plays in the secretory process has not been elucidated. It seems that under normal conditions, Ca^{2+} entry into

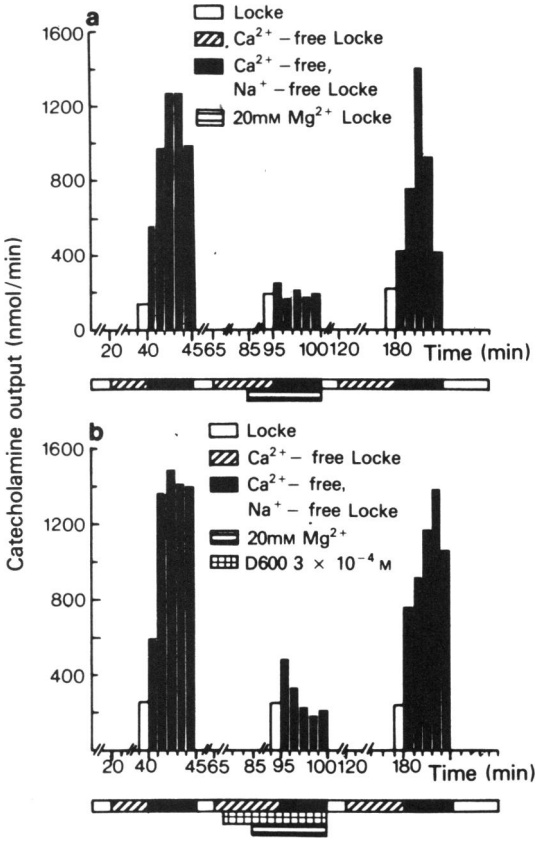

Figure 7 Effect of Mg^{2+} and methoxyverapamil (D600) on the release of catecholamines produced by Na^{+}-deprivation in the absence of extracellular Ca^{2+}. Two bovine adrenal glands were perfused successively with Locke solution (□), Ca^{2+}-free Locke solution (■) and Ca^{2+}-free, Na^{+}-free Locke solution (■). During the second perfusion period with Ca^{+}-free, Na^{+}-free Locke solution, the solution contained 20 mM Mg^{2+} (▭) in (a) and Mg^{2+} plus 3×10^{-4} M D600 (▦) in (b). Similar results were obtained in 7 other experiments. Other conditions were as described in Figure 1.

the chromaffin cell might be the first step in triggering hormone release (Trifaró, 1977).

Baker & Reuter (1975) have demonstrated that in the squid axon and in the mammalian heart, Ca^{2+} enters the tissue in two distinct phases: an early phase, coupled to Na^{+} efflux and inhibited by tetrodotoxin (TTX) and a late phase resistant to TTX, but which is blocked by Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+} and D600. These same ions and D600 also block neurotransmitter and hormone release in several tissues (Douglas, 1974; Russell & Thorn, 1974; Pinto & Trifaró, 1976; Trifaró, 1977). D600 can inhibit the release of vasopressin and oxytocin from the neurohypophysis (Russell & Thorn, 1974; Thorn, 1974), the ^{45}Ca uptake by the neurohypophysis (Dreifuss *et al.*, 1973), the slow Ca^{2+} current in cardiac fibres (Kohlhardt, Bauer, Krause & Fleckenstein, 1972), the influx of Ca^{2+} through the 'late Ca^{2+} channel' of the squid axon (Baker *et al.*, 1973; Baker & Reuter, 1975), and the release of catecholamines in response to acetylcholine stimulation or depolarizing concentrations of K^{+} (Pinto & Trifaró, 1976).

The present results show that D600 is also able to block the release of catecholamines, induced either by Ca^{2+} or Sr^{2+} re-introduction and by Ba^{2+} stimulation. It has been suggested that after a period of perfusion of adrenal glands with a solution devoid of Ca^{2+}, the reintroduction of Ca^{2+} into the perfusion fluid causes the release of catecholamines. This effect has been interpreted as due to a rapid penetration of Ca^{2+} through a highly permeable plasma membrane (Douglas, 1968). D600 not only blocked the release of catecholamines in response to Ca^{2+} reintroduction, but also the ^{45}Ca efflux in response to Ca^{2+} reintroduction. This latter result is in agreement with our previous observations (Aguirre *et al.*, 1977) and it has been interpreted as being due to the fact that the increased ^{45}Ca efflux observed upon Ca^{2+} reintroduction is due to the increased Ca-Ca exchange observed under those circumstances. D600 blocked the entry of Ca^{2+} into the chromaffin cell with the consequent decrease in the Ca-Ca exchange mechanism. Another indication of this effect of D600 can be found in the results obtained in the ^{45}Ca uptake studies presented here. D600 decreased the uptake of ^{45}Ca into adrenal medullary slices induced by K^{+} stimulation. The decrease produced by D600 was 27%, a figure which suggests either that a large percentage of Ca^{2+} enters this tissue by another route, or that there is a large amount of ^{45}Ca bound to extracellular structures. Baker & Rink (1975) also described studies with adrenal medullary slices. These slices were incubated in Ca^{2+}-free media and stimulated with 72 mM K^{+}; ^{45}Ca was introduced into the incubation medium at the beginning of the K^{+} depolarization in one set of slices and after 10 min of K^{+} stimulation, in the other set. The ^{45}Ca uptake in the latter set was 33% lower than in the former (Baker & Rink, 1975). This decrease in the ^{45}Ca uptake, which was of about the same magnitude as that observed in our studies with D600, was interpreted by Baker & Rink (1975) as being caused by inactivation of the 'late Ca^{2+} channel' due to prolonged depolarization. In other words, when ^{45}Ca was added to the second set of adrenal medullary slices, the Ca^{2+} channel was already inactivated.

It is known that Sr^{2+} can effectively substitute for Ca^{2+} in several tissues and maintain the integrity of the Ca^{2+}-dependent processes. Sr^{2+} can replace Ca^{2+}

in the process of quantal release of transmitter from the neuromuscular junction (Miledi, 1966; Dodge, Miledi & Rahamimoff, 1969; Meiri & Rahamimoff, 1970), in the process leading to secretion of oxytocin and vasopressin from the neurohypophysis (Buchs, Dreifuss, Grau & Nordman, 1972) and in the histamine release from mast cells (Foreman & Mongar, 1972). In the adrenal medulla, Sr^{2+} can replace Ca^{2+} and maintain secretion due to acetylcholine and high K^+ stimulation (Douglas, 1968). The present results show that as with Ca^{2+}, the introduction of Sr^{2+} into the perfusion fluid causes catecholamine release and that D600 is able to block this response. Sr^{2+} might trigger catecholamine release by liberating intracellular Ca^{2+} as suggested by Poisner & Hava (1970). However, Sr^{2+} might trigger release itself, since as demonstrated in our experiments, a long perfusion with Ca^{2+}-free Locke solution does not prevent the release of catecholamines when Sr^{2+} is introduced into the perfusion fluid. Furthermore, the responses to successive Sr^{2+} stimulations decline in the same way as in the experiments with Ca^{2+} and short periods of Ca^{2+} reintroduction into the perfusion fluid do not prevent the progressive decline in the responses to Sr^{2+} (Douglas & Rubin, 1964a).

Ba^{2+} can also substitute for Ca^{2+} in the secretory process (Trifaró, 1977). Ba^{2+} depolarizes the chromaffin cell (Douglas, Kanno & Sampson, 1967) and other secretory tissues such as the β cell of the pancreas (Atwater, Biegelman & Ribalet, 1976). The depolarizing effect of Ba^{2+} is probably due to the fact that Ba^{2+} suppresses the outward K^+ current, which is possibly responsible for the repolarization (Hagiwara, Fukuda & Eaton, 1974; Atwater *et al.*, 1976). It has been suggested that, at the neuromuscular junction, Sr^{2+} and Ba^{2+} cause release of transmitter by different mechanisms of action (Zengel & Magleby, 1977), although they may enter the tissue through the same channel. Our results with D600 suggest that Ba^{2+}, like Sr^{2+}, may enter the chromaffin cell through a channel of similar characteristics to the Ca^{2+} channel.

Catecholamine release is also observed when the adrenal medulla is stimulated by perfusion with solution in which Na^+ is substituted by equimolar concentrations of choline or osmotic equivalent amounts of sucrose (Lastowecka & Trifaró, 1974). The release of catecholamines evoked by Na^+-deprivation which occurs even during perfusion with Ca^{2+}-free solutions in not blocked by D600 (Pinto & Trifaró, 1976). However, the catecholamine release produced by Na^+-deprivation is inhibited by Mg^{2+} (10 to 20 mM), either in the presence or in the absence of extracellular Ca^{2+} (Lastowecka & Trifaró, 1974). The inhibitory effect of Mg^{2+} observed during Na^+-deprivation was interpreted as being due to competition between Mg^{2+} and Ca^{2+} at an intracellular site (Lastowecka & Trifaró, 1974). This was one of the reasons for performing the experiments with D600, because it was thought that if Mg^{2+} enters the chromaffin cell through the Ca^{2+} channel, it might be possible to block the inhibitory effect of Mg^{2+} with D600. The results presented in this paper, on the contrary, indicate that D600 did not modify the inhibitory effect of Mg^{2+}. Although we do not have a certain explanation for these results, several possibilities exist: (a) perhaps D600 blocked the entry of Mg^{2+} during perfusion with Na^+-free sucrose Locke solution, but the inhibitory effect of Mg^{2+} was exerted at an extracellular site, either at the plasma membrane level or perhaps by increasing the ionic strength of the extracellular medium. This latter possibility has been suggested by Rink (1977); (b) D600 did partially block the

Table 2 Effect of Mg^{2+} and methoxyverapamil (D600) on the output of catecholamines evoked by Na^+ deprivation.

Expt. No.	*Condition*		*Catecholamine release 2nd stim. as % of release during 1st stim.*
	1st stimulation	*2nd stimulation*	
1	Na^+-free + Mg^{2+} (1.2 mM)	Na^+-free + Mg^{2+} (1.2 mM)	90 ± 10* (n = 10)**
2	Na^+-free + Mg^{2+} (1.2 mM)	Na^+-free + Mg^{2+} (10 mM)	23 ± 5 (n = 4)
3	Na^+-free + Mg^{2+} (1.2 mM)	Na^+-free + Mg^{2+} (10 mM) + D600	24 ± 3 (n = 4)
4	Na^+-free + Mg^{2+} (1.2 mM)	Na^+-free + Mg^{2+} (20 mM)	5.6 ± 1.2 (n = 5)
5	Na^+-free + Mg^{2+} (1.2 mM)	Na^+-free + Mg^{2+} (20 mM) + D600	3.2 ± 2 (n = 4)

* Mean ± s.e. mean; ** number of glands tested.

Bovine adrenal glands were perfused with Ca^{2+}-free Locke solution and catecholamine release was evoked by Na^+-deprivation. During the second stimulation, 10 mM Mg^{2+} was present in the perfusion fluid in experiments 2 and 3 and 20 mM Mg^{2+} was present in experiments 4 and 5. D600 (3×10^{-4} M) was present during the second stimulation in experiments 3 and 5. Other conditions were as described in Figure 1.

Mg^{2+} entry during Na^+ omission. The concentration of D600 used, although high enough to block the entry of Ca^{2+}, Sr^{2+} and Ba^{2+} might not be high enough to block the Mg^{2+} entry. The concentration of D600 could not be increased in the perfusion fluid for solubility reasons. Experiments carried out in the squid axon and in the barnacle muscle indicate that there is a Na-Mg exchange mechanism in these tissues and that during Na^+-deprivation, an increase in Mg^{2+} entry should be observed (Baker & Crawford, 1972; Ashley & Ellory, 1972; Rojas & Taylor, 1975). The efflux of Mg^{2+} in these tissues also depends upon the extracellular Na^+ concentration and during Na^+ omission, there is a decrease in the Mg^{2+} efflux. This effect would produce an increase in the intracellular concentration of Mg^{2+}. Furthermore, these earlier experiments showed that the efflux of Mg^{2+} in these tissues was up to 20 times greater than that of Ca^{2+} (Baker & Crawford, 1972; Rojas & Taylor, 1975); (c) Another possibility is that Mg^{2+} enters the chromaffin cell through a channel other than the D600-sensitive channel. Experiments on barnacle muscles have shown that Mg^{2+} entry is not affected by tetrodotoxin treatment and that there is not a measurable current associated with the entry of Mg^{2+} (Rojas & Taylor, 1975). These findings led Rojas & Taylor (1975) to conclude that the entry of Mg^{2+} may involve the formation of a neutral complex like $MgCl_2$ or that an exchange with some internal cation might take place. Furthermore, although Baker & Crawford (1972) have suggested that perhaps Ca^{2+} and Mg^{2+} enter the squid axon through the same route, they have presented some evidence against a common mechanism of entry for these two ions, and that is, that in some axons, Ca^{2+} efflux was quite insensitive to the removal of extracellular Na^+ whereas in the same axons the efflux of Mg^{2+} was reduced in a normal manner.

In conclusion, the results presented in this paper suggest that Ca^{2+}, Sr^{2+} and Ba^{2+} may enter the chromaffin cell through the same channel and that Mg^{2+} may enter either through another channel which is D600-resistant, or that Mg^{2+} enters through the same Ca^{2+} channel but with a high rate of permeation. Alternatively, the inhibitory effects of Mg^{2+} during stimulation by Na^+-deprivation may be exerted at an extracellular site. Obviously, these aspects of the transport and effects of Mg^{2+} on the chromaffin cell deserve further study and experiments using ^{28}Mg should provide new and fundamental information about this problem.

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