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Calcium Control of Exocytosis and Endocytosis in Bovine Adrenal Medullary Cells [and Discussion]

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Calcium control of exocytosis and endocytosis in bovine adrenal medullary cells

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[Plate 1]

Experimental analysis of the mechanisms of exocytosis and endocytosis has hitherto been hampered by the inaccessibility of the intracellular sites at which they are controlled. We have recently developed a technique that overcomes this problem. Cells are subjected to intense electric fields of brief duration; this renders the plasma membrane permeable without impairing its ability to participate in exocytosis and endocytosis. Working with 'leaky' bovine adrenal medullary cells, catecholamine release has a rather specific requirement for Mg-ATP, is activated by micromolar concentrations of ionized Ca and can be inhibited by Mg, detergents, trifluoperazine, high osmotic pressure and various anions. The mechanism of activation by Ca is discussed in some detail.

Introduction

The formation and fusion of vesicles is an important mechanism for achieving the transport of both membrane-bound and water-soluble substances between different intracellular compartments and between the cell interior and the external medium. The plasma membrane is one of the sites where these processes are especially amenable to study. It has long been known that a host of secretory mechanisms, including the release of nervous transmitter substances as well as many hormones and enzymes, involves the loading of the substances, destined for export, into vesicles and their subsequent release after fusion of the vesicle membrane with the surface membrane of the cell in a process called exocytosis (for reviews see Katz (1969), Baker (1974) and Case (1978)). The resulting increase in membrane area is opposed by a process of endocytosis that returns the same or an equivalent area of plasma membrane to the cell interior (see review by Silverstein *et al.* 1977).

Endocytosis in its simplest form also serves to effect the indiscriminate uptake into the cell of substances that are present in the extracellular fluid; but in many, if not all, cells one form of endocytosis has become highly specialized to effect the internalization of extracellular molecules that have become bound to specific receptors at the cell surface (see Schlessinger *et al.* 1978). Irrespective of physiological function, constancy of surface area requires that any membrane internalized by endocytosis must be replaced, presumably by exocytosis. It therefore seems likely that cycles of exocytosis and endocytosis effect a continuous bidirectional traffic of substances into and across the plasma membrane providing, within a rather simple set of mechanisms, a highly flexible system for establishing communication between cells and their environment.

In order to find out more about the mechanisms of exocytosis and endocytosis we have chosen as our experimental material the bovine adrenal medulla, from which it is possible to obtain

a large number of cells capable of secreting the catecholamines noradrenalin and adrenalin. The secretory materials are packaged within the so-called 'chromaffin granules', membrane-bounded vesicles that contain, in addition to catecholamines, also ATP and a variety of proteins including dopamine-β-hydroxylase and chromogranin, together with the peptides Met- and Leu-enkephalin and their precursors (Schneider *et al.* 1967; Viveros 1975; Schultzberg *et al.* 1978). Stimulation of these cells by the physiological secretagogue acetylcholine causes release of catecholamine along with all the other soluble contents of the chromaffin granules (see Smith *et al.* 1970; Livett *et al.* 1981; Baker & Knight 1980), but fails to release molecules of similar size that are free in the cytosol. This exocytotic release of stored products is dependent on extracellular calcium, a feature that catecholamine release shares with a great many other exocytotic systems (Douglas 1968). A key factor in controlling the frequency of exocytotic events seems to be the concentration of ionized Ca in the cytosol (see Baker 1974; Llinás *et al.* 1981), and most secretory cells have elaborate mechanisms for transducing physiological signals into alterations in cytosolic ionized Ca: a rise in ionized Ca concentration serves to increase the occurrence of exocytosis and a fall to decrease it.

The initiation of exocytosis and endocytosis in intact cells

Isolation of cells

The bovine adrenal medulla is a rather compact structure, and a useful first step in simplifying the analysis of the initiation of exocytosis in this tissue is to work with isolated cells; but it is important to verify whenever possible that these isolated cells retain the properties of the intact gland. We routinely prepare cells by serial treatments of thin slices of medullary tissue with protease (0.2%, Sigma type V) followed by collagenase (0.2%) and hyaluronidase (0.2%). This protocol is preferred to collagenase alone because it gives a higher yield of cells. Either method gives cells that retain most of the properties of the intact tissue (see figure 1*a*). After dissociating the tissue and washing away the enzymes, the isolated cells are normally incubated for 1 h in saline solution containing 0.5% (by mass) bovine serum albumin.

The initiation of exocytosis

Cells prepared in this way lose catecholamine spontaneously very slowly (about 2% per hour) but can be stimulated to secrete by a variety of secretagogues including nicotinic agonists, veratridine and elevated K concentration. These agents seem to evoke exocytosis because all the soluble contents of the chromaffin vesicles are released whereas cytosolic markers such as lactate dehydrogenase are not. Figure 1 illustrates the response to a variety of nicotinic agonists and antagonists. Not shown, but of considerable interest to the overall sequence of events, is the finding that tetrodotoxin (TTX) also blocks the response to nicotinic agonists. This suggests that acetylcholine may bring about secretion through the mediation of Na-dependent and TTX-sensitive action potentials. Direct evidence in favour of this was provided by Brandt *et al.* (1976) who demonstrated TTX-sensitive action potentials in rat adrenal medullary cells maintained in short-term culture. The frequency of these action potentials is very low in resting cells (0.1/s), but can increase roughly 30-fold on exposure to acetylcholine. These findings are consistent with the view that acetylcholine brings about a small depolarization that serves to increase the frequency of action potentials and that these action potentials are an essential link between the acetylcholine receptor and secretion. At the neuromuscular junction the nicotinic post-junctional channels permit Ca to enter the muscle and, given that a rise in cytosolic Ca

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concentration is likely to cause secretion, it is somewhat surprising that catecholamine release from adrenal medullary cells seems not to be activated directly by nicotinic agonists. Such a direct effect may be possible with very high concentrations of acetylcholine because cultured cells have been reported to release catecholamine in a TTX-sensitive manner at low concentrations of acetylcholine and in a TTX-insensitive manner at high concentrations of

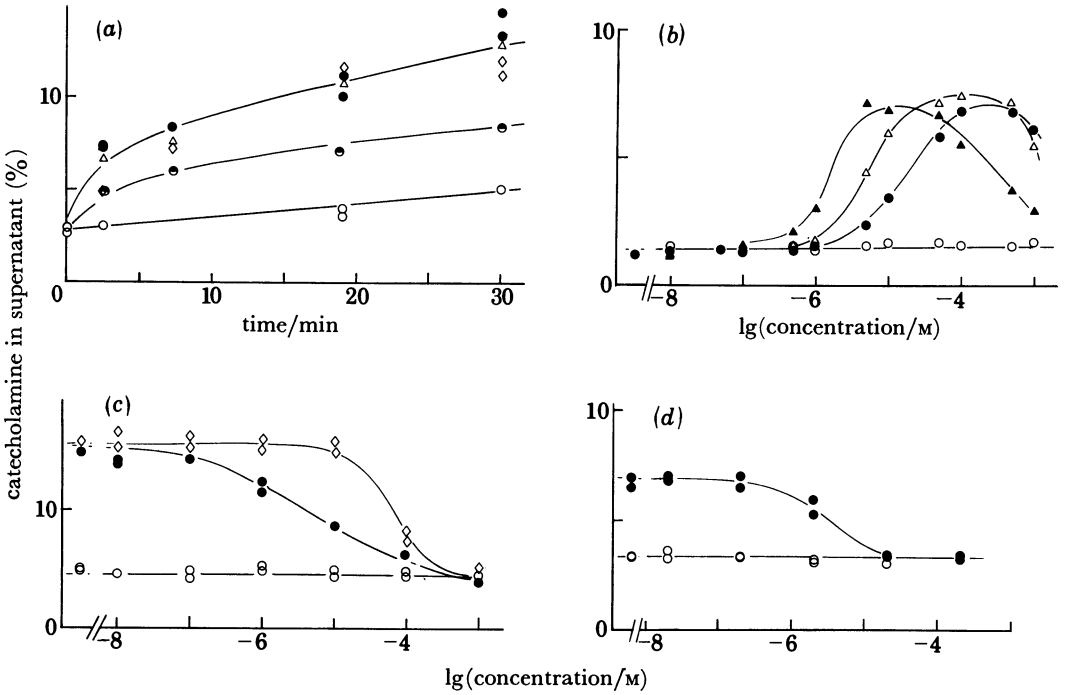

FIGURE 1. Catecholamine release from cells exposed to nicotinic agonists in saline solution containing 3.6 mM $CaCl_2$ at 37 °C. Ordinate: catecholamine in the supernatant expressed as a percentage of the total in the cell suspension. (*a*) Catecholamine release from cells isolated by Sigma protease type V (●), Sigma protease type VI (△) and Sigma collagenase (◇) exposed to 10^{-4} M carbamylcholine, ⊖, protease type V cells exposed to 10^{-5} M carbamylcholine; ○, unstimulated cells protease type V. (*b*) Cells exposed for 15 min to nicotine (▲), acetylcholine (△), carbamylcholine (●) and methacholine (○). (*c*) Cells incubated in hexamethonium (●) or atropine (◇) for 3 min and then exposed to 10^{-4} M carbamylcholine for 10 min. ○, Unstimulated cells. (*d*) Cells incubated with D600 for 3 min and then challenged with 10^{-4} M carbamylcholine for 10 min. ○, Unstimulated cells; ●, stimulated cells.

secretagogue (Kilpatrick *et al.* 1981). The underlying electrophysiology of these effects has yet to be analysed.

Electrical activity seems to facilitate Ca entry by a route that is sensitive to D600, a well known blocker of Ca channels. In the presence of TTX, secretion can still be elicited by high K concentration and remains sensitive to D600, suggesting that TTX and D600 act at different sites. By analogy with other systems it seems likely that the action potential serves to open voltage-sensitive and D600-sensitive channels through which Ca enters the cell (Baker *et al.* 1971; Baker & Glitsch 1975). Studies with ^{45}Ca provide direct evidence for an increased Ca inflow in response to secretagogues, but the interpretation of the data is complicated because Ca efflux also changes.

Figure 2 summarizes the effects of a variety of blocking agents on secretion elicited by the three secretagogues carbamylcholine, veratridine and high K concentration. Also included are

the effects of the Ca ionophore A23187 and of the calmodulin antagonist trifluoperazine. Although A23187 liberates catecholamine in a Ca-dependent manner it also brings about a quantitatively similar release of lactate dehydrogenase, which suggests that cells may be damaged. This is supported by the appearance of the cells, by their stainability with trypan blue and by the failure of trifluoperazine to block A23187–induced secretion whereas it blocks

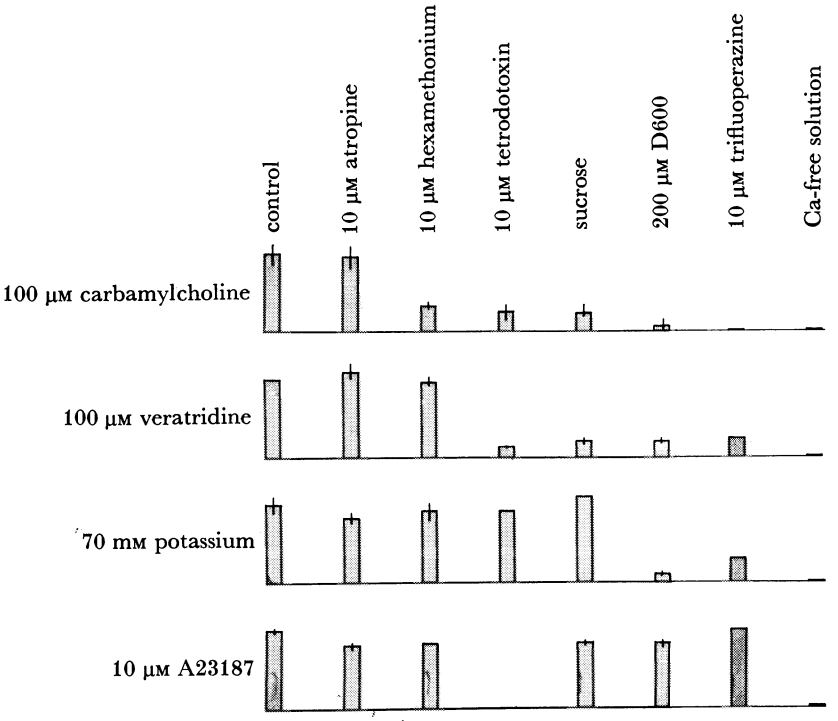

FIGURE 2. Summary of effects of various inhibitors on response of bovine adrenal medullary cells to different secretagogues. Cells in saline solution containing $CaCl_2$ at 3.6 mM (except in the last column where the saline was Ca-free) were incubated with the inhibitors as shown for 3 min before exposing them to the secretagogues. The column labelled 'sucrose' corresponds to cells in a sucrose solution containing 3.6 mM $CaCl_2$ and challenged as above. Cells were exposed to the secretagogues for 10 min and the percentage of the total cellular catecholamine released was measured. The results are expressed as the amounts of catecholamine released relative to that released by control cells that had not been incubated with the inhibitors. The error bars represent the range of between two and five experiments.

that induced by the other secretagogues. The data are all consistent with a rather simple chain of events: under physiological conditions, (1) acetylcholine released from the splanchnic nerve acts on nicotinic receptors to bring about a small depolarization; (2) this causes an increased frequency of firing of Na-dependent and TTX-sensitive action potentials; (3) the large

DESCRIPTION OF PLATE 1

FIGURE 3. Transmission electron micrographs of intact and 'leaky' adrenal medullary cells fixed in ice-cold glutaraldehyde (20 g l^{-1}), post-fixed with osmium tetroxide (20 g l^{-1}) and stained with uranyl acetate and alkaline lead citrate. (*a*) Unstimulated intact cell in saline solution. (*b*) Intact cell challenged with 500 μM carbamylcholine for 20 min before fixing. The challenge evoked a release of 10% of the catecholamine in the bulk of the cells. Note the appearance of apparently empty vacuoles within the cytoplasm. (*c*, *d*) A population of cells was exposed to intense electric fields (exposures to 2000 V cm^{-1}, τ = 200 μs) in a solution similar to that described in table 1 (except that the K glutamate was replaced isosmotically by sucrose), challenged with 10 mM EGTA, ionized Ca in the region of 10 nM (*c*), or in 10 mM Ca EGTA ionized Ca in the region of 10 μM (*d*), and fixed 20 min later. The 10 μM Ca^{2+} evoked a release of 25% of the catecholamine in the bulk of the cells. Note the appearance in (*d*) of apparently empty vacuoles within the cytoplasm.

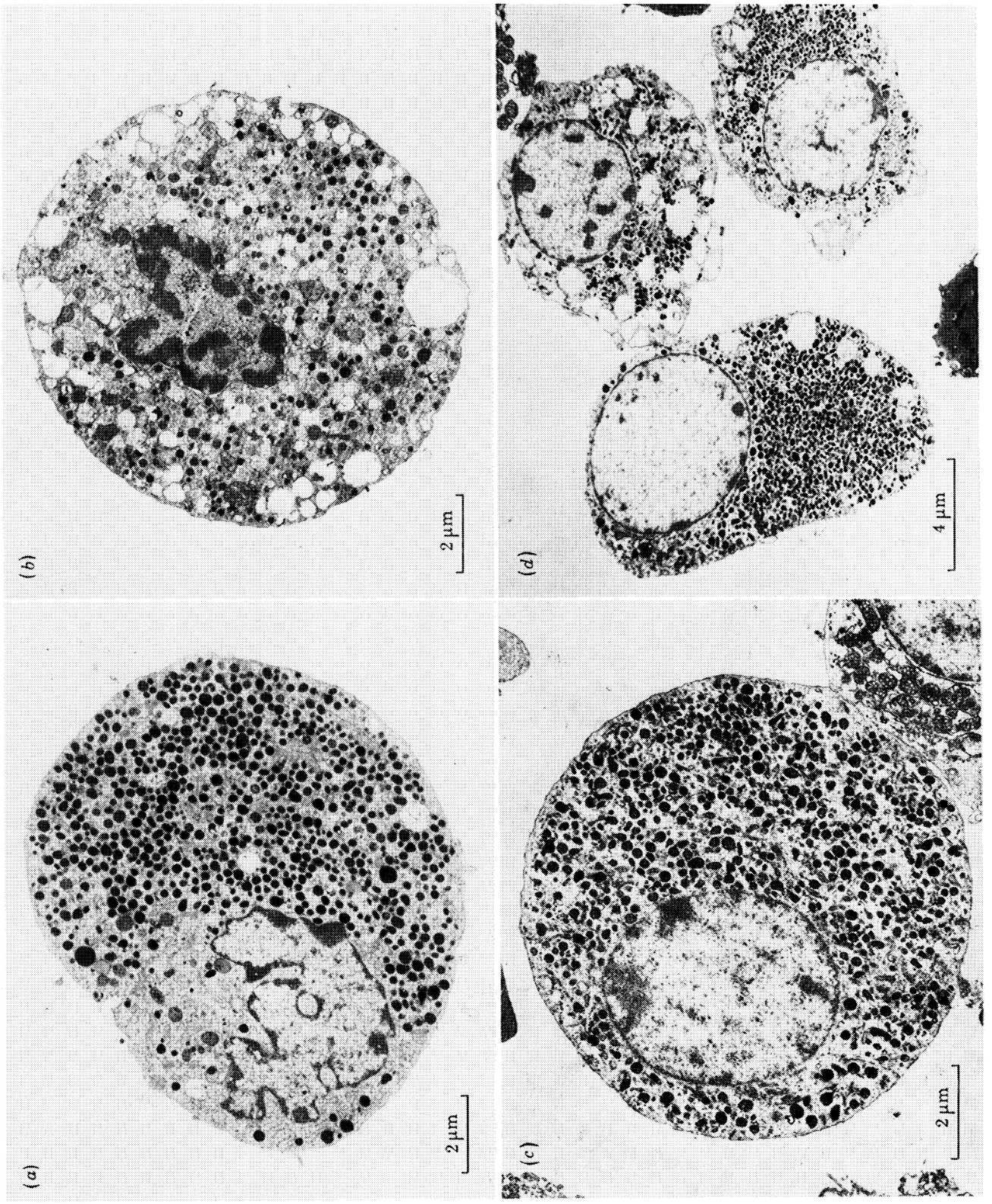

FIGURE 3. For description see opposite.

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depolarization during each action potential serves to open up D600-sensitive Ca channels through which Ca enters the cytosol; (4) the elevated cytosolic Ca concentration activates exocytosis in a reaction that is sensitive to the antipsychotic drug trifluoperazine. Separate acetylcholine-gated channels, voltage-sensitive Na, K and Ca channels as well as Ca-gated K channels have recently been demonstrated in cultured bovine adrenal medullary cells with the aid of the patch clamp technique (Fenwick *et al.* 1981; Marty 1981).

Two further interesting features of stimulation–secretion coupling in adrenal cells are (1) the transient nature of catecholamine release from cells subjected to a maintained depolarization, and (2) the reduction in secretion brought about by a variety of peptides including substance P, somatostatin and enkephalins. Baker & Rink (1975) presented evidence that the transient release of catecholamine in response to high K concentration does not reflect depletion of some releasable store of transmitter, but may rather reflect failure or inactivation of calcium entry (but see later). The mechanism of action of the various peptides has not yet been investigated electrophysiologically, but the peptides only seem effective when the stimulus for secretion is acetylcholine and not when it is high K concentration (Mizobe *et al.* 1979; Kumakura *et al.* 1980). This suggests that they may inhibit secretion by reducing the effectiveness of acetylcholine either by exerting a direct effect on the nicotinic receptors or by acting at another site so as to minimize the effectiveness of acetylcholine as a depolarizing agent.

The initiation of endocytosis

A single exposure to acetylcholine releases about 6% of the total catecholamine in the cell. The exocytotic release of this amount of catecholamine might increase the cell surface by approximately 16%. From what was said in the introduction one might expect to find that stimulation of exocytosis will be associated with an increased rate of membrane retrieval by endocytosis, and this seems to be so (Nagasawa & Douglass 1972). Examination by transmission electron microscopy of thin sections of adrenal medullary cells that have recently been stimulated to secrete reveals a number of large membrane-bounded vesicles located mainly in the peripheral cytoplasm (see figure 3, plate 1). If stimulation is performed in the presence of horseradish peroxidase (5 mg ml^{-1}), reaction product is subsequently found in these peripheral vesicles, suggesting that they have been exposed to the extracellular fluid. They seem to become cut off from the extracellular space rather rapidly because when cells are exposed to secretagogues in the presence of [^{3}H]sucrose and subsequently thoroughly washed, stimulated cells contain significantly more sucrose than unstimulated cells, suggesting that secretion stimulates the internalization of extracellular fluid. This pattern of events resembles that described in the posterior pituitary (Nordmann *et al.* 1974).

Exocytosis and endocytosis under conditions of intracellular solute control

A high-voltage technique for gaining access to the cell interior

A major obstacle to elucidating the mechanisms of exocytosis and endocytosis is that they are controlled by events occurring at the inner face of the plasma membrane, which is relatively inaccessible to experimental manipulation. We have recently developed an electrical technique that permits the plasma membrane barrier, and the physiological mechanisms for initiating exocytosis, to be bypassed without impairing exocytosis and endocytosis (Baker & Knight 1978, 1980). This is achieved by exposing a suspension of cells to one or more high-voltage discharges

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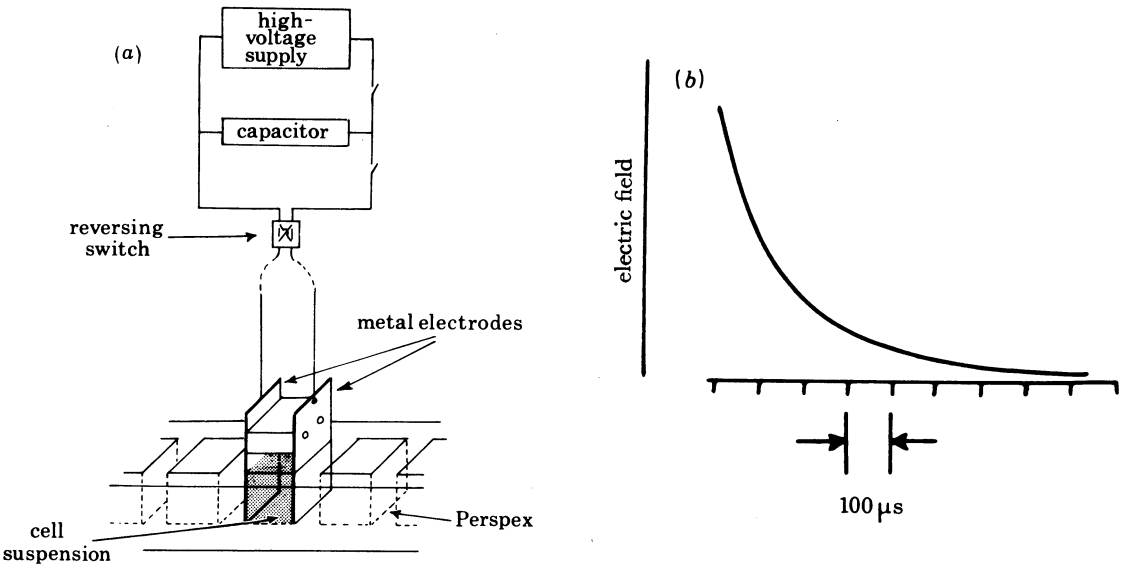

FIGURE 4. (*a*) The experimental set-up used to expose a suspension of cells to a brief electric field. (*b*) The time course of the electric field through the cell suspension made up in a glutamate solution as described in table 1 when the applied high voltage is 2 kV and the capacitor 2 μF. The metal electrodes were stainless steel.

of brief duration. The experimental set up is shown in figure 4. It is a modification of that used by Zimmerman and his colleagues on red blood cells (Zimmerman *et al.* 1975). The externally applied field required to effect dielectric breakdown in the surface membrane of a sphere increases as the diameter of the sphere decreases. It follows that by suitable choice of field it is easy to cause localized breakdown of the plasma membrane without affecting the intracellular organelles. By exposing cells to a series of discharges it is possible to create 6–10 pores each of effective diameter 4 nm. The resulting 'leaky' cells equilibrate rapidly with substances of low molecular mass placed in the external medium. Such substances include catecholamine, EGTA and ATP. Although erythrocytes subsequently reseal if incubated at 37 °C, the bovine adrenal medullary cells do not reseal and remain fully accessible for up to 1 h.

TABLE 1. COMPOSITION OF MEDIUM IN WHICH CELLS ARE RENDERED 'LEAKY'

medium		comments
potassium glutamate	140 mM	K can be replaced by Na, but the nature of the major anion is important
Mg-ATP	5.0 mM	essential
PIPES, pH 6.6	20.0 mM	pH can be varied from 6.0 to 8.0
EGTA	0.4 mM	can be increased to at least 50 mM without altering the results
free Mg^{2+}	2.0 mM	
ionized Ca	< 100 nM	
glucose	5.0 mM	not essential

The choice of intracellular solutions

If the 'leaky' cells are to remain competent to undergo exocytosis and endocytosis, the composition of the medium in which the discharges are delivered proves to be most important. Table 1 lists the composition of the solution that we have used, together with comments on the importance or otherwise of its various components. Exocytosis proves to have a very specific requirement for Mg-ATP and to be rather sensitive to the nature of the major anion. Provided these constraints are met, the release of catecholamine from 'leaky' cells depends entirely on the ionized Ca concentration to which they are exposed.

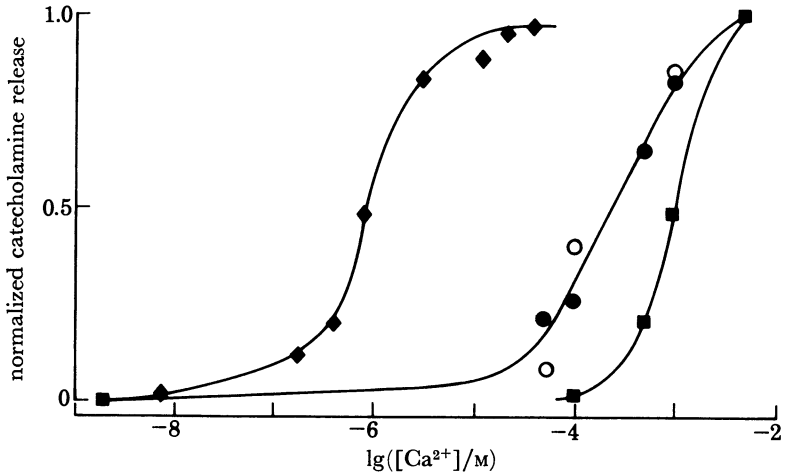

FIGURE 5. Comparison of the Ca dependence of catecholamine release in intact and 'leaky' cells. Intact cells in saline solution containing 2 mM Mg were exposed for 15 min to 500 μM carbamylcholine (●), 500 μM veratridine (■) or 70 mM K^+ (○). Cells in a K glutamate medium as described in table 1 were challenged with Ca for 15 min after being rendered 'leaky' by ten exposures to 2 kV cm^{-1} (τ = 200 μs) (◆). Ordinate: catecholamine release normalized to that in 5 mM Ca for the intact cells and to the maximum response of the 'leaky' cells. Temperature 37 °C.

The dependence on ionized calcium

At an ionized Ca concentration of 10 nM or lower, less than 1% of the total cellular catecholamine is released on making the cells 'leaky'; but if the ionized Ca concentration is subsequently raised into the micromolar range, up to 30% of the cellular catecholamine appears in the external medium. This rather simple experiment permits two important conclusions: (1) there is very little free catecholamine in the cytosol, and certainly not enough to account for the 5–10% of total catecholamine released from intact cells exposed to secretagogues such as carbamylcholine or K, and (2) the Ca concentration required to evoke catecholamine release from 'leaky' cells is almost three orders of magnitude lower than in intact cells (figure 5). Figure 6 shows an extension of this type of experiment. Cells are first exposed to radioactive noradrenalin, washed well, rendered 'leaky' and finally challenged with 10 μM Ca. Measurements of total and labelled catecholamine released reveal few differences except that the specific activity in the cytosol is somewhat higher than that in the whole cell presumably because the isotope had not equilibrated fully with all the cellular catecholamine.

The suggestion has often been made that the concentration of ionized Ca immediately internal to the surface membrane of intact secretory cells may rise to levels well in excess of micromolar, perhaps even to millimolar, and it might be argued that even in 'leaky' cells the

weakly buffered micromolar concentrations of Ca that evoke secretion are effecting the release of much higher concentrations of Ca locally. This seems highly unlikely because in 'leaky' cells the Ca-activation curve shows no change over a range of Ca buffer concentrations from 0.4 to 50 mM. Our data on activation by Ca are best fitted on the assumption that 2 Ca ions cooperate to effect secretion.

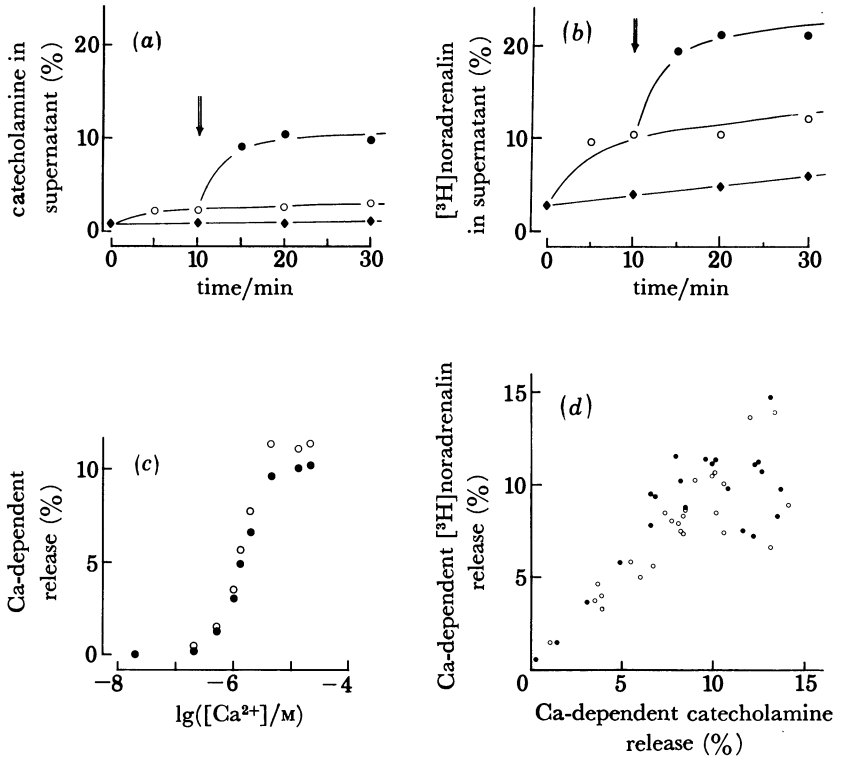

FIGURE 6. Release of labelled and unlabelled catecholamine from 'leaky' cells. Cells in saline solution were incubated for 90 min in L-[7,8-^{3}H]noradrenalin and then washed in a glutamate solution as described in table 1. (*a*, *b*). Cells were subjected to ten exposures of 2 kV cm^{-1} (τ = 200 μs) in the presence of 10 nM Ca^{2+} (○) and 10 min later were challenged with 10 μM Ca^{2+} (arrows) (●). ◆, Cells not exposed to the field. Ordinate: amount of (*a*) unlabelled catecholamine, (*b*) labelled noradrenalin in the supernatant expressed as a percentage of the total in the suspension. (*c*) Cells were challenged with Ca EGTA buffers for 20 min after first subjecting them to ten exposures of 2 kV cm^{-1} (τ = 200 μs). The Ca-dependent release is expressed as a percentage of the total in the cells. ○, Labelled noradrenalin; ●, unlabelled catecholamine. (*d*) Cells in glutamate solution (table 1) were challenged with a range of Ca^{2+} concentration (0.2–20 μM) after first subjecting them to ten exposures of 2 kV cm^{-1} (τ = 200 μs) and the amounts of labelled and unlabelled catecholamine secreted determined (●). Also included are some data for intact cells immersed in saline solution containing 3.6 mM Ca^{2+} and exposed to a range of acetylcholine concentrations for various times (○). The axes are shown as the amounts secreted as a percentage of the total in the cells. Temperature throughout, 37 °C.

Evidence for exocytosis and endocytosis

The reasons for believing that Ca-dependent release of catecholamine from 'leaky' cells occurs by exocytosis are as follows.

1. All the soluble contents of the storage vesicles seem to be released in a Ca-dependent fashion, whereas cytosolic markers of similar molecular mass are not.

2. Of the soluble contents of the storage granules that we have examined, which range in size from catecholamine (M_r = 330) to dopamine-β-hydroxylase (M_r = 290000) the time course of their appearance in the extracellular medium is the same (see figure 7). This is not consistent with vesicle breakdown within the cell and subsequent escape of the stored products

by diffusion through the holes in the plasma membrane because the escape through these same holes of the cytosolic marker lactate dehydrogenase is very much slower. It is, however, precisely what would be expected if release occurred by exocytosis. Figure 8 is of particular interest because it shows that release of the peptide Met-enkephalin has properties that are virtually identical to the release of catecholamine. There is still uncertainty about whether these two molecules are packaged in the same or different vesicles. The data of figure 8 could be consistent with either possibility, although if separate populations of vesicles are involved, release of their contents would seem to have very similar properties.

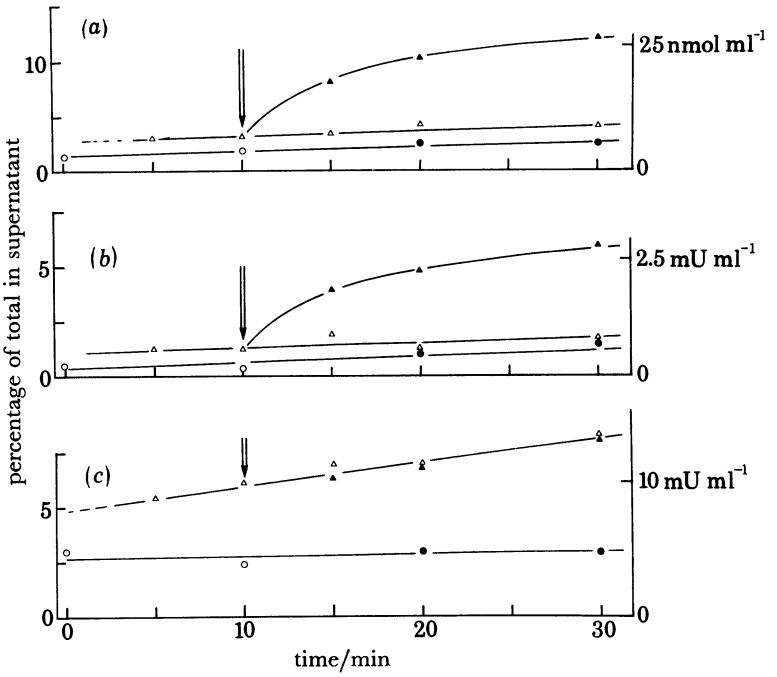

FIGURE 7. The time course of release of (*a*) catecholamine (*b*) dopamine-β-hydroxylase, and (*c*) lactate dehydrogenase in response to a Ca challenge. Cells were suspended in a medium similar to that shown in table 1, except that the glutamate had been isosmotically replaced by sucrose and the EGTA concentration was 0.4 mM, and were subjected to ten exposures of 2 kV cm^{-1} (τ = 200 μs) (△); 10 mins after rendering them leaky, 0.4 mM $Ca(OH)_2$ was added to the cell suspension (arrows) (▲). Cells from the same population not rendered leaky (○) also had $Ca(OH)_2$ added at the same time (●). Temperature 37 °C. Ordinates: amounts of catecholamine, dopamine-β-hydroxylase and lactate dehydrogenase in the supernatant expressed as a percentage of their total cellular content and also in units of concentration.

3. The ultrastructure of 'leaky' cells that have been challenged with Ca closely resembles that of intact cells that have been exposed to nicotinic agonists, veratridine or high K concentration (see figure 3). The periphery of the cell contains a number of large membrane-bounded vacuoles. Horseradish peroxidase (5 mg ml^{-1}) only enters 'leaky' cells very slowly, but when these cells are challenged with 10 μM Ca, reaction product is visible in the membrane-bounded vacuoles. Likewise, if 'leaky' cells are immersed in [^{3}H]sucrose, sucrose enters the cells. Provided that the medium contains enough EGTA to stabilize an ionized Ca concentration of less than 10 nM, most of this sucrose can subsequently be washed out of the cells. If, however, the cells are exposed to 10 μM Ca, part of the sucrose behaves as if it is bound and is very difficult to remove by repeated washing: a finding consistent with trapping of sucrose in endocytotic vacuoles. The dependence of the binding on ionized Ca concentration is very similar to that of exocytosis.

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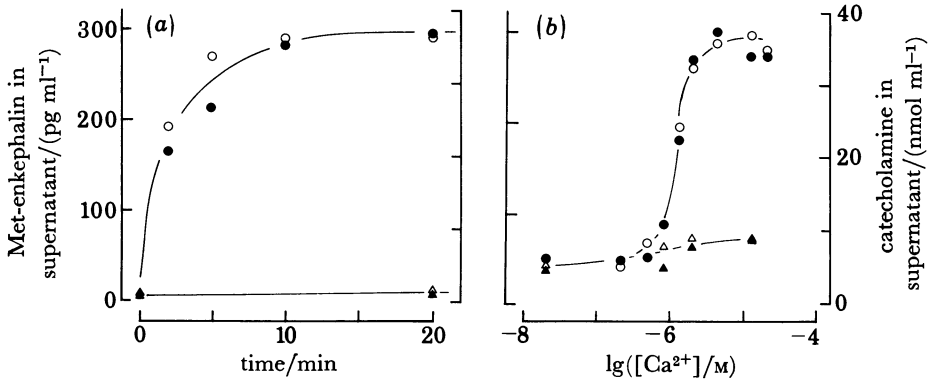

FIGURE 8. Comparison of the time course and Ca-dependence of catecholamine and Met-enkephalin release from 'leaky' cells.

(*a*) Cells in K glutamate medium (table 1) containing 10 mM Ca EGTA, corresponding to 10 μM Ca^{2+}, were subjected to ten exposures of 2 kV cm^{-1} ($\tau = 200$ μs) and the catecholamine (○) and Met-enkephalin (●) in the supernatant determined at the times shown. (Supernatants were diluted by a factor of 10 with distilled water for both assays.) Catecholamine (△) and Met-enkephalin (▲) in the supernatant from control cells not exposed to the field. Ordinate: Met-enkephalin and catecholamine expressed in units of concentration; 300 pg ml^{-1} of Met-enkephalin corresponded to 5.7% of the total assayable enkephalin in the cells and 40 nmol ml^{-1} of catecholamine corresponded to 22% of the cellular content. Temperature 37 °C.

(*b*) Cells in K glutamate medium (table 1) containing 0.4 mM EGTA were subjected to ten exposures of 2 kV cm^{-1} ($\tau = 200$ μs) and then immediately challenged with 10 mM Ca EGTA buffers corresponding to a range of Ca^{2+} concentrations. After 20 min the supernatants were assayed for catecholamine (○) and Met-enkephalin (●). Cells in K glutamate medium (table 1) but lacking Mg-ATP were rendered 'leaky' and challenged in an identical fashion. △, Catecholamine; ▲, Met-enkephalin. Ordinate as (*a*). Temperature 37 °C.

TABLE 2. PROPERTIES OF Ca-DEPENDENT CATECHOLAMINE RELEASE FROM 'LEAKY' ADRENAL MEDULLARY CELLS

(1) activation half-maximal at an ionized Ca concentration of 1 μM

(2) requirement for Mg-ATP is very specific. Half-maximal activation requires 1 mM

(3) unaffected by:
- (*a*) agonists and antagonists of acetylcholine receptors including acetylcholine, nicotine and hexamethonium (1 mM)
- (*b*) Ca channel blocker D600 (100 μM)
- (*c*) agents that bind to tubulin (colchicine, vinblastine, 100 μM)
- (*d*) cytochalasin B (1 mM)
- (*e*) inhibitors of anion permeability (SITS, DIDS, 100 μM).
- (*f*) protease inhibitor TLCK (1 mM)
- (*g*) cyclic nucleotides (cyclic AMP, cyclic GMP, 1 mM)
- (*h*) *S*-adenosyl methionine (5 mM)
- (*i*) phalloidin (1 mM)
- (*j*) vanadate (1 mM)
- (*k*) Leu- and Met-enkephalins, substance P (100 μM)
- (*l*) somatostatin (1 μM)
- (*m*) NH_4Cl (30 mM)

(4) inhibited by:
- (*a*) chaotropic anions: SCN > Br > Cl
- (*b*) detergents (complete inhibition after 10 min incubation with 10 μg/ml of digitonin, Brij 58 or saponin)
- (*c*) trifluoperazine (complete inhibition with 20 μg/ml)
- (*d*) high Mg concentration: small increase in apparent K_m for Ca accompanies large reduction in V_{max}
- (*e*) high osmotic pressure: large reduction in V_{max} but no significant changes in the affinity for Ca
- (*f*) carbonylcyanid *p*-trifluormethoxyphenylhydrazone (FCCP) (45% inhibition by 10 μM)

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Taken together, these findings provide very strong evidence that Ca can evoke exocytosis in 'leaky' cells. In addition, the electron microscopy and [^{3}H]sucrose uptake data suggest that endocytosis also occurs in response to a Ca challenge. This was unexpected and greatly complicates the analysis of our results because, if exocytosis and endocytosis take place at the same site and if there are a limited number of these sites, inhibition of catecholamine release could result either from an action on exocytosis or on endocytosis. So far we have not found any simple way to uncouple these two Ca-triggered events.

Factors affecting Ca-*dependent release of catecholamine from 'leaky' cells*

Table 2 summarizes a number of properties of catecholamine secretion in 'leaky' bovine adrenal medullary cells and the response to a number of potential inhibitors. Some of the more interesting findings are discussed below.

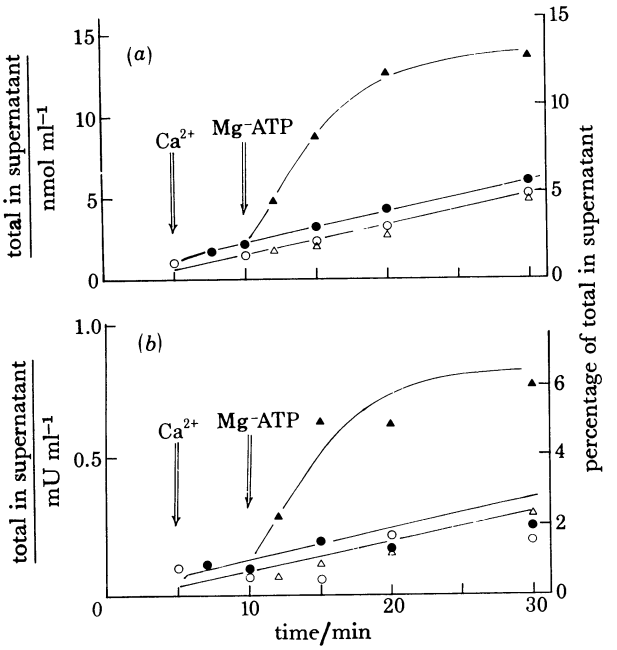

FIGURE 9. The triggering of secretion by Mg ATP. Cells in K glutamate medium (table 1) containing 0.4 mM EGTA but lacking Mg-ATP were subjected to ten exposures of 2 kV cm^{-1} ($\tau = 200$ μs); 5 min later they were challenged with 5 mM EGTA, corresponding to *ca.* 10 nM Ca^{2+} (○), and 5 mM Ca EGTA, corresponding to 10 μM Ca^{2+} (●). After a further 5 min, 5 mM Mg-ATP was added to both the 10 nM Ca^{2+} (△) and the 10 μM Ca^{2+} (▲) cells. At the times shown the cells were spun down and the supernatant assayed for catecholamine (*a*) and dopamine-β-hydroxylase (*b*). (It was necessary to dilute the supernatant elevenfold with distilled water in order to overcome the inhibitory effects of glutamate and EGTA on the dopamine-β-hydroxylase assay.) The ordinates are expressed in both units of concentration and as percentages of the total cellular content. Temperature 37 °C.

Requirement for Mg-ATP

In order to observe activation of exocytosis by micromolar concentrations of Ca, it is essential that Mg-ATP also be present and in the absence of Mg-ATP even millimolar concentrations of Ca fail to evoke catecholamine release. In the absence of Mg, ATP alone is ineffective and Mg-ATP cannot be replaced by a variety of other nucleotides both hydrolysable and non-hydrolysable. Mg-ADP on a molar basis is half as effective as Mg-ATP in supporting Ca-dependent exocytosis, but this occurs through synthesis of ATP as ADP becomes completely ineffective when myokinase is inhibited. Figure 9 shows that if 'leaky' cells are first depleted

of Mg-ATP they can be exposed to high concentrations of Ca without evoking catecholamine release. Subsequent addition of Mg-ATP precipitates the parallel release of both catecholamine and dopamine-β-hydroxylase, providing rather convincing evidence that under these conditions Mg-ATP is triggering exocytosis. Figure 10*a* reveals that as the concentration of Mg-ATP is reduced, the extent of exocytosis becomes less but there is no detectable change in affinity for Ca.

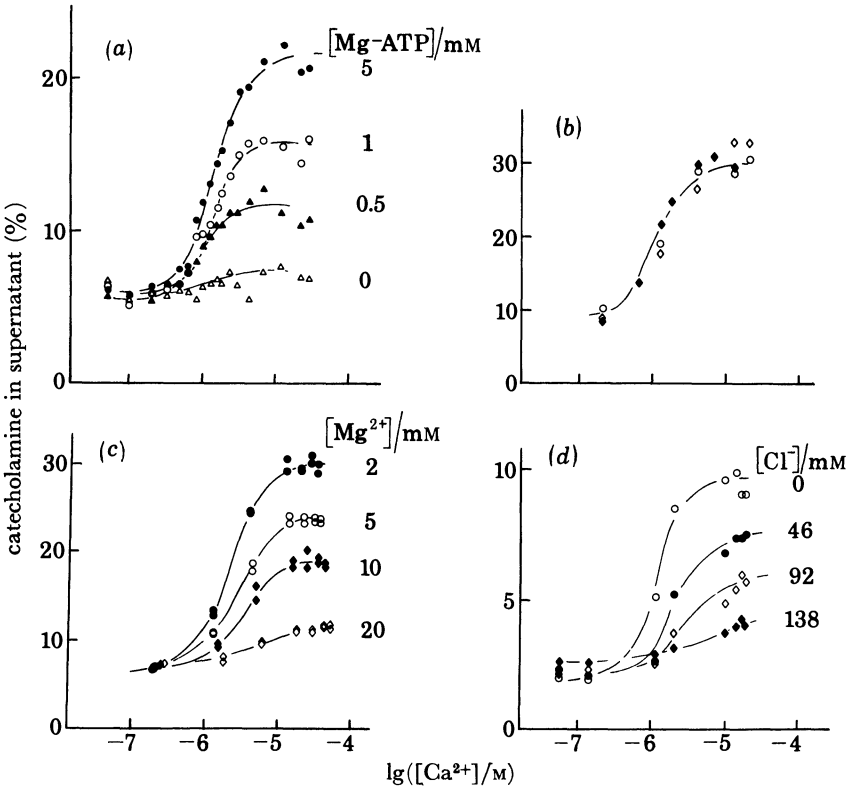

FIGURE 10. (*a*) Dependence of catecholamine release on Mg-ATP. Cells in K glutamate medium (table 1) lacking Mg-ATP were rendered leaky by ten discharges of 2 kV cm^{-1} (τ = 200 μs) and immediately diluted into similar solutions containing various concentrations of Mg-ATP. After 10 min the cells were challenged with 10 mM Ca EGTA buffers and the catecholamine in the supernatant was determined 15 min later. Temperature 37 °C.

(*b*) Effect of Na^+, K^+ and low ionic strength on Ca-sensitive release of catecholamine. Isosmotic replacement of K glutamate by Na glutamate or sucrose. Cells in a K glutamate solution (table 1) were rendered leaky by ten exposures to 2 kV cm^{-1} (τ = 200 μs) and immediately diluted into either an identical K solution (◇), or into one in which the K had been replaced by Na to give a final concentration of 120 mM Na^+ (○), or into one in which the K glutamate had been replaced by sucrose to give a final concentration of 300 mM (●); after 3 min the cells were challenged with 10 mM Ca EGTA buffer. The catecholamine in the supernatant was determined 15 min later. Temperature 37 °C.

(*c*) Effect of Mg acetate on Ca-sensitive release of catecholamine. Cells on a K glutamate solution (table 1) were rendered 'leaky' by ten exposures to 2 kV cm^{-1} (τ = 200 μs) and immediately transferred to similar solutions containing various concentrations of free Mg^{2+} (Mg acetate used). After 5 min incubation the cells were challenged with 10 mM Ca EGTA buffer and the catecholamine was determined 10 min later. Temperature 37 °C.

(*d*) Ca activation curves at different chloride concentrations. Cells in a K glutamate medium (table 1) (*ca.* 10 nM Ca^{2+}) were rendered permeable by ten exposures to 2 kV cm^{-1} (τ = 200 μs) and then injected into similar solutions in which the glutamate had been wholly or partly replaced by chloride. After 5 min the cells were challenged with 10 mM Ca EGTA buffer and the catecholamine in the supernatant was determined 10 min later. Temperature 37 °C.

Influence of Na, Mg *and anions*

Figure 10 also shows the effect of altering the ionic composition of the medium that has access to the cell interior. Isosmotic replacement of K by Na or sucrose has no detectable effect on the Ca-activation curve, but raising the concentration of Mg reduces both the extent of exocytosis and its apparent affinity for Ca. Progressive replacement of glutamate by chloride has three discernible effects: a small increase in the basal release of catecholamine, a decrease in the apparent affinity for Ca and a fall in the extent of catecholamine release. Chloride also inhibits the Ca-dependent release of dopamine-β-hydroxylase, showing that it is acting on exocytosis. This action is not restricted to chloride but seems to be common to a number of chaotropic anions, and the order of effectiveness of anions at inhibiting Ca-dependent exocytosis follows the order of the lyotropic series, with SCN^- the most potent inhibitor and acetate the least (figure 11*a*).

Effects of other agents

Of the various agents tested, many had no detectable effect on the activation of exocytosis by Ca (table 2). This included a variety of agonists and antagonists of the nicotinic receptor, a number of peptides, the Ca channel blocker D600, anion channel inhibitors, microtubule disrupters, cytochalasin B, phalloidin, protease inhibitors, cyclic nucleotides, the methylating agent *S*-adenosyl methionine, and vanadate. Of those that did affect exocytosis, in addition to the chaotropic anions and Mg, the most notable are detergents, trifluoperazine and high osmotic pressure. In addition, high concentrations of FCCP bring about partial inhibition.

Detergents

Inhibition by the detergents digitonin, saponin and Brij 58 (figure 11*b*) is perhaps not surprising; but of particular significance is the finding that it occurs at very low concentrations, much lower than those used routinely to 'permeabilize' cells (Fiskum *et al.* 1980; Murphy *et al.* 1980; Cande *et al.* 1981). It would seem that although some aspects of cell function persist in cells permeabilized by detergents, exocytosis is not one of these.

Trifluoperazine

Figure 11*c* shows that the antipsychotic drug trifluoperazine has two effects on leaky cells: in the presence of EGTA, high concentrations of the drug increase the basal rate of catecholamine release, and in the presence of 10 μM Ca, low concentrations of trifluoperazine bring about some reduction in Ca-dependent catecholamine release but inhibition then gives way to increased release. The difference curve is consistent with half-maximal inhibition of Ca-dependent release by 10 μM trifluoperazine. As trifluoperazine binds to calmodulin (Weiss & Levin 1978), this effect may imply the involvement in exocytosis of calmodulin or a related protein. Although this would be consistent with the high affinity of exocytosis for Ca and its inhibition by Mg, data based solely on the action of inhibitors must be considered inconclusive. It may, perhaps, be no coincidence that qualitatively and quantitatively the action of trifluoperazine closely resembles that of detergents.

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Osmotic pressure

Inhibition of Ca-dependent exocytosis by an increase in osmotic pressure is quite dramatic (figure 11*d*) and may reflect the involvement of an osmotically sensitive step in the exocytotic process. Such a possibility has been postulated by Pollard *et al.* (1977) and shown to play an important part in model systems (Zimmerberg & Finkelstein 1980). In addition, during exocytotic discharge of the cortical granules in sea urchin eggs the granules seem to swell slightly before they disappear. It is possible that an osmotically sensitive step may precede fusion, and the analysis of the sensitivity of the 'leaky' adrenal cells to osmotic pressure may prove rewarding.

FCCP *and the possible role of the vesicle membrane potential*

Inhibition by FCCP is only partial and as the concentration required is an order of magnitude greater than that needed to uncouple mitochondria, it most likely originates in some rather non-specific effect. Nevertheless, an alternative possibility merits careful examination. Work on isolated chromaffin granules (see Salama *et al.* 1980) has revealed that they accumulate H^+ in an ATP-dependent fashion and the operation of this ATPase maintains the interior of the vesicles positive with respect to their cytosolic face. If a hydrogen ion gradient or a vesicle potential of normal magnitude and size is important for it to participate in exocytosis, a number of our observations might be explained, including the requirement for ATP, inhibition by chaotropic anions, which can permeate the vesicle and short-circuit the potential, and inhibition by FCCP, which should render the vesicles selectively permeable to H^+ and thereby reverse the sign of the potential. Although attractive, this explanation seems unlikely for the following reasons.

1. Ca-dependent exocytosis is unaffected by exposing 'leaky' cells to 30 mM NH_4Cl, which should dissipate the vesicle H^+ gradient.

2. The nucleotide requirement for exocytosis is much more specific than that for the vesicle H^+-pumps. (Pollard *et al.* 1976).

3. Inhibition of the vesicle H^+-pump by trimethyl tin (1 mM) or DCCD (1 mM) only reduces exocytosis by 30%, and this inhibition is not reduced further by NH_4Cl. Although these agents are known to collapse the potential of isolated vesicles, it is possible that *in situ* the vesicles are more resistant to inhibition. We have attempted to assess the effectiveness of the various inhibitors by measuring the distribution of trace amounts of $[^{14}C]SCN^-$. This should be accumulated in 'leaky' cells by virtue of the positive internal potential of the vesicles. Collapse or reversal of the vesicle potential should result in less thiocyanate associated with the cells. The data are consistent with accumulation because in the presence of Mg-ATP the SCN^- space exceeds that of ^3H_2O. This accumulation is virtually abolished by FCCP and markedly reduced after application of trimethyl tin or DCCD under conditions that fail to block Ca-dependent exocytosis.

Our tentative conclusion is that although we cannot rule out the possibility that the vesicle potential plays a small part in exocytosis, changes in potential seem unlikely to account for the whole of the inhibitory effects of ATP removal, chaotropic anions or FCCP.

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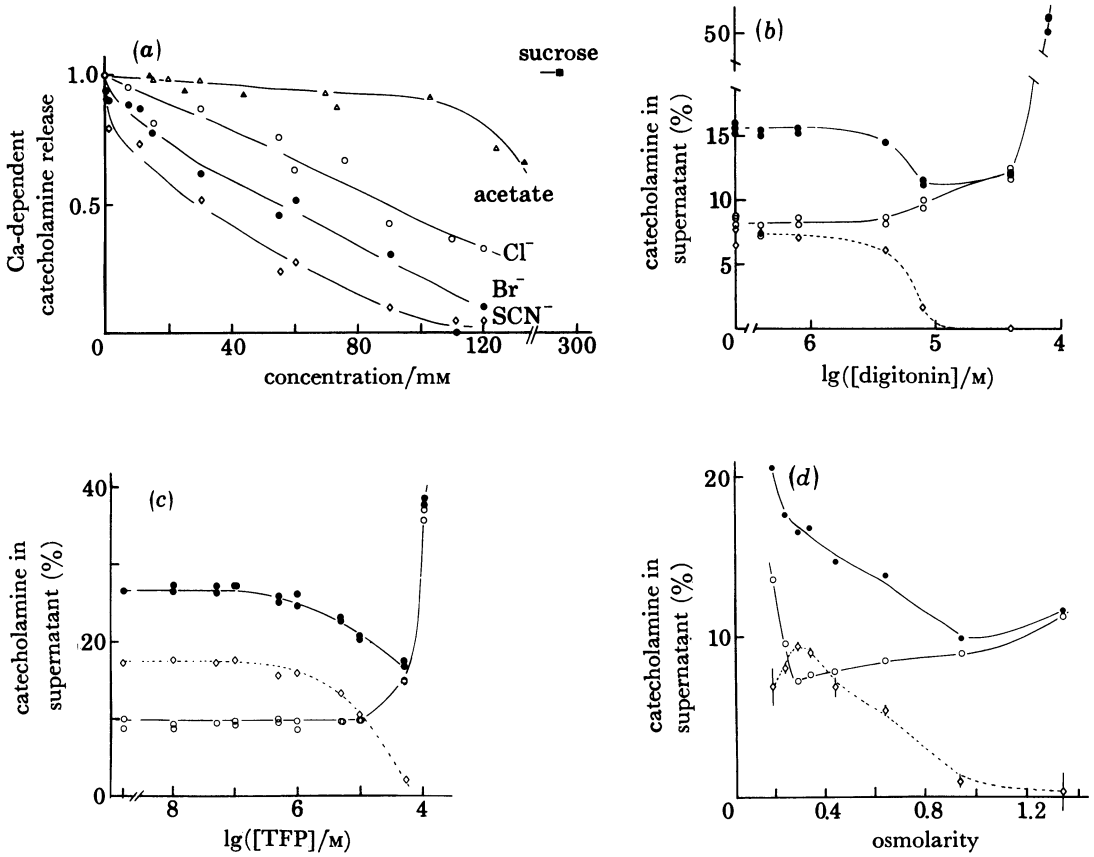

FIGURE 11. (*a*) The influence of anions on Ca-dependent secretion. Cells in a K glutamate solution (table 1) were rendered 'leaky' by ten exposures to 2 kV cm^{-1} ($\tau = 200$ μs) and then diluted into similar solutions in which the glutamate had been wholly or partly replaced by one of several anions. After 3 min the cells were challenged with 10 μM Ca^{2+} for 15 min or kept at 10 nM Ca^{2+}. The amount of catecholamine secreted due to the 10 μM Ca^{2+} challenge (i.e. secretion in 10 μM Ca^{2+} minus that in 10 nM Ca^{2+}) is shown, the ordinate being normalized to that secreted in the presence of glutamate alone. ◇, Thiocyanate; ●, bromide; ○, chloride; ▲, acetate; ■, sucrose. Temperature 37 °C.

(*b*) Effect of digitonin on secretion. Cells (less than 10^6/ml) in K glutamate medium (table 1) were rendered 'leaky' by ten exposures to 2000 V cm^{-1} ($\tau = 200$ μs) and incubated for 3 min with various concentrations of digitonin before being challenged with 10 μM Ca^{2+} and the catecholamine in the supernatant was determined 15 min later. ●, Cells challenged with 10 μM Ca^{2+}; ○, cells held at 10 nM Ca^{2+}; ◇– – –◇, difference curve, Ca-dependent release. Results very similar to these were also obtained with saponin and Brij 58. Temperature 37 °C.

(*c*) Effect of trifluoperazine (TFP) on secretion. 'Leaky' cells in a K glutamate medium (table 1) were incubated with various concentrations of trifluoperazine for 5 min before being challenged with 10 μM Ca^{2+} and the catecholamine in the supernatant was determined 10 min later. ●, Cells challenged with 10 μM Ca^{2+}; ○, cells held at 10 nM Ca^{2+}; ◇– – –◇, Ca-dependent release. Temperature 37 °C.

(*d*) The influence of osmolarity on secretion. Cells, in a medium similar to the K glutamate solution described in table 1 but where the K glutamate had been replaced isosmotically by sucrose, were rendered 'leaky' and diluted into solutions of various osmolarities. (Sucrose concentration varied.) After 5 min the cells were challenged with 10 μM Ca^{2+} and the catecholamine in the supernatant was determined 20 min later (●). ▫, Cells not challenged; ◇– – –◇, Ca-dependent release. Error bars represent standard error of four determinations.

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The kinetics of exocytosis in 'leaky' cells

Two striking features merit consideration: (1) the quantity of catecholamine released is a function of the concentrations of both Ca^{2+} and Mg-ATP, and (2) the maximum amount released in the presence of saturating concentrations of Ca and Mg-ATP is rarely greater than 30% of the total in the cell. Under the conditions of our experiments, no further catecholamine can be released even if the cells are returned to EGTA and subsequently rechallenged with Ca. We do not know why Ca is unable to release more than 30% of the cellular catecholamine, but it may reflect the distribution of vesicles within the cell: perhaps only 30% of the total catecholamine is in vesicles located close enough to the plasma membrane to react. If the 'leaky' cell lacks mechanisms for transporting vesicles within the cytosol, vesicles located some distance from the cell surface may be unable to participate in exocytosis.

As exocytosis of each individual vesicle is an all-or-none event, one might expect that submaximal concentrations of Ca or Mg-ATP would eventually discharge the whole of the releasable pool of catecholamine, the rate of discharge being determined by the concentrations of Ca and Mg-ATP. This is, however, not observed. The experimental finding is quite clear: submaximal concentrations of Ca and Mg-ATP release submaximal amounts of catecholamine and the whole of the releasable pool is not discharged. Failure to effect the discharge of the whole releasable pool does not result from some sort of inactivation or desensitization of the release mechanism to Ca or Mg-ATP because raising the concentration of the activator that is limiting immediately promotes more secretion (figure 12*a*). Nor does the failure reflect populations of vesicles with different, but fixed, thresholds for Ca because once secretion in the presence of a subthreshold concentration of Ca has stabilized, returning the cell to EGTA and subsequently rechallenging with the *same* Ca concentration gives another burst of release, implying that vesicles sensitive to this Ca concentration are still present in the cells (figure 12*b*).

One way to explain these findings is to postulate that Mg-ATP can be used to phosphorylate some key site and that the extent of this phosphorylation depends on the concentration of ionized Ca. It follows that the level of phosphorylation will be a saturating function of the concentrations of Ca^{2+} and Mg-ATP. All that is needed to explain our findings is to assume that the chances of exocytosis are greatly increased while the level of phosphorylation is increasing. Two interesting corollaries of this suggestion are as follows.

1. Secretion from the adrenal medulla should be transient even in the presence of a sustained elevation in cytosolic Ca concentration. It is well known that in response to maintained depolarization, secretion from the bovine adrenal is only transient and although this might reflect a transient Ca entry (Baker & Rink 1975) it could also be consistent with the above hypothesis. Another observation that is consistent with this hypothesis is that at the squid giant synapse transmitter release fails while the ionized Ca concentration is still elevated (Miledi & Parker 1981). On the other hand, some preparations, for example the frog neuromuscular junction, maintain an elevated rate of exocytosis for many hours when partly depolarized.

2. Changes in phosphorylation should accompany the stimulation of exocytosis. This is certainly so. Stimulation of catecholamine release by a variety of secretagogues increases the extent of phosphorylation of at least two proteins (DeLorenzo *et al.* 1979; Amy & Kirshner 1981), but it is not yet clear whether phosphorylation precedes exocytosis or whether it is associated with endocytosis or some other recovery mechanism, for example the Ca pump. An

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analysis of phosphorylation in 'leaky' cells may provide answers to these questions and permit a more detailed examination of the mechanisms underlying the rather characteristic kinetics of exocytosis in 'leaky' adrenal cells.

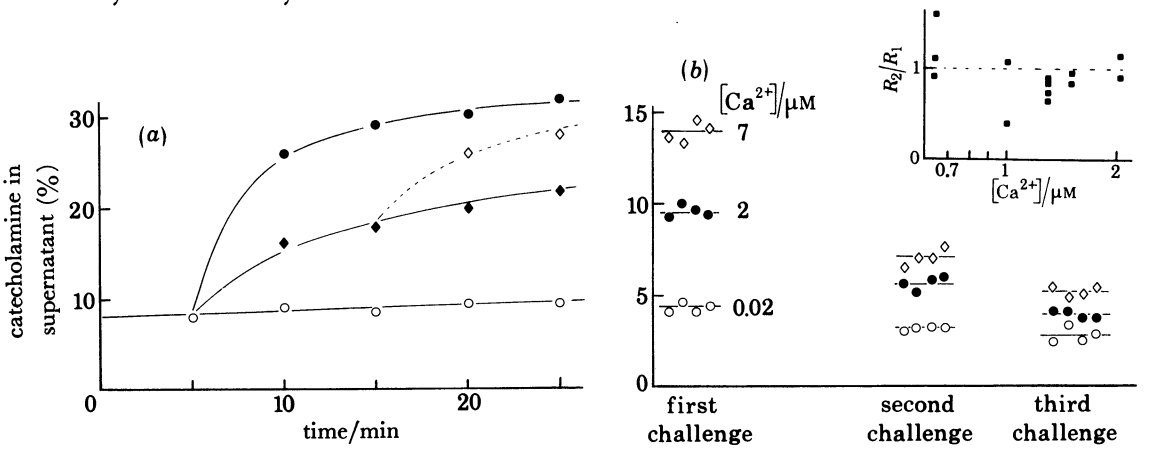

FIGURE 12. Catecholamine release in response to a calcium challenge.

(*a*) Catecholamine release as a function of time. Cells were rendered 'leaky' by ten exposures to 2 kV cm^{-1} ($\tau = 200$ μs) in a K glutamate solution (table 1) containing 0.2 mM EGTA. After 5 min the cells were challenged by adding 0.4 mM Ca EGTA buffer to give a final calculated free Ca concentration 10 nM (○), 1 μM (◆), and 10 μM (●). After a further 10 min the Ca level of an aliquot of the 1 μM cells was raised to 10 μM by the addition of 10 mM Ca EGTA (◇). Temperature 37 °C.

(*b*) Ca dependence of catecholamine release from cells previously challenged with micromolar levels of calcium. Cells in a K glutamate solution (table 1) containing 0.4 mM EGTA and a calculated free Ca^{2+} concentration of 0.02 μM were rendered 'leaky' by exposing them ten times to 2 kV cm^{-1} ($\tau = 200$ μs) and challenged for the first time with 10 mM Ca EGTA buffers corresponding to 2 and 7 μM Ca; the catecholamine released over 10 min was measured (C_2 and C_7 respectively). The cells in the buffer corresponding to 2 μM Ca^{2+} were then washed three times in 10 ml of solution (table 1) containing 0.4 mM EGTA (calculated Ca^{2+} concentration 0.02 μM), this procedure taking 20 min. These washed cells were challenged for a second time with Ca buffer corresponding to 2 and 7 μM Ca and the catecholamine released over 10 min was measured. Cells challenged a second time with 2 μM Ca were washed again by the same procedure, before being challenged with Ca^{2+} for a third time. ○, Cells in 0.02 μM Ca; ●, 2 μM Ca; ◇, 7 μM Ca. Temperature 37 °C. The relative amount released by the first 2 μM Ca challenge is defined as

$$R_1 = (C_2 - C_{0.02})/(C_7 - C_{0.02}),$$

where $C_{0.02}$ is the amount in the supernatant at 0.02 μM Ca. The relative amount released by the second challenge (R_2) is defined similarly. R_2/R_1 represents the response of cells to a particular Ca challenge compared with their response to a similar previous challenge. This ratio is shown in the inset of (*b*) over a range of Ca challenges from 0.64 to 2 μM.

REGULATION OF CYTOSOLIC IONIZED CALCIUM CONCENTRATION

Bound and free Ca *in adrenal cells*

The experiments on 'leaky' cells suggest that, in the presence of Mg-ATP, the extent of exocytosis is determined by the level of ionized Ca: it is very small at an ionized Ca concentration of 100 nM or lower and increases rapidly as the ionized Ca concentration is raised into the micromolar range. A resting ionized Ca concentration of 100 nM is close to that found in a variety of cells (see Baker 1972, 1978), but measurements on adrenal cells are lacking. One established method for estimating cytosolic ionized Ca concentration is to lyse cells with digitonin or saponin in the presence of different levels of ionized Ca (Murphy *et al.* 1980; Becker *et al.* 1980). The ionized Ca concentration at which no gain or loss of Ca occurs is assumed

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to be the same as that in the cytosol. Unfortunately, as mentioned earlier, concentrations of digitonin or saponin that render adrenal cells permeable also release appreciable amounts of catecholamine, presumably owing to lysis of chromaffin granules. As these granules also contain appreciable quantities of Ca, the digitonin method will seriously overestimate the ionized Ca concentration because of release into the cytosolic pool of vesicular Ca. An alternative approach is to use the Ca-sensitive dye Quin-2 (Tsien 1980), which can be rendered lipid-soluble by conversion to its acetomethoxy ester (Tsien 1981). This ester permeates cells, but once inside is hydrolysed to the free dye, which becomes trapped. Measurements with the use of Quin-2 give values for the cytosolic ionized Ca concentration in the range 0.05–0.1 μM. These values must be considered an upper limit for the resting ionized Ca concentration because Quin-2 will also enter intracellular organelles such as mitochondria and chromaffin vesicles. Fully satisfactory measurements have not yet been made on secreting cells, but it does not seem at all improbable that stimulation may raise the ionized Ca concentration into the micromolar range, which is adequate to activate exocytosis in 'leaky' cells. If this is so, one factor in terminating catecholamine release will be the rate at which the cytosolic ionized Ca concentration is returned to its resting level.

Measurement of the Ca content of adrenal cells reveals about 1–2 mmol/kg, so if the concentration of ionized Ca in resting cells is only 0.2 μM, the bulk of the Ca must be bound. Two questions immediately arise.

1. Where is the bound Ca located in the cell and what reactions are responsible for maintaining equilibrium between bound and free Ca?

2. Why is the Ca content stabilized at only 1–2 mmol/kg? With a membrane potential of about −60 mV inside, the Nernst relation predicts that the internal Ca should be at least 100 mM, and much greater if the bulk of the intracellular Ca is bound.

Characterization of intracellular Ca *binding sites*

We have made use of the 'leaky' cell preparation to examine the question of intracellular binding. Our main findings are summarized in figure 13. In the absence of Mg-ATP or in the presence of Mg-ATP plus the mitochondrial uncoupling agent FCCP, there is very little binding: less than 200 μmol/kg over the Ca concentration range 10 nM–10 μM. In the presence of Mg-ATP, appreciable binding is seen in the micromolar range of Ca concentrations. All of the ATP-dependent binding seems to be released by FCCP, suggesting that the binding sites are mitochondria, although uptake into the chromaffin vesicles cannot be excluded.

The extrusion of calcium from adrenal cells

If the ionized Ca concentration increases during secretion, some of this Ca will presumably be taken up by intracellular binding systems and the rest extruded across the plasma membrane. In the long term, the Ca content of cells remains rather constant, implying that any Ca entering the cells must be balanced by extrusion. Ca extrusion from adrenal cells seems to involve three routes: (1) exocytosis, (2) Na-dependent Ca efflux, and (3) uncoupled, presumed ATP-dependent, Ca efflux. To take each in turn:

1. Calcium is sequestered within the secretory vesicles and the Ca is, presumably, released during exocytosis. Very little is known about the mechanisms accumulating Ca in the secretory vesicles, but this could clearly represent a significant route for Ca loss.

2. Although Na-dependent Ca efflux is present in many nerve cells (Baker 1972) and has

been described in slices of the bovine adrenal medulla (Rink 1977), there is very little evidence for it in isolated cells (Pocock 1980).

3. The efflux of radioactivity from cells loaded with ^{45}Ca is largely insensitive to external Ca and Na concentrations but can be increased by FCCP and inhibited by low temperatures and the cardiac glycoside ouabain (Pocock 1980). This flux may represent the operation of a Ca-ATPase and is similar to that known to exist in red blood cells and many other cells, including squid axons. The sensitivity of the Ca efflux from adrenal cells to ouabain is particularly interesting because exposure of intact adrenal cells to cardiac glycosides is known to increase the basal rate of catecholamine release, and this might result rather directly from inhibition of Ca efflux.

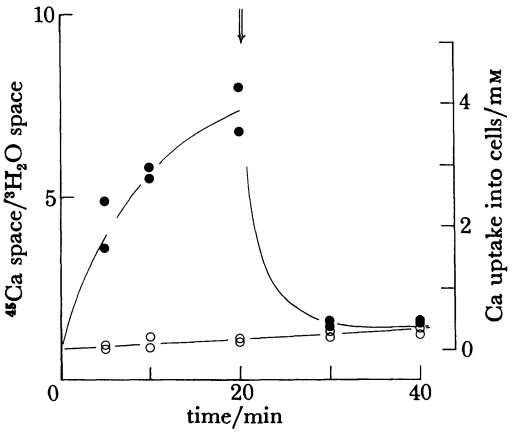

FIGURE 13. Sensitivity of Ca binding to the uncoupling agent FCCP. Cells in K glutamate medium (table 1) containing 0.2 mM EGTA and trace amounts of $^{3}H_2O$ were rendered 'leaky' by ten exposures to 2 kV cm^{-1} (τ = 200 μs). The cells were exposed to 0.6 mM Ca EGTA, which raised the free Ca concentration to 10 μM and the ^{45}Ca and ^{3}H spaces associated with the cell pellets determined at different times afterwards (●). FCCP (7 μM) was added to the cell suspension 20 min after exposure to Ca (arrow). ○, Cells in the presence of FCCP throughout. The left ordinate shows the ^{45}Ca space relative to the ^{3}H space. The accessibility of the Ca EGTA to the cell is expected to be about 0.7 of the cell volume, the Ca space above this level reflecting Ca uptake and equilibration of radioactive marker with existing Ca stores. This uptake and equilibration are expressed as millimolar levels of calcium in the cells (right ordinate). The presence of the same concentrations of FCCP did not alter the Ca-sensitive secretory response in this batch of cells. Temperature 37 °C.

CONCLUSION

The analysis of catecholamine release from cells of the bovine adrenal medulla offers many advantages for studies of stimulus–secretion coupling. In particular, the 'leaky' cells seem an excellent preparation for investigating the mechanisms both of exocytosis and endocytosis and of Ca binding. The advantages of an electrical technique for rendering the cells 'leaky' are that it is both quick and clean. It seems preferable to the use of detergents that inhibit exocytosis, and possibly other membrane functions, at extremely low concentrations.

The picture that emerges is a rather simple one. The release of acetylcholine from the splanchnic nerve acts on nicotinic receptors to increase the frequency of TTX-sensitive action potentials, which in turn open specific D600-sensitive channels through which Ca enters the cell, raising the cytosolic concentration of ionized Ca from approximately 0.2 μM into the micromolar range. In the presence of Mg-ATP this rise in ionized Ca increases the rate of membrane turnover by exocytosis and endocytosis. In the short term the resting ionized Ca concentration is probably re-established by Ca binding within the cell, but in the longer term

any Ca that has entered is pumped out across the surface membrane. In all these respects the adrenal medullary cell seems to resemble most other nerve cells and has much in common with many non-nervous secretory cells (see Baker *et al.* 1980; Knight & Scrutton 1980), and the elucidation of the molecular basis of these various mechanisms as well as their modulation by physiological and pharmacological agents poses an exciting challenge.

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Discussion

J. R. Garrett (*King's College Hospital Dental School, London, U.K.*). A. V. Edwards at Cambridge has found that *in vivo* dopamine-β-hydroxylase and catecholamines are not necessarily secreted in parallel (Edwards, A. V., Furness, P. N. & Helle, K. B. 1980 *J. Physiol., Lond.* **308**, 15–27). Would Professor Baker comment about this?

P. F. Baker. This is certainly a puzzle, but it is possible that a molecule of the size of dopamine-β-hydroxylase ($M_r \approx 300000$) may not easily enter the venous effluent of the gland.

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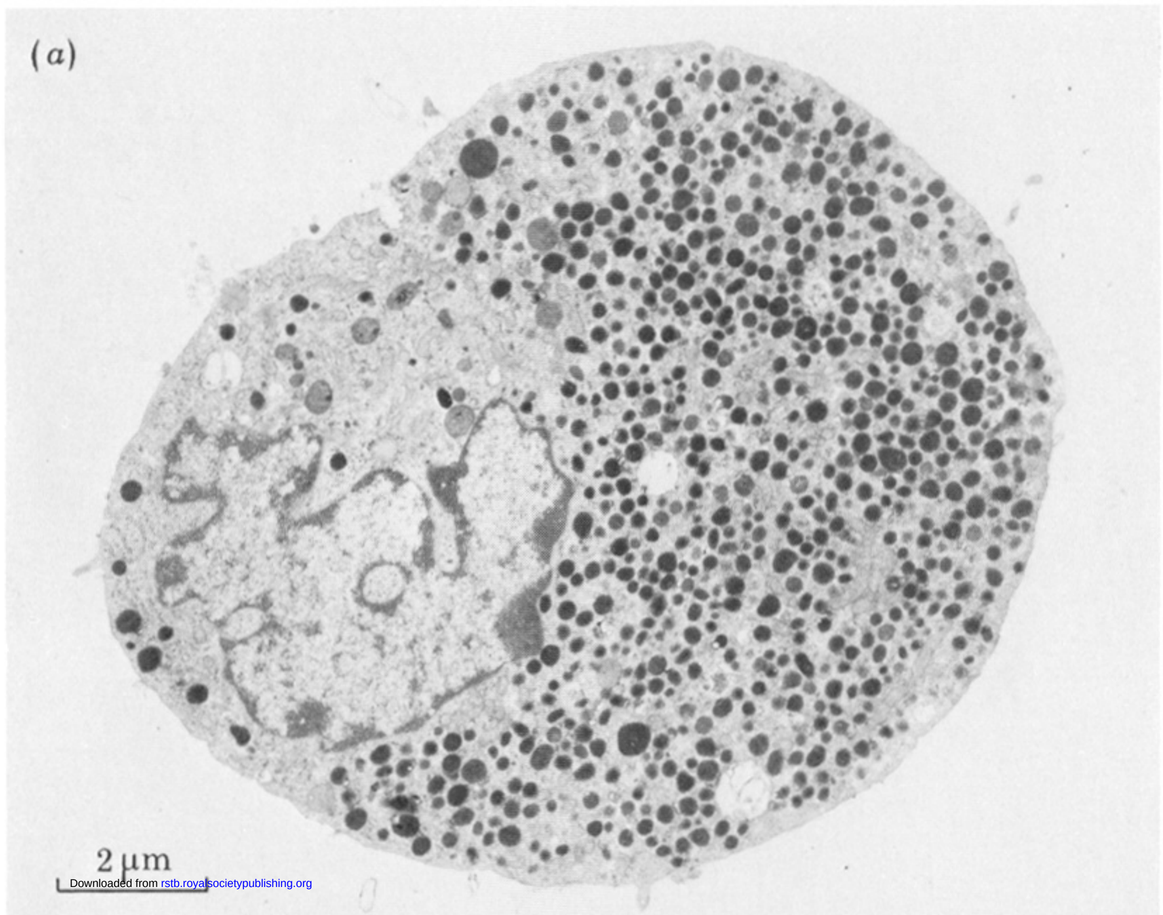

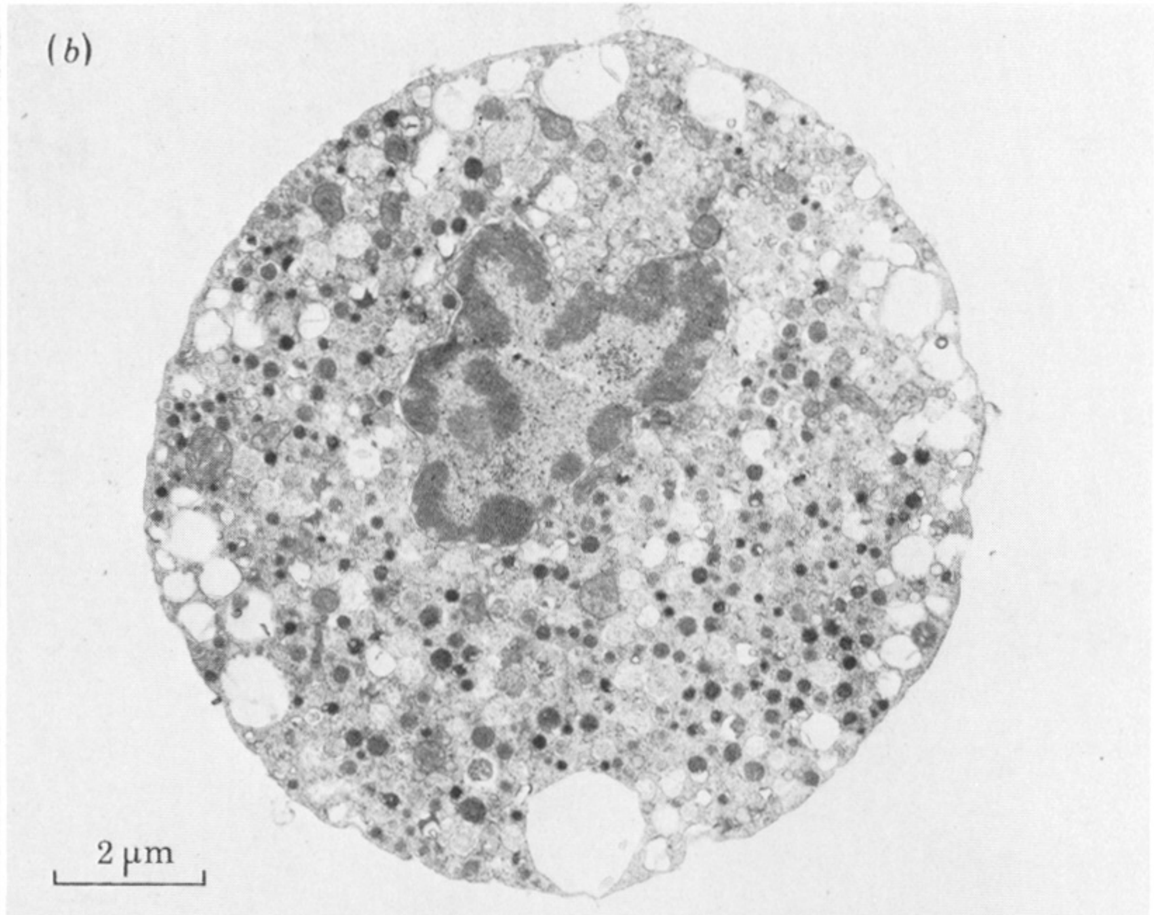

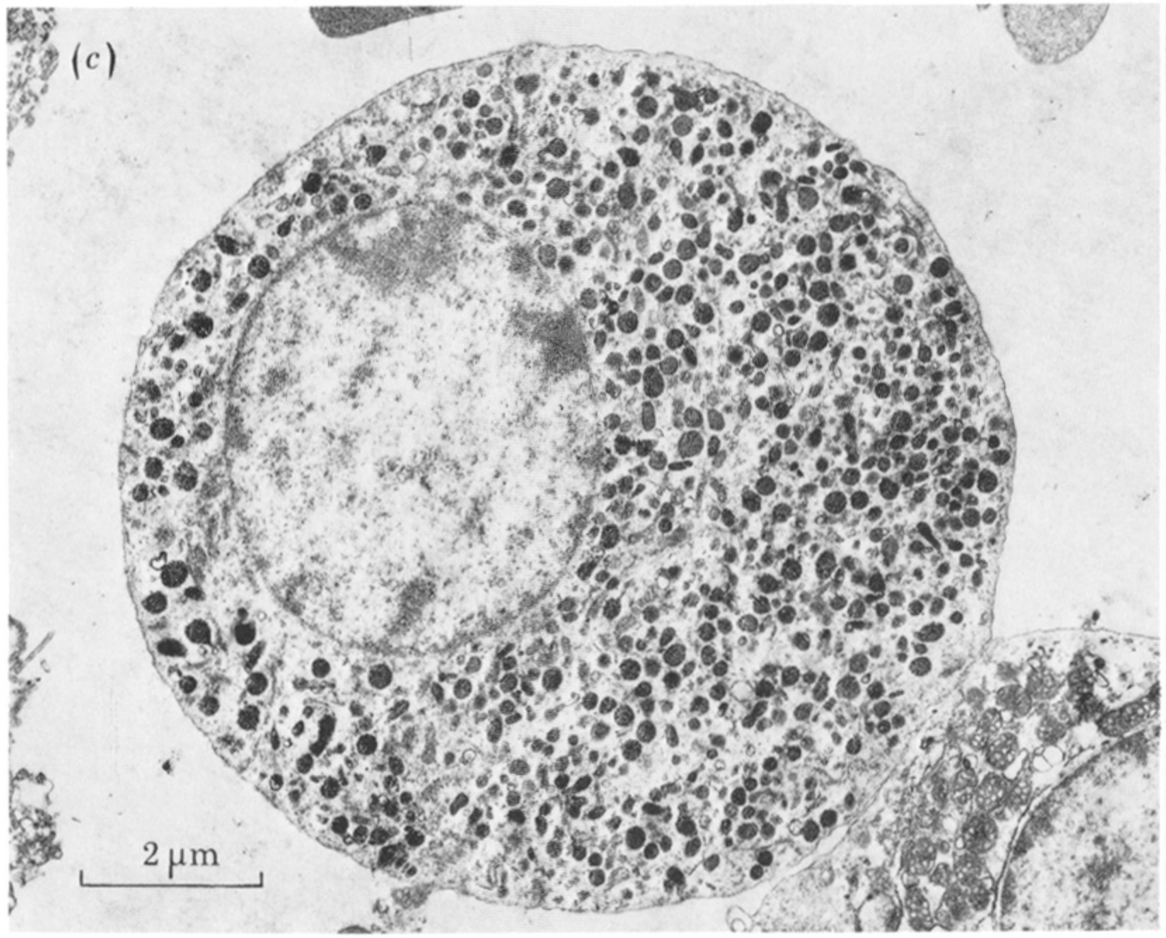

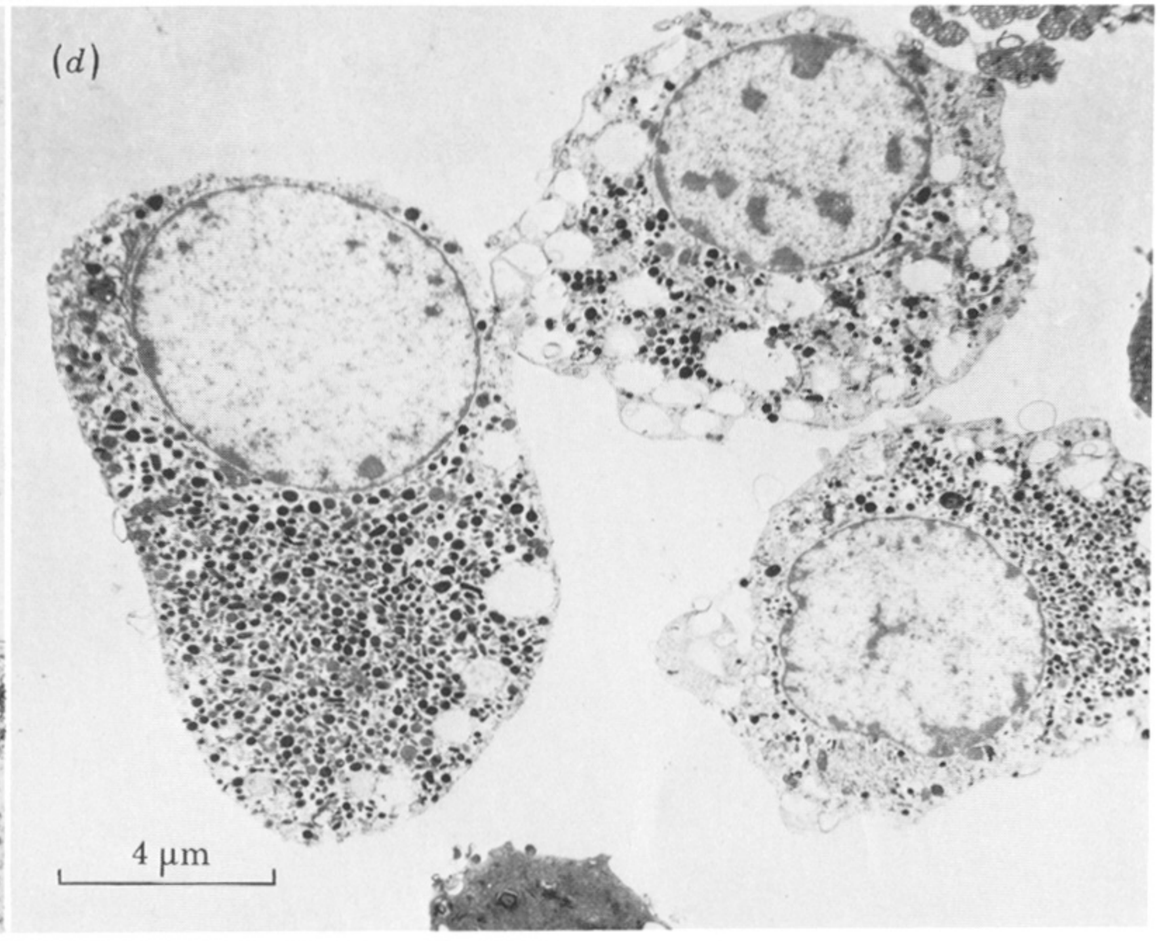

(Facing p. 86)

FIGURE 3. For description see opposite.

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