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EXOCYTOTIC SECRETION OF CATECHOLAMINES FROM THE CAT ADRENAL MEDULLA BY SODIUM DEPRIVATION: INVOLVEMENT OF CALCIUM INFLUX MECHANISM

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1 Cat adrenal glands were perfused with Ca-deficient medium and secretion of catecholamines (CA) was induced by perfusion with Na-free medium in which NaCl was replaced by an osmotically equivalent amount of sucrose.

2 Release of CA and dopamine-β-hydroxylase (DBH), but not that of phenylethanolamine-*N*-methyltransferase, was concomitantly found in the effluents when the adrenals were stimulated, indicating that secretion was due to exocytosis.

3 Secretion of CA induced by Na-free (sucrose) medium was dependent on the concentration of Ca and was saturated at 0.5 mM of Ca.

4 Sr or Ba substituted for Ca in maintaining secretion by Na-free (sucrose) medium.

5 The addition of Na, Li or alkali metal ions to Na-free (sucrose) medium containing Ca reduced the response to a variable extent but this inhibition was reversed by raising the concentration of Ca in the Na-free medium.

6 All of the Na substitutes used induced secretion only when this medium contained Ca. However, different Na substitutes released different amounts of CA; sucrose was most effective, K, Tris and choline were moderately and Li least effective.

7 Secretion of CA by Na-free (sucrose) medium was strongly inhibited by D-600, tetracaine or divalent cations such as Co, Ni, Zn and Mg. The inhibition by Co was partially reversed by raising the concentration of Ca in the Na-free medium.

8 Secretion of CA from bovine isolated chromaffin cells was induced by Na-deficient (sucrose) medium and was dependent on the concentrations of ionized Ca involved.

9 All the Na substitutes tested increased secretion of CA and ^{45}Ca uptake, in a parallel fashion.

10 A correlation between secretion and ^{45}Ca uptake was found under various experimental manipulations which reduced secretion of CA.

11 These results demonstrated that unlike the perfused bovine adrenals, the Ca influx mechanism is essential for secretion by Na deprivation in the perfused cat adrenals as it is in bovine isolated chromaffin cells.

12 It is suggested that Na deprivation increases Ca entry through the Ca channels by eliminating the competition between Na and Ca, and possibly by activating Ca influx linked with Na efflux.

Introduction

Studies with chromaffin cells revealed that the action potentials generated by the physiological transmitter, acetylcholine (ACh) (Brandt, Hagiwara, Kidokoro & Miyazaki, 1976; Biales, Dichter & Tischler, 1976) or depolarization caused by a number of agents including K-enriched medium, promote the influx of Ca and that a prompt rise in the intracellular Ca provides the immediate stimulus for exocytotic secretion of catecholamines (CA) (Douglas, 1975). The evidence supporting this view has been obtained in other secretory cells. Iontophoretic injection of Ca releases the neurotransmitter from the squid giant axon (Miledi, 1973) and there is a degranulation (exocytosis) of mast cells (Kanno, Cochrane & Douglas, 1973). In the light of the Ca hypothesis, intracellular Ca bound to the membrane is also essential for the secretion which persists in the absence of extracellular Ca (Rahman & Borowitz, 1973; Lastowecka & Trifaro, 1974; Rink, 1977). Douglas & Rubin (1961) observed that in the absence of extracellular Ca, there was an increase in CA secretion when NaCl in the medium was replaced by sucrose. This secretion was biochemically shown

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to reflect an exocytosis and it was suggested that the response depended on the availability of intracellular Ca (Lastowecka & Trifaró, 1974).

The present study was undertaken on both cat and bovine adrenals in order to acquire further information on the mechanism of this secretion. The results suggest that the Ca influx mechanism is critically involved in this secretion in both species and that Na deprivation increases the Ca entry into the chromaffin cells thereby inducing the secretion.

Methods

Perfusion

Adrenal glands of the cat were retrogradely perfused at room temperature (24 to 26°C) through the adrenal vein by the procedure developed by Douglas & Rubin (1961). The only modification was that the glands were perfused by means of a peristaltic pump at a constant rate of flow of 0.6 to 1.0 ml/min. Two basic Ca-deficient perfusion media were used, either Tris-buffered solution containing (mM): NaCl 150, KCl 5, Tris(aminomethane)-maleate buffer (pH 7.2) 5 and glucose 11, or phosphate buffered solution containing (mM): NaCl 150, KCl 2, K phosphate buffer (pH 7.2) 3 and glucose 11. There was no discernible difference in the responses of the glands with the differently buffered solutions. Na-deficient and Na-free solutions were of the same composition as the main perfusion medium except that NaCl was partially or totally replaced by osmotically equivalent amounts of sucrose, choline Cl, LiCl, KCl or Tris Cl. When Ca-EGTA buffer (0.1 or 1.0 mM) was used to adjust the concentrations of ionized Ca contained in Na-free solution, 20 mM Tris-maleate buffer was used so that the final pH became 6.8 and in this solution, NaCl or Na substitute was reduced by an osmotically equivalent amount. HEPES or PIPES buffer with a pK_a near the working pH could not be used because of the increase in concentration of Na or K in the medium. These solutions were equilibrated with pure O_2. Effluents were collected in test tubes containing 10 µl of acetic acid. When dopamine-β-hydroxylase activity (DBH) was measured, non-acidified samples were divided into two parts: one for DBH assay was transferred to test tubes containing bovine serum albumin (BSA, final 0.25%) and the other for CA assay to test tubes containing acetic acid.

Bovine isolated chromaffin cells

Chromaffin cells were isolated by the method of Fenwick, Fajdiga, Howe & Livett (1978). Usually 3 to 4 bovine adrenals were used and approx. 2 to 3 × 10^7 cells were obtained. Chromaffin cells were purified and finally suspended in BSA (0.2%)-containing Tris-buffered solution used for perfusion experiments. More than 95% of the cells was found to be viable by exclusion of trypan blue.

For release experiments, 20 µl samples of cell suspensions, containing 2 to 5 × 10^5 cells, were added to plastic tubes containing 0.5 to 1.0 ml of Ca-deficient basic solution (mM: NaCl 135, KCl 5, Tris-maleate 20, pH 7.2 and glucose 11) or 0.5 to 1.0 ml of Na-free solution containing various concentrations of Ca adjusted by 1 mM Ca-EGTA buffer. Following incubation at 37°C for 10 min, the samples were chilled on ice, the cells were removed by centrifugation at 800 *g* for 5 min and the CA content in the supernatant was determined.

For ^{45}Ca uptake experiments, 20 µl samples of cell suspensions were added to plastic tubes containing 0.5 ml basic solution (or Na-free solution) and 0.1 mM of $^{45}CaCl_2$ (1 to 2 µCi). After 5 min incubation, the reaction was terminated by adding 1.5 ml of ice-cold basic solution, and the cell suspensions were layered over 0.2 ml of ice-cold basic solution containing 5% BSA which was kept in other plastic tubes, and centrifuged at 800 *g* for 5 min at room temperature. The resulting supernatant was discarded, and the tubes were freed of adhering solution with filter paper. The cell pellets were successively dispersed in 2 ml of toluene-Triton X-100 (2:1) scintillator twice and in 4 ml of toluene scintillator once. The radioactivity was measured by conventional scintillation counting. The net uptake of ^{45}Ca was calculated by subtracting the value obtained after incubation in an ice-water bath.

Analytical procedure

CA in the effluents was determined fluorimetrically by the conventional trihydroxyindole method (Anton & Sayre, 1962). DBH activity was measured radiochemically by the method of Molinoff, Weinshilboum & Axelrod (1971) as described previously (Sorimachi & Yoshida, 1979). Ca concentrations in the medium were measured by atomic absorption spectrophotometry (Gimblet, Marney & Bonsnes, 1967). NaCl-based solution was concentrated by a rotary evaporator at 40°C or by heating at 70°C in an oven, and sucrose-containing solution was ashed under reduced pressure at 120°C in a Hitachi Plasma Asher ASH-15R. Ca levels in Ca-deficient medium and in the effluents were found to be comparable (approx. 1 to 2 µM), indicating that little Ca leached into the solution out of the glands, even when a solution of low Ca content was perfused.

Chemicals

The chemicals were obtained from the following sources: tetracaine, from Sigma chemical Co. D-600

(methoxyverapamil) was a generous gift from Dr K. Yamatsu (Eisai Pharmaceutical Co., Japan). D-600 was dissolved in ethanol (80 to 160 mg/ml) and the final concentration of ethanol was 0.025 to 0.05%. All other chemicals were of analytical grade.

Results

Experiments with the perfused cat adrenal glands

Secretion of catecholamines induced by Na-free solution (replaced by sucrose) in the absence of Ca When the adrenals were perfused with Ca-deficient medium, the secretion of CA in response to ACh (0.1 mM) was greatly diminished (Figure 1). On the other hand, repeated secretion of CA was observed immediately after the perfusion medium was changed to a Na-free solution, in which sucrose was substituted for NaCl. The adrenals were therefore routinely perfused with Ca-deficient medium and the response to Na deprivation in these conditions was investigated. Figure 1 also shows the concomitant increase in the secretion of CA and DBH after stimulation by Na deprivation. On the other hand, phenylethanolamine-*N*-methyltransferase, a cytoplasmic enzyme converting noradrenaline to adrenaline, was never detected in the effluents. These results confirmed the observations by others (Lastowecka & Trifaró, 1974) indicating that the secretion is due to exocytosis.

However, the response to the Na-free (sucrose) medium was readily abolished by a chelating agent (EGTA) (Figures 1, 2) and these findings are in sharp contrast to those observed in the case of perfused bovine adrenals (Lastowecka & Trifaró, 1974). This inhibition was readily reversed by the removal of this agent (Figure 1) or by the addition of Ca to Na-free-EGTA solution (Figure 2), suggesting that the blocking effect of EGTA is not due to depletion of Ca bound to the surface membrane, but rather to chelation of contaminating Ca involved in Na-free solution (approx. 2 to 3 μM according to atomic absorption analysis). Indeed, the response was considerably reduced when the solution contained sucrose which had been through the cation exchanger, Amberlite IR-120[H^+], to remove any contaminating cations. In the following experiments, therefore, deionized sucrose was routinely used to replace NaCl.

Quantitative relation between secretion and Ca concentration of the Na-free (sucrose) solution There was a quantitative relation between the amounts of CA released by Na-free medium and the concentrations of Ca in this medium, over a wide range. This was shown by experiments in which the response to Na-free medium containing 10 μM and other concen-

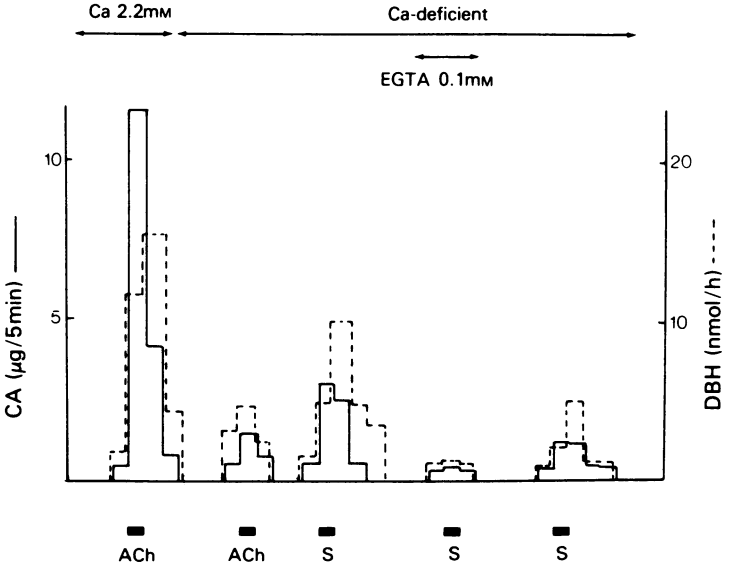

Figure 1 The effect of perfusion with Ca-deficient medium or Ca-free medium (containing 0.1 mM EGTA) on catecholamine (CA) and dopamine-β-hydroxylase (DBH) secretion from the cat adrenal. Secretion was induced by acetylcholine (ACh, 0.1 mM) or by Na-free medium(S) in which isotonic sucrose was substituted for NaCl. The height of the columns represents the amount of DBH (dashed line) and CA (continuous line) in the effluents collected every 5 min. The filled rectangles represent the period during which the gland was perfused with medium containing ACh or sucrose.

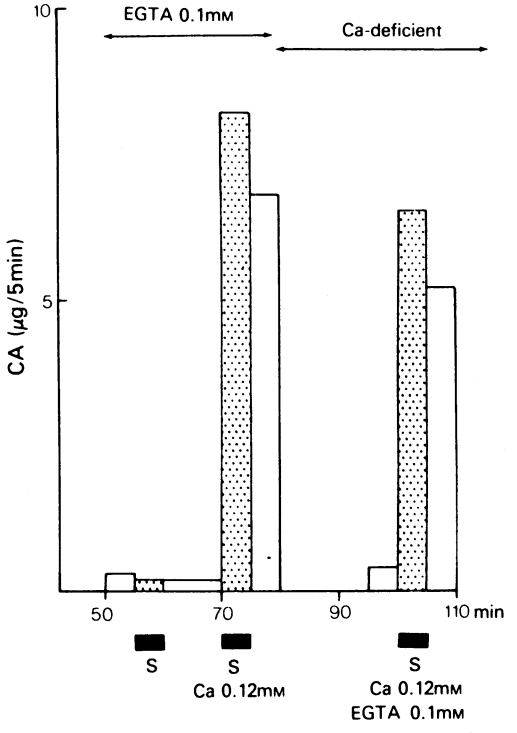

Figure 2 Inhibitory effect of EGTA on catecholamine (CA) secretion induced by Na-free medium (replaced by sucrose) and its reversal by the addition of Ca to Na-free medium containing EGTA. The cat adrenal was perfused throughout with Ca-deficient medium with or without EGTA (0.1 mM) and was stimulated by perfusion of either Na-free medium containing EGTA or containing both EGTA and $CaCl_2$ (0.12 mM) for a 5 min period (indicated by filled rectangles under the figure). The height of the columns represents the amount of CA in the effluents collected every 5 min. A representative result is shown and 3 additional experiments produced similar results.

trations (1 to 50 μM) of ionized Ca (concentrations were adjusted by Ca-EGTA buffer) were tested alternately. One of four such experiments is illustrated in Figure 3. Control experiments indicated that the magnitude of the response to Na-free medium containing 0.5 mM Ca decreased successively when tested at 25 min intervals; when the first response was referred to as 100%, the second response was 75.5 ± 4.1% (mean ± s.e. mean, $n = 6$), the third was 45.1 ± 4.7%, the fourth was 32.0 ± 3.0% and the fifth was 19.6 ± 2.5%. Thus, the second and the fourth responses were nearly equal to the mean of the first and the third response and to the mean of the third and fifth response, respectively. Therefore, the tested responses to Na-free medium containing Ca (1 to 50 μM) are expressed as percentages of the mean of the preceding and the following response observed in the presence of 10 μM Ca (Figure 4b). In another series of experiments, the tested responses to Na-free medium containing Ca (50 μM to 0.5 mM) were similarly expressed as percentages of the mean of the two responses in the presence of 2 mM Ca (Figure 4a). Figure 4 shows that the minimal concentration of Ca required for secretion over the resting level was about 1 μM and the secretion was saturated at 0.5 mM Ca.

The absolute amounts of CA released by Na-free medium containing the fixed level of Ca varied in different glands. When the glands were first stimulated with Na-free medium containing 10 μM ionized Ca, the amounts of CA released during the 5 min stimulation and the 5 min post-stimulation period ranged from 2.8 to 25.1 μg (mean 14.3 μg, $n = 12$). When the Ca level was increased to 100 μM, the range was 21.0 to 47.5 μg (mean 30.6 μg, $n = 11$), that is, 10 to 20% of total CA stored in the gland was released. These amounts are comparable to those released in response to ACh (0.1 mM) in the presence of 2 mM Ca, although the two kinds of response cannot be validly compared in the same gland as the response to ACh was greatly diminished when several periods of Na deprivation were interpolated, as described previously (Douglas & Rubin, 1961).

Time course of secretion induced by Na-free medium As shown in Figure 5a, the rate of secretion per min reached a maximum at 2 to 5 min when the glands were stimulated for 5 min with Na-free medium containing 10 μM Ca. In the presence of Na, reintroduction of such a low concentration of Ca into Ca-deficient medium never induced CA release (cf. Figure 8). When the Ca concentration in Na-free medium was increased to 2 mM, the maximal rate was attained more rapidly (Figure 5b). In the same figure is also included the rate of secretion in response to reintroduction of 2 mM Ca into Ca-deficient medium; the peak was observed during the first min after which secretion rapidly decreased (Figure 5c).

The ability of Sr and Ba to substitute for Ca in secretion induced by Na-free medium Sr and Ba have been shown to mimic Ca in that each induced secretion when introduced during perfusion with Ca-deficient medium and restored the responses to ACh and excess K otherwise absent in this solution (Douglas & Rubin, 1964). As shown in Figure 6, Sr or Ba could substitute for Ca to maintain secretion by Na-free medium in a concentration which, in the presence of Na, never induced secretion.

Effect of Na substitutes other than sucrose When Na was replaced by Li, choline or K in the absence of Ca, no secretion of CA occurred, as has been demonstrated in bovine slices (Rink, 1977). However,

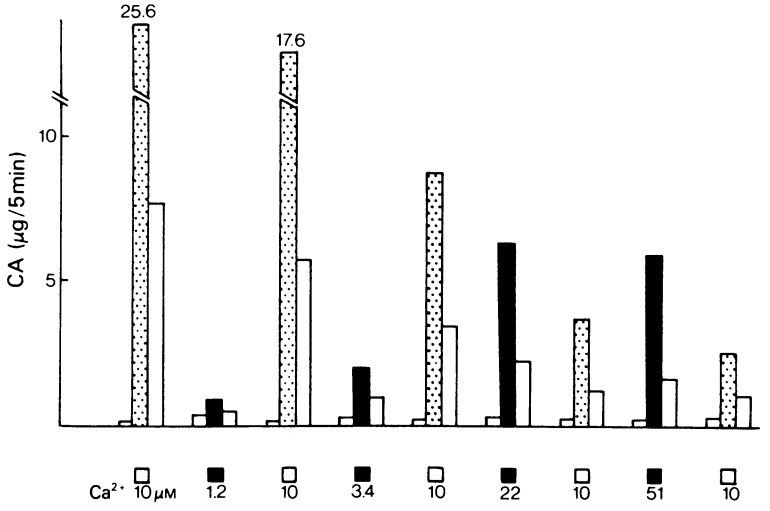

Figure 3 Ca-dependence of catecholamine (CA) secretion in response to Na-free (sucrose) medium. Ca-deficient medium was perfused throughout except for a 5 min period of stimulation (open or filled squares under the figure). The responses to Na-free medium containing 10 μM (open squares and dotted column) or other concentrations of ionized Ca (adjusted by 0.1 mM of Ca-EGTA buffer; filled squares and columns) were tested alternately at 25 min intervals. Re-introduction of ionized Ca (up to, at least, 50 μM) did not induce secretion in the presence of Na.

marked secretion was observed when the various concentrations of Ca were added to these Na-free solutions. In Table 1, the responses induced by solutions with four different Na substitutes containing various amounts of Ca were compared with those induced by sucrose-substituted medium containing a given concentration of Ca. It is apparent that different Na substitutes produced quantitatively different effects, but the rate of secretion was similar for any Na substitute used; secretion was highest during the 5 min stimulation period, followed by that during the 5 min post-stimulation period and then fell to the level comparable to that during the pre-stimulation period. Although replacing Na and Tris was shown to produce a gradual increase of secretion in bovine slices (Rink, 1977), this replacement produced an immediate secretion in the perfused cat adrenals.

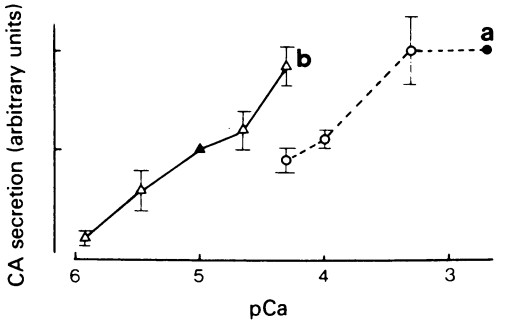

Figure 4 Ca-dependence of catecholamine (CA) secretion induced by Na-free (sucrose) medium. As shown in Figure 3, the tested responses to Na-free medium containing various amounts of ionized Ca were compared to the mean of two control responses to Na-free medium containing 10 μM ionized Ca (b). In another series of similar experiments, the tested responses were compared to the mean of two control responses to Na-free medium containing 2 mM Ca (a). In (a) the concentrations of Ca were adjusted by adding Ca to sucrose medium without the use of Ca-EGTA buffer. Each point and bar represents the mean and s.e. mean, respectively ($n = 4$).

Inhibition of the response to Na deprivation by Na, Li or K, and its reversal by raising the concentration of Ca The response to Na-free (sucrose) medium containing 3.4 μM Ca was markedly reduced when 10 mM Na was added to this solution (Figure 7; isotonicity was maintained by reducing sucrose to 20 mM). In four experiments, the response in the presence of Na was 26.3 ± 5.6% (mean ± s.e. mean) of the mean of the preceding and the following responses in the absence of Na. This effect of Na could be, at least partially, reversed by increasing the concentration of Ca contained in the Na-free medium; the higher the concentrations of Ca, the less the inhibition (Figures 7, 8). When the concentrations of Na were increased, a more marked inhibition was observed, but a similar reversal of inhibition was observed when increasing the concentrations of Ca, regardless of the concentration of Na used (Figure 8).

It should be noted that in Ca-deficient conditions, introduction of Ca alone (if increased to over 0.5 mM)

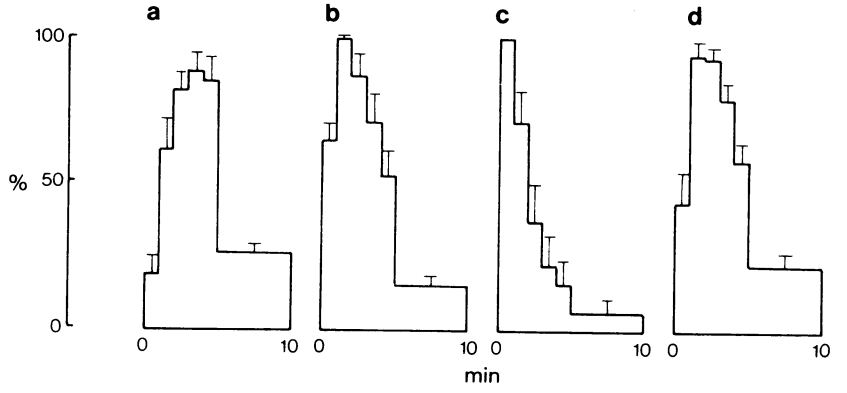

Figure 5 Time course of catecholamine (CA) secretion induced by Na-free (sucrose) medium or by re-introduction of Ca. In (a to c), Ca-deficient medium was perfused throughout except for a 5 min stimulation period and secretion was induced by perfusion of Na-free medium containing 10 μM (a) or 2 mM (b) Ca or by perfusion of Na-based conventional medium containing 2 mM Ca (c). In (d) adrenals were perfused throughout with medium containing 2 mM Ca and secretion was induced by perfusion of Na-free medium containing 2 mM Ca for 5 min. In each experiment, rate of secretion per min was expressed as percentage of the maximal rate. Each column and bar represents the mean and s.e. mean, respectively (n = 4 to 7).

induced secretion, even in the presence of Na, a phenomenon which was first described by Douglas & Rubin (1961). This finding raises the question as to whether or not it is the increased permeability of the cell membrane, induced by the omission of Ca from the perfusion medium (Douglas & Rubin, 1961) and not Na deprivation *per se*, which is responsible for secretion. Na deprivation may enable the lower concentrations of Ca, which are otherwise ineffective, to induce secretion. Experiments were therefore done to determine whether the presence of Ca or Mg in the perfusion medium would prevent secretion induced by Na deprivation. We found that Na-free medium containing 2 mM Ca induced secretion even after the gland was perfused with Ca (2 mM)-containing medium (time course of secretion is shown in Figure 5d). However, the magnitude of secretion was much smaller than that in the absence of Ca; the response in the presence of Ca (2 mM) was only 19.6 ± 4.0% (n = 4) of the mean of the preceding and the following responses to Na-free medium containing 3.4 μM Ca, which were obtained after the glands had been exposed to Ca-deficient medium for 15 min. There was a similarly reduced response when the glands were previously perfused for 20 min with Mg (2 mM)-containing medium and were stimulated with Na-free medium containing 50 μM Ca and no Mg. In three experiments, the response after prior exposure to Mg-containing medium was 35.1 ± 10.0% of the mean of the two control responses obtained in the absence of both Ca and Mg.

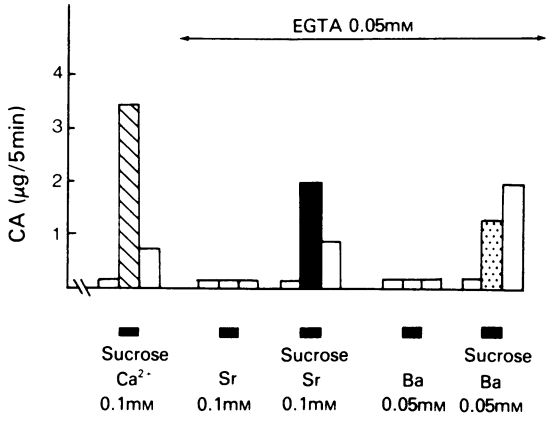

Figure 6 The effect of Na-free (sucrose), Ca-free medium containing Sr or Ba on catecholamine (CA) secretion. Either ion restored the response to Na deprivation in a concentration which failed to induce secretion in the presence of Na. EGTA was added to Ca-deficient medium in the presence or absence of Na to rule out the possible secretion due to contaminating Ca. Adrenals were stimulated with Na-free medium several times before starting this experiment, thus the response to Na deprivation was weakened. A representative result is shown and two additional experiments produced similar results.

The addition of Li to Na-free (sucrose) medium containing 3.4 μM Ca was also found to diminish secretion to 21.0 ± 4.7% (n = 3) or 14.0 ± 5.8% (n = 3) of the mean of two control responses at concentrations of 10 mM and 20 mM, respectively. The addition of alkali metal ions (K, Rb or Cs) to Na-free medium containing 3.4 μM Ca inhibited secretion, but to a lesser extent than Li; the response was

Table 1 The effects of different Na substitutes on catecholamine (CA) secretion

Na substitutes	*Concentrations of ionized Ca(mM)*				
	0.01	0.05	0.1	0.5	2.0
K	71 ± 15(3)	81 ± 10(3)	—	—	—
Choline	53 ± 8(5)	39 ± 4(5)	—	—	—
Li	12 ± 3(7)	30 ± 3(9)	—	11 ± 5(4)	8(2)
Tris	—	—	55 ± 11(3)	43 ± 6(3)	—

Ca-deficient medium was perfused throughout except for a 5 min period of stimulation with medium containing different Na substitutes and with different concentrations of ionized Ca. In each experiment, the responses to Na-free medium with Na replaced by sucrose and by other substitutes containing a given concentration of Ca were tested alternately and values are expressed as percentages of the mean of two responses to sucrose-substituted medium. Values represent the mean ± s.e. mean and numbers of experiments are indicated in parentheses.

57.0 ± 4.7% (n = 6) and 49.5 ± 5.9% (n = 6) of mean controls in the presence of 20 mM and 40 mM, respectively (relative potencies of inhibiting secretion were similar among these ions, and the data were combined). However, the inhibition by Li or alkali metal ions was reversed by raising the concentration of Ca added to Na-free medium; inhibition was only 32.0% (mean of two experiments) in the presence of 20 mM Li, and no significant inhibition was found in the presence of 20 mM K (two experiments) when the concentration of Ca was raised to 10 μM in each case.

The effects of Ca channel blockers The results described above strongly suggest that the Ca influx mechanism is critically involved in secretion induced by Na-deprivation in the cat adrenals. We therefore investigated the effects of Ca channel blockers on this secretion to determine whether or not Ca entry through the specific channels is responsible for secretion (Douglas & Kanno, 1967; Jaanus, Miele & Rubin, 1967; Baker, Meves & Ridgway, 1973; Pinto & Trifaró, 1976).

The response to Na-free (sucrose) medium was strongly inhibited by both the organic blockers D-600 and tetracaine (Figure 9, Table 2) and divalent ions such as Co, Ni, Zn (each 0.1 mM) and Mg (1 mM) either when the gland was treated for 10 min prior to stimulation (organic agents) or when directly added to Na-free medium (divalent ions). The responses in the presence of divalent ions ranged from 0 to 32% of the mean of two control responses when stimulated with medium containing 3.4 μM Ca (more than two experi-

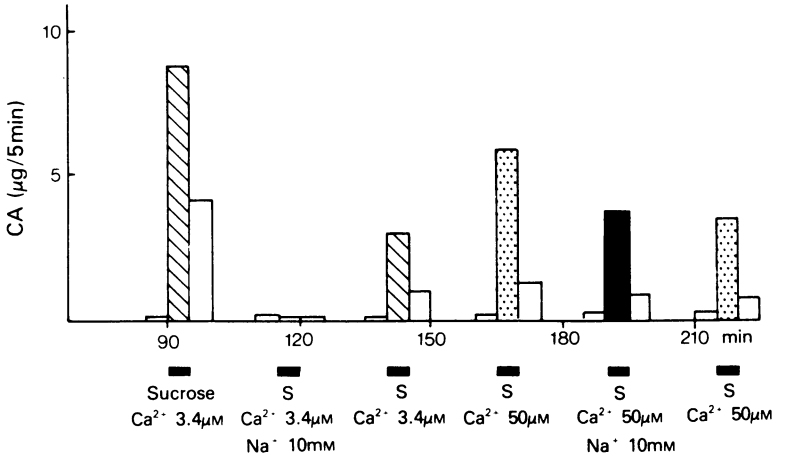

Figure 7 The inhibitory effect of Na on catecholamine (CA) secretion in response to Na deprivation, and its reversal by raising the concentration of Ca contained in Na-deficient medium. Ca-deficient medium was perfused throughout except for a 5 min period of stimulation (filled rectangles under the figure). The adrenal gland was stimulated with Na-free (sucrose) or Na-deficient medium containing 3.4 μM ionized Ca (left half of the figure) or 50 μM Ca (right half of the figure).

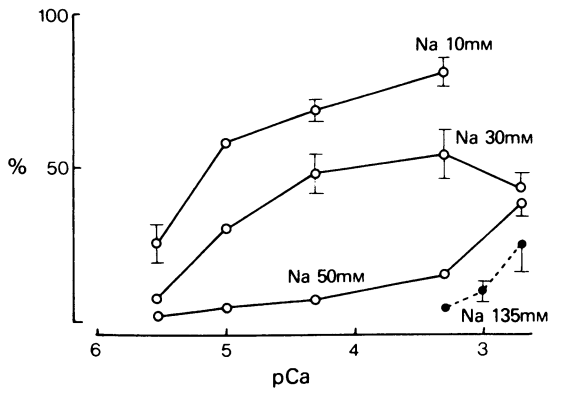

Figure 8 The inhibition by Na of catecholamine (CA) secretion induced by Na-free medium (sucrose) and its reversal by raising the concentration of Ca contained in the Na-deficient medium. At each concentration of Ca in the Na-deficient medium (abscissae), the responses to Na-deficient medium containing the various amounts of Na were expressed as percentages of the mean of two responses to Na-free (sucrose) medium as shown in the representative experiment in Figure 7. The points without a bar represent the mean of two experiments, and those with bars are the mean of 3 to 4 experiments with s.e. mean. Note that the adrenals were perfused throughout with Ca-deficient medium: therefore the Ca-dependent secretion was obtained in the presence of a conventional concentration of Na (135 mM).

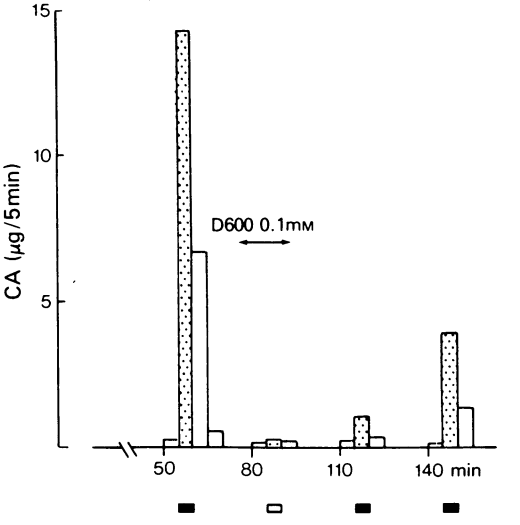

Figure 9 The effect of D-600 (0.1 mM) on catecholamine (CA) secretion induced by Na-free (sucrose) medium containing 0.1 mM Ca. Ca-deficient medium was perfused throughout except for a 5 min period of stimulation and for a 20 min period of exposure to D-600. Two control experiments showed that 0.05% ethanol used to dissolve D-600 did not affect the response to Na deprivation. Note the slow reversibility of the blocking effect of D-600.

ments were done for each divalent ion). The inhibition by tetracaine or divalent ions was easily reversed by removal of the agent but a long washing period was required for the recovery of the response after the removal of D-600 (Figure 9, Table 2).

The inhibition by Co was, at least partially reversed by raising the concentration of Ca added to Na-free medium (Figure 10). The addition of 70 μM Co to Na-free medium containing 3.4 μM Ca completely blocked secretion, while the response in the presence of the same concentration of Co was 35.7 ± 5.2% (n = 3) of the controls when the level of Ca was raised to 50 μM.

Experiments with bovine isolated chromaffin cells

The chromaffin cells isolated from the bovine medulla contained 1 μg CA per $2.11 \pm 0.17 \times 10^4$ cells (n = 13). The preparations purified in the absence of Ca responded poorly to ACh stimulation unless pretreated with Ca at 37°C (Fenwick *et al.*, 1978) but these cells secreted CA in response to excess K or Na

Table 2 The effects of tetracaine and D-600 on catecholamine (CA) secretion induced by Na-free (sucrose) medium containing 3.4 μM Ca

Test agents	*% of the first response* 2nd response	3rd response
Tetracaine (0.33 mM)	7.4 ± 4.5 (7)	35.6 ± 5.0 (7)
D-600 (0.04–0.08 mM)	7.8 ± 4.0 (7)	10.9 ± 3.8 (7)

Adrenals were perfused during the first and third stimulation with Na-free medium containing 3.4 μM Ca. Ten min before the second stimulation, test agents were added to Ca-deficient medium. Test agents were removed from the medium 5 min after the second stimulation. CA secretion during the 10 min collection period after the first stimulation is referred to as 100% and that in the second and third stimulation is expressed as a percentage of the first response (mean ± s.e. mean). Numbers of experiments are shown in parentheses.

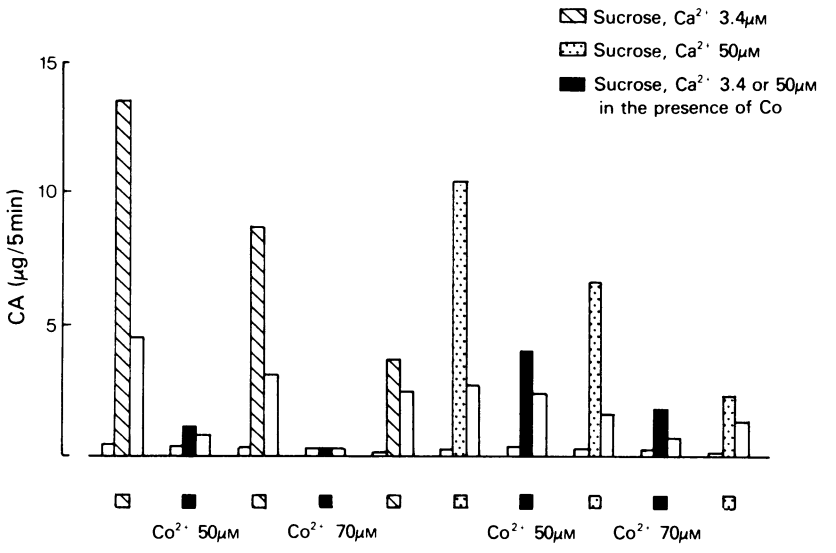

Figure 10 The inhibitory effect of Co on catecholamine (CA) secretion by Na-free (sucrose) medium and its partial reversal by raising the concentration of Ca contained in Na-free medium. The adrenal gland was stimulated for 5 min with Na-free medium containing 3.4 μM Ca in the presence (filled column) or absence (shaded column) of Co (left half of the figure) and with Na-free medium containing 50 μM Ca in the presence (filled column) or absence (dotted column) of Co (right half of the figure).

deprivation in the presence of Ca, without Ca pretreatment.

Catecholamine release Some features of secretion by these cells were noted; the temperature-dependent resting secretion of CA was higher and the secretion induced by either excess K or Na deprivation in the presence of 2 mM Ca was smaller than that in the perfused adrenals of the cat. CA release during 10 min stimulation by Na deprivation never exceeded 5 to 6% of the total cellular content, while in the cat perfused adrenals the release often reached 15 to 20% with the same stimulation. These results suggest that these cells could be useful for investigating the secretory mechanism, but that some deterioration of cellular function might occur during the purification procedure.

All of the Na substitutes induced secretion over the resting level in the presence of Ca (Figure 11). The secretion was dependent on the concentrations of Ca and also on the Na substitute used, as it was in the cat adrenals.

^{45}Ca uptake When the isolated cells were incubated at 37°C in the presence of Na, the temperature-dependent uptake of ^{45}Ca (0.1 mM) slowly increased with time (Figure 12). On the other hand, incubation of the cells in Na-deficient solution replaced by sucrose resulted in a rapid and a marked increase in the uptake of Ca (Fig. 12). Na deprivation caused a 10 to 20 fold stimulation of Ca uptake during 5 min incubation period, indicating that the Ca influx mechanism is involved in the secretion induced by Na deprivation.

Correlation between secretion and Ca uptake To determine the exact correlation between secretion and Ca uptake, in some experiments the cells were divided into two parts, one of which was used for release experiments and the other for Ca uptake experiments. Both were incubated in the presence of 0.1 mM Ca under the same conditions and these results are shown in Table 3. Any of the Na substitutes tested increased Ca uptake or CA release in a parallel fashion. Tris was found to have equal potency to sucrose, followed by choline and Li in increasing both Ca uptake and CA release.

The addition of Na to Na-free (sucrose) medium dose-dependently inhibited both Ca uptake and CA release, in a parallel fashion. Similar tendencies were also observed in the inhibitory effects of Mg and Co on the two parameters. These ions thus inhibit secretion by blocking inward Ca movement, although the possibility has been raised that in some secretory cells, including the adrenal medulla, that the inhibitory action of Mg is exerted at the intracellular Ca sites (Douglas & Poisner, 1964; Rahman, Borowitz & Miya, 1973; Lastowecka & Trifaró, 1974; Cochrane

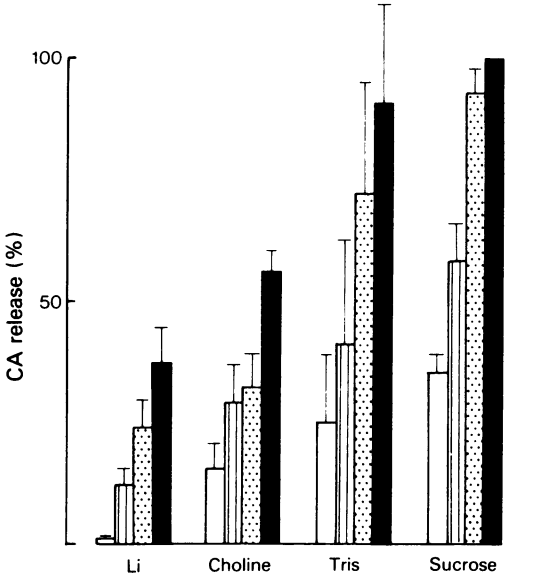

Figure 11 Catecholamine (CA) secretion from bovine isolated chromaffin cells in response to different Na substitutes, and its dependence on the concentrations of ionized Ca (adjusted by 1 mM of Ca-EGTA buffer). CA secretion induced by sucrose-substituted medium containing 5.6 mM Na and 0.5 mM Ca is taken as 100% in each experiment and the responses to solutions with different Na substitutes and containing 5.6 mM Na and different concentrations of ionized Ca are expressed as percentages. CA secretion induced by Na-deficient medium containing EGTA was comparable to the spontaneous secretion in the presence of Na. The responses to Na-deficient medium containing 10 μM (open column), 50 μM (shaded column), 0.2 mM (stippled column) and 0.5 mM (solid column) are shown from left to right in each group of columns. Each column and bar indicates the mean and s.e. mean. (n = 3), respectively.

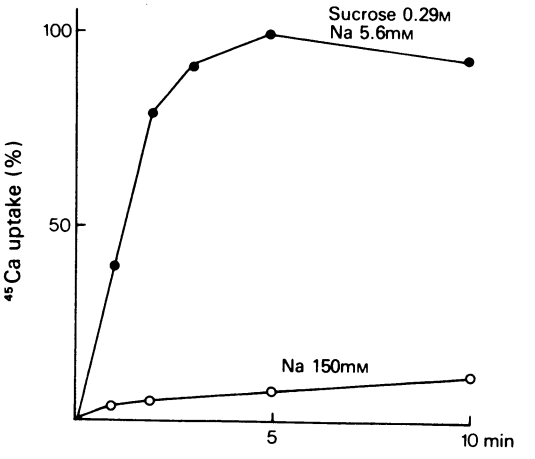

Figure 12 Time course of ^{45}Ca uptake by bovine isolated chromaffin cells. Cells were incubated at 37°C for 1 to 10 min with Na-deficient (sucrose) medium (●) or Na-based medium (○) containing 0.1 mM ^{45}Ca. Uptake is expressed as a percentage of the maximal uptake. Values are the means of comparable duplicate determinations and two additional experiments produced similar results.

& Douglas, 1976). We did not investigate the effects of D-600 and tetracaine as both agents were found to increase the spontaneous secretion of CA.

Discussion

The requirement of extracellular Ca for secretion of CA in the adrenal medulla is well established (Douglas, 1975). There is also evidence that secretion can be induced in the absence of Ca under certain experimental conditions (Rahman *et al.*, 1973; Rahman & Borowitz, 1973; Lastowecka & Trifaró, 1974; Rink, 1977). Evidence favouring exocytotic secretion is lacking in most cases with the exception that this mechanism was indicated in the secretion observed when sucrose replaced NaCl in the bovine adrenals (Lastowecka & Trifaró, 1974). Trifaró and coworkers considered that this secretion depended on the availability of intracellular Ca (Lastowecka & Trifaró, 1974; Pinto & Trifaró, 1976; Aguirre, Pinto & Trifaró, 1977). Rink (1977) also investigated this mechanism of secretion using the same animal species and suggested that secretion is not due to Na deprivation, but to the sucrose solution substituted for NaCl as he found no secretion when Li or choline replaced Na in the absence of Ca.

However, in sharp contrast to these observations we found that in the cat adrenals, secretion with a sucrose-substituted solution was dependent on the concentration of extracellular Ca, and that all the Na substitutes could induce secretion only when extracellular Ca was available, thus indicating that Na deprivation *per se* is closely related to the marked secretion. An essentially similar dependency of secretion on Ca was observed in bovine isolated chromaffin cells (Figure 11). The discrepancy between the results with the perfused adrenals (Lastowecka & Trifaró, 1974) and those with isolated cells of the same species has not been explored but it is possible that the intact cells use both intracellular and extracellular Ca for secretion induced by Na deprivation, and that some properties of cells are altered during the enzyme digestion so that secretion depends only on extracellular Ca.

The minimal concentration of Ca required for secretion by Na-free (sucrose) medium is about 1 μM,

Table 3 Correlation between catecholamine (CA) secretion and ^{45}Ca uptake by bovine isolated chromaffin cells

Medium	*^{45}Ca uptake(%)*	*CA secretion(%)*
Na 150 mM	9.8 ± 0.9 (6)	22.9 ± 2.5 (5)
K 60 mM	26.6 ± 3.3 (5)	30.3 ± 2.0 (3)
Sucrose 0.29 M, Na 5 mM	100	100
As above, plus Mg 1 mM	48.9 ± 2.5 (3)	67.2 ± 3.6 (3)
As above, plus Mg 5 mM	24.5 ± 2.3 (3)	31.9 ± 2.2 (3)
As above, plus Co 1 mM	16.2 ± 0.9 (3)	24.6 ± 3.1 (3)
Sucrose 0.27 M, Na 15 mM	60.7 ± 4.1 (3)	70.5 (2)
Sucrose 0.234 M, Na 33 mM	25.8 ± 0.8 (3)	38.5 (2)
Sucrose 0.198 M, Na 51 mM	17.6 ± 0.9 (3)	23.0 (2)
Li 0.145 M, Na 5.6 mM	44.8 ± 3.1 (4)	55.7 ± 7.4 (4)
Choline 0.145 M, Na 5 mM	48.0 ± 6.1 (4)	52.0 ± 3.9 (4)
Tris 0.185 M, Na 5 mM	90.3 ± 19.7 (4)	127.5 ± 25.3 (4)

The media contained 0.1 mM Ca. The values for CA secretion and Ca uptake obtained under the appropriate conditions are expressed as percentages of those obtained with sucrose (0.29 mM)-based medium containing 5.6 mM Na. Incubations were carried out at 37°C for 10 min for CA secretion and for 5 min for Ca uptake. In each experiment, values (mean ± s.e. mean) were obtained by duplicate determinations, and the number of experiments is shown in parentheses.

and the secretion was saturated at a concentration of 0.5 mM Ca. When the adrenals were stimulated with ACh, secretion was not saturated even at a concentration of 2 mM since secretion increased further when the concentrations of Ca were raised (Douglas & Rubin, 1961). Thus, the apparent affinity of the secretory process for Ca is increased when sucrose replaces NaCl. This may not be due to increase in the affinity for Ca of the intracellular Ca sites as it was reported that in cells rendered freely permeable to Ca, secretion was saturated at about 10 μM Ca (Baker & Knight, 1978), but is probably due to the saturation of these sites with increase in the entry of ionized Ca (see below).

Experiments carried out on the endocrine pancreas have also shown that Na deprivation produced an immediate rise in insulin secretion (Lambert, Henquin & Malvaux, 1974; Griffey, Conaway & Whitney, 1974) and that this is Ca-dependent (Griffey *et al.*, 1974). It was suggested that secretion in this tissue may be due to an increase in the level of intracellular ionized Ca as a result of a decrease in Ca efflux following the removal of extracellular Na. The existence of a Na-dependent Ca extrusion mechanism has been demonstrated in the adrenal medulla (Rink, 1977; Aguirre *et al.*, 1977) as in other excitable tissues (Baker & Reuter, 1975) and Na deprivation was expected to produce a decrease in the net Ca efflux even in the presence of Ca (Aguirre *et al.*, 1977). A decrease in the rate of Ca extrusion in the absence of Na would explain the failure of the transient secretion to return quickly to the basal level (compare Figure 5b and c), but it seems unlikely that this mechanism is directly involved in secretion induced by Na deprivation. Na-dependent Ca efflux was shown to be little affected by Mg and Co (Rink, 1977), which strongly inhibited secretion by Na deprivation. In experiments on isolated chromaffin cells, we found that ^{45}Ca uptake was rapidly promoted by Na deprivation and that this uptake is fairly well correlated with secretion, under various experimental conditions.

These results favour the view that Na deprivation promotes Ca entry into the cells either by activation of Ca influx linked with Na efflux (Baker, Blaustein, Hodgkin & Steinhardt, 1969; Baker, 1972; Baker & Reuter, 1975) or by suppression of competition between the two cations (Niedergerke, 1963; Dean & Matthews, 1970; Droograms & Casteels, 1979). As shown in Figure 8, the introduction of relatively high concentrations of Ca alone induced secretion, even in the presence of a conventional concentration of Na. This is not consistent with the former mechanism which includes an increase in the $[Na]_i/[Na]_o$ ratio, and raises the possibility that the increased permeability of the plasma membrane, induced by Ca removal, is a prerequisite for the secretion induced by Na deprivation and that Na competes with Ca to penetrate the plasma membrane. This view is not incompatible with the findings that the secretion induced by Na deprivation was markedly reduced when permeability of the cell membranes was maintained at a normal level by perfusion with a medium containing conventional concentrations of Ca or Mg. If this is feasible, then the different effects of various Na substitutes in producing secretion could be explained by assuming that some of the Na substitutes mimic Na, to a variable extent, in preventing Ca movement. This assumption is reasonable if we con-

sider that Li can substitute for Na in producing action potentials (Hodgkin & Katz, 1949; Keynes & Swan, 1959). Indeed, the addition of Li or K to Na-free (sucrose) medium significantly inhibited secretion when the level of Ca was 3.4 μM, but this inhibition was readily reversed by raising the concentration of Ca to 10 μM.

On the other hand, it should be kept in mind that, unlike the reintroduction of Ca (Douglas & Rubin, 1963), Na deprivation continued to induce a significant secretion even when the plasma membrane was stabilized with Ca or Mg. Thus, the increase in Ca influx probably involves mechanisms other than suppression of competition between Na and Ca. Although Rink (1977) did not demonstrate the existence of a Ca influx mechanism in exchange for internal Na, nevertheless, this mechanism may be related to the residual secretion in the presence of Ca or Mg. Related investigations are in progress in our laboratory.

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References

AGUIRRE, J., PINTO, J.E.B. & TRIFARÓ, J.M. (1977). Calcium movements during the release of catecholamines from the adrenal medulla: effects of methoxyverapamil and external cations. *J. Physiol.*, **269**, 371–394.

ANTON, A.H. & SAYRE, D.F. (1962). A study of the factors affecting the aluminum oxide trihydroxyindole procedure for analysis of catecholamines. *J. Pharmac. exp. Ther.*, **138**, 360–375.

BAKER, P.F. (1972). Transport and metabolism of calcium ions in nerve. *Progr. Biophys. molec. Biol.*, **24**, 177–223.

BAKER, P.F., BLAUSTEIN, M.P., HODGKIN, A.L. & STEINHARDT, R.A. (1969). The influence of calcium on sodium efflux in squid axons. *J. Physiol.*, **200**, 431–458.

BAKER, P.F. & KNIGHT, D.E. (1978). Calcium-dependent exocytosis in bovine adrenal medullary cells with leaky plasma membranes. *Nature*, **276**, 620–622.

BAKER, P.F., MEVES, H. & RIDGWAY, E.B. (1973). Effects of manganese and other agents on the calcium uptake that follows depolarization of squid axons. *J. Physiol.*, **231**, 511–526.

BAKER, P.F. & REUTER, H. (1975) In *Calcium Movement in Excitable Cells* pp. 1–100. Oxford: Pergamon.

BIALES, B., DICHTER, M. & TISCHLER, A. (1976). Electrical excitability of cultured adrenal chromaffin cells. *J. Physiol.*, **262**, 743–753.

BRANDT, B. L., HAGIWARA, S., KIDOKORO, Y. & MIYAZAKI, S. (1976). Action potentials in the rat chromaffin cell and effects of acetylcholine. *J. Physiol.*, **263**, 417–439.

COCHRANE, D.E. & DOUGLAS, W.W. (1976). Histamine release by exocytosis form rat mast cells on reduction of extracellular sodium: a secretory response inhibited by calcium, strontium, barium or magnesium. *J. Physiol.*, **257**, 433–448.

DEAN, P. M. & MATTHEWS, E.K. (1970). Electrical activity in pancreatic islet cells: effects of ions. *J. Physiol.*, **210**, 265–275.

DOUGLAS, W.W. (1975). Secretomotor control of adrenal medullary secretion: synaptic, membrane, and ionic events in stimulus-secretion coupling. In *Handbook of Physiology*, section 7, vol. 6, *Endocrinology*. pp. 367–388. Washington: American Physiological Society.

DOUGLAS, W.W. & KANNO, T. (1967). The effect of amethocaine on acetylcholine-induced depolarization and catecholamine secretion in the adrenal chromaffin cell. *Br. J. Pharmac.*, **30**, 612–619.

DOUGLAS, W.W. & POISNER, A.M. (1964). Calcium movement in the neurohypophysis of the rat and its relation to the release of vasopressin. *J. Physiol.*, **172**, 19–30.

DOUGLAS, W.W. & RUBIN, R.P. (1961). The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol.*, **159**, 40–57.

DOUGLAS, W.W. & RUBIN, R.P. (1963). The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. *J. Physiol.*, **167**, 288–310.

DOUGLAS, W.W. & RUBIN, R.P. (1964). The effects of alkaline earths and other divalent cations on adrenal medullary secretion. *J. Physiol.*, **175**, 231–241.

DROOGRAMS, G. & CASTEELS, R. (1979). Sodium and calcium interactions in vascular smooth muscle cells of the rabbit ear artery. *J. gen. Physiol.* **74**, 57–70.

FENWICK, E.W., FAJDIGA, P.B., HOWE, N.B.S. & LIVETT, B.G. (1978). Functional and morphological characterization of isolated bovine adrenal medullary cells. *J. cell Biol.*, **76**, 12–30.

GIMBLET, E.G., MARNEY, A.F. & BONSNES, R. (1967). Determination of calcium and magnesium in serum, diet and stool by atomic absorption spectrophotometry. *Clin. Chem.*, **13**, 204–214.

GRIFFEY, M.A., CONAWAY, H.H. & WHITNEY, J.E. (1974). Insulin secretion induced by Na^+ deprivation. *Endocrinology*, **95**, 1469–1472.

HODGKIN, A.L. & KATZ, B. (1949). The effects of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.*, **108**, 37–77.

JAANUS, S.D., MIELE, E. & RUBIN, R.P. (1967). The analysis of the inhibitory effect of local anaesthetics and propranolol on adrenomedullary secretion evoked by calcium or acetylcholine. *Br. J. Pharmac. Chemother.*, **31**, 319–330.

KANNO, T., COCHRANE, D.E. & DOUGLAS, W.W. (1973). Exocytosis (secretory granule extrusion) induced by injection of calcium into mast cells. *Can. J. Physiol. Pharmac.*, **51**, 1001–1004.

KEYNES, R.D. & SWAN, R.C. (1959). The permeability of frog muscle to lithium ions. *J. Physiol.*, **147**, 626–638.

LAMBERT, A.E., HENQUIN, J.C. & MALVAUX, P. (1974). Cationic environment and dynamics of insulin secretion. I. Effect of low concentrations of sodium. *Endocrinology*, **95**, 1069–1077.

LASTOWECKA, A. & TRIFARÓ, J.M. (1974). The effect of sodium and calcium ions on the release of catecholamines from the adrenal medulla: sodium deprivation induces release by exocytosis in the absence of extracellular calcium. *J. Physiol.*, **236**, 681–705.

MILEDI, R. (1973). Transmitter release induced by injection of calcium ions into nerve terminals. *Proc. R. Soc. B.*, **183**, 421–425.

MOLINOFF, P.B., WEINSHILBOUM, R. & AXELROD, J. (1971). A sensitive enzymatic assay for dopamine-β-hydroxylase. *J. Pharmac. exp. Ther.*, **178**, 425–431.

NIEDERGERKE, R. (1963). Movements of Ca in frog heart ventricles at rest and during contractures. *J. Physiol.*, **167**, 515–550.

PINTO, J.E.B. & TRIFARÓ, J.M. (1976). The different effects of D-600 (methoxy-Verapamil) on the release of adrenal catecholamines induced by acetylcholine, high potassium or sodium deprivation. *Br. J. Pharmac.*, **57**, 127–132.

RAHMAN, R.G., BOROWITZ, J.L. & MIYA, T.S. (1973). The role of intracellular calcium in catecholamine secretion from the bovine adrenal medulla. *J. Pharmac. exp. Ther.*, **184**, 106–118.

RAHMAN, R.G. & BOROWITZ, J.L. (1973). Mechanisms of stimulus-secretion coupling in adrenal medulla. *J. pharm. Sci.*, **62**, 1911–1923.

RINK, T.J. (1977). The influence of sodium on calcium movements and catecholamine release in thin slices of bovine adrenal medulla. *J. Physiol.*, **226**, 297–325.

SORIMACHI, M. & YOSHIDA, K. (1979). Exocytotic release of catecholamines and dopamine-β-hydroxylase from the perfused adrenal gland of the rabbit and cat. *Br. J. Pharmac.*, **65**, 117–125.

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