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Calcium-Dependence of Catecholamine Release from Bovine Adrenal Medullary Cells after Exposure to Intense Electric Fields

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Summary. By subjecting isolated adrenal medullary cells to intense electric fields of brief duration it is possible to gain access to the cell interior without impairing the ability of the cell to undergo exocytosis. After a single exposure to a field of 2 kV/cm, $\tau = 200$ μsec, adrenal medullary cells behave as if their plasma membrane contains two pores of effective radius 2 nm. At 37 °C these 'equivalent pores' remain patent for up to 1 hr. The formation and stability of these 'pores' is not affected by the Ca content of the bathing solution. The 'pores' permit externally applied catecholamine and Ca-EGTA to equilibrate rapidly with the cell water.

Cells rendered 'leaky' in K glutamate medium containing 5 mM Mg-ATP and EGTA to give an ionized Ca close to 10^{-8} M release less than 1% of their total catecholamine. These same cells can release up to 30% of their catecholamine when exposed to 10^{-5} M Ca. This Ca-dependent release is unaffected by Ca-channel blockers such as D600. Catecholamine release in response to a calcium challenge only seems to occur during the first few minutes whilst the Ca concentration is changing, and the extent of release depends on the final Ca concentration achieved. Half-maximal release occurs at about 1 μM Ca, and this value is independent of the EGTA concentration used to buffer the ionized Ca. The relation between ionized Ca and catecholamine release is best fitted by a requirement for 2 Ca ions.

Calcium-evoked release of catecholamine is associated with the release of dopamine-β-hydroxylase (DβH) but not lactate dehydrogenase. The ratio DβH/catecholamine released is the same as that in stimulated intact cells and perfused glands. The time course of appearance in the external medium of DβH and catecholamine is identical. Transmission electron microscopy of 'leaky' cells exposed to 10^{-8} M Ca reveals no marked differences from unstimulated intact cells. The cytoplasm of 'leaky' cells exposed to 10^{-5} M Ca contains large membrane-bounded vacuoles. When secretion is caused to take place in the presence of horseradish peroxidase, this marker is found within the vacuoles.

Ca-dependent release of both catecholamine and DβH requires Mg-ATP. Cells equilibrated with Ca in the absence of Mg-ATP can be triggered to undergo exocytosis by the addition of Mg-ATP. In the absence of Mg, ATP alone is ineffective. Of a variety of other nucleotides tested, none is as effective as ATP. Mg-ATP affects the extent of exocytosis and not its apparent affinity for Ca.

Replacement of glutamate as the major anion by chloride results in a marked reduction in Ca-dependent release of both catecholamine and DβH. Chloride causes a small increase in Ca-independent release of catecholamine, a large reduction in the extent of exocytosis, and a decrease in the apparent affinity of exocytosis for Ca. Of a variety of anions examined, their order of effectiveness at supporting Ca-dependent exocytosis is glutamate$^-$ > acetate$^-$ > Cl^- > Br^- > SCN^-.

Exocytosis is not obviously affected by replacing K by Na or sucrose or by altering the pH over the range pH 6.6 to 7.8. Raising the free Mg concentration reduces the extent of Ca-dependent exocytosis and also its apparent affinity for calcium. Calcium-dependent exocytosis in 'leaky' cells is largely unaffected by (i) a variety of agonists and antagonists of the nicotinic receptor; (ii) agents that disrupt microtubules and microfilaments; (iii) phalloidin; (iv) vanadate; (v) inhibitors of anion permeability; (vi) protease inhibitors; and (vii) agents that dissipate the vesicle pH gradient and potential. It is partially inhibited by (i) certain antipsychotic drugs; (ii) a rise in osmotic pressure, (iii) lowering the temperature below 20 °C, and (iv) N-ethyl maleimide.

Key words exocytosis · calcium · adrenal medulla

Introduction

Secretion of many neurotransmitter substances, hormones, and enzymes seems to occur in two stages. The substances to be released are first packaged into vesicles within the cell, and the contents of these vesicles are subsequently released into the external medium in a reaction that involves fusion of the limiting membrane of the vesicle with the plasma membrane of the cell. This fusion reaction is called exocytosis, and in many secretory cells the rate of exocytosis is calcium-dependent. Studies of the voltage-dependence of transmitter release at the squid giant synapse (Katz & Miledi, 1967; Kusano, Livengood & Werman, 1967), experiments with calcium ionophores in a variety of cells (Foreman, Mongar & Gomperts, 1973; Feinman & Detwiler, 1974; Eimerl, Savion, Heichal & Selinger, 1974; Steinhardt & Epel, 1974), and in a few favorable instances direct injection of calcium (Miledi, 1973; Kanno, Cochrane & Douglas, 1973) all suggest that the site at which calcium acts to control exocytosis is intracellular. This view is supported by experiments with calcium-sensitive photoproteins which reveal a

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rise in intracellular ionized calcium associated with exocytosis (Llinas, Blinks & Nicholson, 1972; Llinas & Nicholson, 1975; Gilkey, Jaffe, Ridgway & Reynolds, 1978). Because of the relative inaccessibility of this intracellular site, virtually nothing is known of the quantitative relation between intracellular ionized Ca and exocytosis — nor of the other requirements, if any, of the exocytotic reaction.

In this paper we describe a new experimental procedure by which the plasma membrane barrier can be by-passed without interfering with the ability of the remaining membrane to take part in exocytosis. This new technique permits experimental control over the intracellular site at which exocytosis takes place. Although this paper is addressed specifically to the application of the method to elucidating the properties of exocytosis in bovine adrenal medullary cells, the technique seems to have wide applicability and has already been used by us to gain access to the interior of sea urchin eggs (Baker, Knight & Whitaker, 1980), human platelets (Knight & Scrutton, 1980), and *Paramecium*, and by Pace et al. (1980) to the pancreatic β cell. The most important conclusions from this work are that secretion, in a variety of tissues, has a rather specific requirement for Mg-ATP and is activated by Ca concentrations in the micromolar range.

Some of our results on adrenal medullary cells have already been described briefly (Baker & Knight, 1978*a*, *b*; 1979*a*, *b*; 1981).

Theory

When a suspension of cells is exposed to a high electric field, their membranes become permeable to solutes (Sale & Hamilton, 1968; Neumann & Rosenheck, 1972; Zimmermann, Pilwat & Riemann, 1974; Kinosita & Tsong, 1977; Baker & Knight, 1978*a*, Jeltsch & Zimmermann, 1979; Baker et al., 1980; Knight & Scrutton, 1980). It is thought that the voltage developed across the membrane as a result of exposure to an electric field causes the membrane to break down and become permeable. The theory predicting the magnitude of this voltage developed across the surface membrane by such a field has been briefy mentioned by other authors (Sale & Hamilton, 1968; Neumann & Rosenheck, 1972; Zimmermann et al., 1974; Schwan, 1977; Jeltsch & Zimmermann, 1979; Knight, 1981), the original theory being due to J.C. Maxwell (1892). Very simply, it is as follows:

If a cell, which may be considered as a hollow thin walled sphere, is exposed to an electric field, the magnitude of the potential difference imposed across its membrane will depend on the intensity of the field (E volts cm^{-1}), and the radius (b) of the cell and will vary around the cell's circumference. With reference to Fig. 1 the voltage (volts) at point $P(Vp)$ across the membrane of a spherical cell is given by the simplified equation

$$Vp = C \cdot b \cdot E \cdot \mathrm{Cos}\,\theta \tag{1}$$

where the value of the constant term C depends on the relative electrical conductivities of the extracellular fluid, the cytosol and the membrane, the size of the cell, and is given by the relation (Zimmermann et al., 1974)

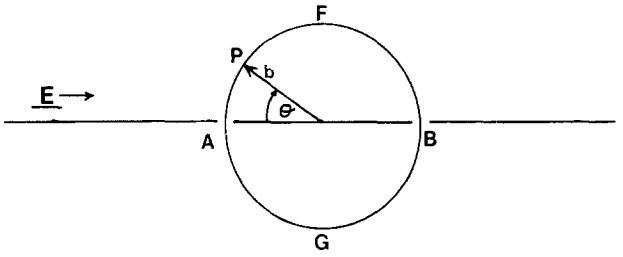


Fig. 1. Diagrammatic representation of a cell in an electric field E (*see* text)

$$C = \frac{3}{b(2+\beta)} \times \frac{b - a + \dfrac{\alpha - 1}{1+\alpha} a^3 (a^{-2} - b^{-2})}{1 + 2\left(\dfrac{1-b}{2+\beta}\right)\left(\dfrac{1-\alpha}{2+\alpha}\right)\left(\dfrac{a}{b}\right)^3} \tag{2}$$

where $\beta = \lambda_1/\lambda_2$ in which λ_1 is the conductance of the cytosol, and λ_2 is the membrane conductance, $\alpha = \lambda_2/\lambda_3$ in which λ_3 is the conductance of the extracellular fluid and a and b are the internal and external radii respectively ($b - a$ is the membrane thickness).

Adrenal medullary cells in suspension are roughly spherical of radius 10 μm. The total intracellular ionic content is similar to that of the extracellular medium, and so the specific conductivity of the cytosol may be of the same order as that of the extracellular fluid, i.e., $\lambda_1 = \lambda_3 \sim 0.01$ mho cm^{-1}. The membrane thickness ($b - a$) in Eq. (2) is not known but could range from, say, 5 to 10 nm. The membrane resistance of the adrenal cell seems to be between 1 and 4 kohm cm^2 (Brandt, Hagiwara, Kidokoro & Miyazaki, 1976). Considering a membrane thickness of between 5 and 10 nm, this corresponds to a specific conductance of between 10^{-9} and 10^{-11} mho cm^{-1}. If the excitable cell was fully depolarized the specific membrane conductance could rise to, say, 10^{-7} mho cm^{-1}. The value of β in Eq. (2) could therefore range from 10^5 to 10^9.

Table 1 shows the values of C calculated from Eq. (2) over this range of β. For membrane thicknesses of 5 and 10 nm, they do not differ significantly from a value of 1.5. This value of 1.5 is an asymptotic value of C in Eq. (2) under the conditions that the conductances of the extra- and intracellular fluids are much greater than that of the membrane and that the dimensions of the cell are much greater than the membrane thickness. The voltage imposed across the membrane of the adrenal medullary cell exposed to an electric field may therefore be written from Eq. (1) as:

$$V_p = 1.5\, bE \,\mathrm{Cos}\,\theta. \tag{3}$$

From this equation it can be seen that the potential difference imposed across the membrane will be a maximum at the points A and B in Fig. 1 corresponding to $\mathrm{Cos}\,\theta = \pm 1$ and will decrease around the cell to positions F and G where the electric field has no influence on the membrane ($\mathrm{Cos}\,\theta = 0$). If, therefore, the 20 μm diameter adrenal medullary cell is placed in an electric field of, say, 2000 V cm^{-1}, a transmembrane potential of 3 V ($1.5 \times 10^{-3} \times 2000$) would be generated at opposite ends of the cell in line with the electric field, i.e., at points A and B. It is this large potential difference that is thought to cause the membrane to break down and become leaky to solutes. Studies on nerve, muscle, and artificial membranes suggest that a potential difference of 0.3 V is sufficient to cause a membrane to break down in as much as the membrane exhibits nonohmic properties. Other studies, however, using cell suspensions, artificial membranes, and giant plant cells (Sale & Hamilton, 1968; Zimmermann et al.,

Table 1. Calculated values for the constant C in Eq. (2) for different conductances and membrane thicknesses.

		β	
		10^5	10^9
$b-a$ (nm)	5	1.456	1.499
	10	1.478	1.499

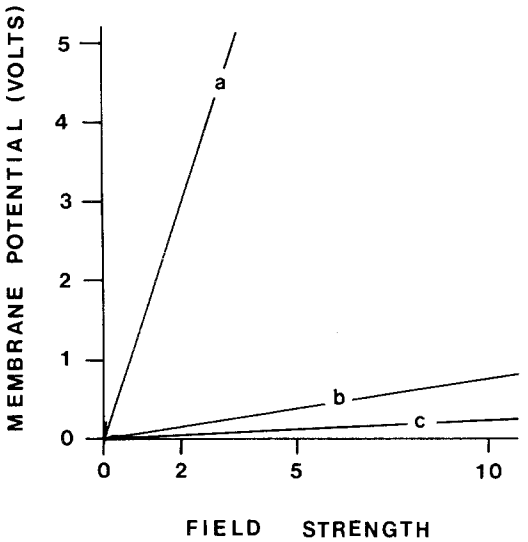


Fig. 2. The expected maximum *trans* membrane potential (from Eq. (3)) across the limiting membranes of (*a*) whole adrenal medullary cells 20 μm diam, (*b*) mitochondria 1 μm diam, and (*c*) chromaffin granules 0.2 μm diam, as a result of exposure to various field strengths

1974; Coster & Zimmermann, 1975*a*, *b*; Jeltsch & Zimmermann, 1979; Benz, Beckers & Zimmermann, 1979) point strongly to a membrane potential of about 1 V being necessary to cause breakdown. The difference in these voltages, and durations over which they must be applied, might reflect different breakdown mechanisms or different criteria upon which breakdown is deemed to have occurred (Crowley, 1973; Benz et al., 1979; Benz & Zimmermann, 1980). Permeability changes to small molecular weight solutes seem to be associated with transmembrane voltages of about 1 V. Under the influence of a 2000 V cm^{-1} field, therefore, the adrenal cell should be breached at the poles of the cell (*A* and *B* in Fig. 1), as it is at these points we calculate that the voltage developed across the membrane is greatest (approximately 3 V).

Figure 2 describes the maximum voltage imposed across the membranes of the adrenal cell and its organelles as a result of exposing them to electric fields of different strengths [from Eq. (3)]. It shows that if the adrenal medullary cell is exposed to a field of, say, 2000 V cm^{-1} the resultant maximum transmembrane potential of 3 V should bring about collapse of the plasma membrane, whereas two of the intracellular organelles — the vesicles (approximately 0.2 μm diameter) containing the secretory product catecholamine, and the mitochondria (approximately 1 μm diameter) are of such sizes that the voltage imposed across their membranes would only be 30 and 150 mV, respectively, and insufficient to render them leaky. Indeed in the case of the mitochondrion with its double membrane, it could be expected that the voltage necessary to induce mechanical collapse would be twice that necessary in a similarly sized organelle bounded by a single membrane.

The dependence of membrane breakdown on particle size suggests that a field of 2000 V cm^{-1} might permit access to the cytosol without disturbing the intracellular organelles and hence their function. For a voltage to be imposed across the membrane by an externally applied electric field, the duration of the applied field must be at least as long as the time taken for the membrane to charge up. If the electric field is imposed as a step function, i.e., it does not decay with time, then the voltage across the membrane will increase exponentially, the time constant τ_m sec being given by (Schwan, 1957; Sale & Hamilton, 1968; Jeltsch & Zimmermann, 1979)

$$\tau_m = b C_1 \left[\frac{\lambda_3 + 0.5\lambda_1}{\lambda_1\lambda_3 + b\lambda_2(\lambda_3 + 0.5\lambda_1)} \right]$$

in which C_1 is the capacitance of the membrane ($F\,cm^{-2}$), and b, λ_1, λ_2 and λ_3 are as defined before, having the units of cm and mho cm^{-1}.

Assuming $\lambda_1 \approx \lambda_3 \sim 10^{-2}$ mho cm^{-1}, $C_1 \approx 2\,\mu F\,cm^{-2}$ (Brandt et al., 1976), $b \approx 10^{-3}$ cm, and $\lambda_2 \leqq 10^{-7}$, the time constant for the charging up of the membrane should be about 0.3 μsec. To achieve a voltage across the membrane, therefore, the applied electric field must last longer than 0.3 μsec.

Materials and Methods

Chemicals and Solutions

All chemicals were of A.R. grade (Sigma, Fisons, or BDH). Disodium ATP (vanadium free) was obtained from Sigma. In a few experiments dipotassium ATP was used, but there was no difference between these experiments and those experiments using disodium ATP.

Three solutions were used:

A. (Physiological saline) (mM): NaCl, 136; KCl, 2.7; glucose, 5; Na-HEPES, 8; $MgCl_2$, 1.8; and $CaCl_2$, 1.8; pH 7.2.

B. (Glutamate solution) (mM): potassium glutamate, 138.7; glucose, 5; K-PIPES, 20; ATP, 5; Mg acetate, 7; EGTA or Ca-EGTA, 0.4–50; pH 6.6.

C. Sucrose solution (mM): sucrose, 270; K acetate, 20; glucose, 5; K-PIPES, 20; ATP, 5; Mg acetate, 7; EGTA or Ca-EGTA 0.4–50; pH 6.6.

All solutions were equilibrated with 100% O_2 before being used. In a few experiments which investigated the calcium and ATP dependence of secretion, glucose was omitted from the solutions. There was no difference, however, between these and control experiments that included glucose.

Preparation and Handling of Medullary Cells

Bovine adrenal glands were obtained from the local abattoir where they were excised within 15 min of death, freed from peripheral fat, perfused through the adrenal vein with ice-cold physiological saline solution containing 0.5% bovine serum albumin (BSA), and transported back to the laboratory on ice.

The adrenal cortex, which is visually distinguishable from the medulla, was removed and discarded. Thin slices (less than 1 mm thick) were cut from the isolated medulla using stacked single edged razor blades and any obvious islands of cortical tissue removed. The medullae of three glands were usually used for each experiment. After washing with ice-cold oxygenated solution the slices were brought to 37 °C and incubated with saline (approx. 5 ml/g of tissue) containing 0.2% protease (Type V Sigma). The

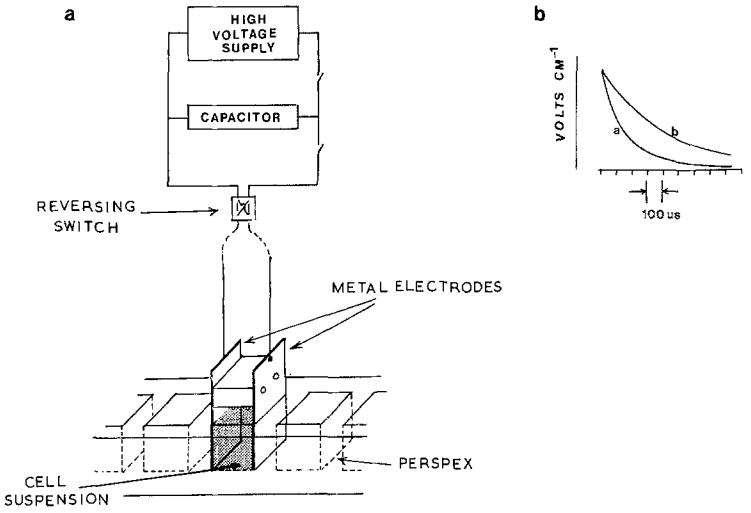


Fig. 3. (*a*): The experimental set-up used to expose a suspension of cells to a brief electric field. (*b*): The time course of the electric field through the cell suspension made up in (*a*) solution B and (*b*) solution C (p. 109) when the applied high voltage is 2 kV and the capacitor 2 μF

enzyme digestion was carried out in a large flat bottomed 250 ml conical flask, which was agitated gently. The depth of the fluid was approximately 3 mm, and oxygen, saturated with water, was passed over the surface. After 30 min the solution was replaced by a similar 0.2% protease solution and the tissue slowly disaggregated by gently drawing through a wide bore (4 mm id) plastic pipette tip. After a further 30 min an equal volume of saline containing 0.2% collagenase (Type 1, Sigma) and 0.2% hyaluronidase (Type 1S, Sigma) together with 0.5% BSA was added. Dissaggregation by pipetting was continued throughout this last 30-min period of enzyme digestion, and the cells were then filtered through a double layer of muslin.

The cells were washed by layering the suspension on top of saline solution containing 7 mM dextran (mol wt 9400), spinning them through at $100 \times g$ for 4 min and resuspending the loose pellet in fresh saline solution. This procedure was not usually repeated more than twice, by which time the extracellular fluid had been 'cleaned' to the extent that it contained less than 5% of the total catecholamine measured in the suspension. Cells were washed several times in either solution *B* or *C* containing EGTA to give a calculated ionized Ca less than 3×10^{-8} M.

Method of exposing cells to electric fields. Cells suspended in solution *B* or *C* were exposed to a short duration electric field by discharging a capacitor through the cell suspension. The typical set-up is shown in Fig. 3*a*. The 2-μF capacitor was charged to a known voltage, isolated from the supply, and discharged through 1 ml of cell suspension between steel electrodes (area 1 cm^2) positioned 1 cm apart. The electric field in the cell suspension decayed exponentially with a time constant given by the product of the capacitance and the resistance of the cell suspension. A 2-μF capacitor discharged through solution *B* produced a field decaying with time constant of about 200 μsec. As explained, the two main solutions we used were either based on potassium glutamate or on sucrose. This latter solution has a higher resistivity than the glutamate solution, and therefore with this solution the duration of the electric field to which the cells are exposed will be correspondingly longer. Figure 3*b* shows the time courses of the electric field through the cell suspension. This was measured by monitoring the voltage across a low resistance in series with the suspension. When a series of discharges was given, the reversing switch was used to reduce the effects of electrical polarization. In practice, however, for such short pulse durations and large voltages the polarization effects were negligible, i.e., less than 5% change in voltage or time course over 30 repeated discharges.

The temperature rise in the cell suspension as a result of the electric fields was measured using a thermistor. For ten discharges of a 2-μF capacitor (2 kV) the temperature rose by 6 °C. Before cells were exposed to a series of discharges, the suspension in the chamber was allowed to cool so that the subsequent heating effect did not raise the temperature above 37 °C. Immediately after the discharges the electrodes were withdrawn and the cells either left in the chamber or transferred to a test tube in a water bath. In the former case, the temperature could not always be maintained at 37 °C but occasionally fell to about 30 °C. However, no differences in the properties of the leaky cells over this temperature range were observed. A series of 10 rapid discharges took 30 sec to apply.

A simple brief field exposure is expected to produce breakdown of the cell membrane at two oppositely positioned sites. The cells in suspension will rotate because of random motion, and therefore if another brief exposure is given when the sites of initial breakdown are at 90° to the field, the field should see an intact sphere and the membrane again be breached in another two places. It is possible to influence the membrane in an identical fashion when the field again sees an intact sphere, i.e., when the four sites of breakdown are in a plane orthogonal to the field. If the field is not at 90° to the sites of breakdown, however, it would not see an intact sphere but rather a sphere with localized highly conducting regions through which the field lines could be routed.

It is to be expected, therefore, that successive discharges will initially increase the permeability of the cell by increasing the number of effective pores made, but the ability to increase the permeability further might decrease as the number of discharges increases. The reduced effect of successive discharges would depend on the size and number of the pores already in the membrane and their effectiveness in acting as a sink for the field lines. If the effective pores are very small compared with the size of the cell, their perturbing effect on the field will be small enough to allow further discharges to create more pores.

As will be shown in the results section, the strength and duration of the electric field used seems to render the cells permeable with an equivalent pore diameter of between 2 and 4 nm. Such small pores do not act as effective sinks, as a number of discharges seemed to bring about a correspondingly larger number of pores of about the same size (Table 4).

Preparation of Granules from 'Leaky' Cells

Leaky cells were resuspended in a small volume of solution C (p. 109), containing 10 mM EGTA, at 4 °C and then homogenuzed with sand pre-washed with the same solution. The debris and sand were removed by centrifuging at $4{,}000 \times g$ for 10 min and the granule fraction spun out at $15{,}000 \times g$ for 15 min and resuspended in solution C containing 0.2 mM EGTA.

The Permeability of the Plasma Membrane after Exposure to the Electric Field

The effect of a single discharge is expected to produce two pores in the membrane and "m" such discharges might produce "$2m$" holes. Calculation of the approximate pore size is possible by measuring the rates of either influx or efflux of various solutes into and out of the cells. Consider the efflux of a solute. The rate of loss of a solute from a cell will be limited by (i) the rate at which it can diffuse through the pores and also (ii) the rate with which it can diffuse towards the pores from the interior of the cell. The diffusion time for the second process is of the order of b^2/D where b is the radius of the cell and D is the diffusion coefficient of the solute in the cytosol. For the 20-μm diameter adrenal cell the time for solutes to reach the pores is less than 1 sec when their diffusion coefficients are about 10^{-5} cm^2 sec^{-1}. If, therefore, the rate of efflux is very much slower than this it would suggest that the rate-limiting step is the rate at which the solute diffuses through the pores and that the concentration of the solute throughout the cytosol should be constant. Under these conditions the calculation of the average pore size is simplified. Suppose the cell has a radius of "b" cm and "m" circular holes in it, each of radius r cm. Suppose further that the concentration of the solute in the cell at time t was "c" and that outside it was zero. The concentration gradient across the membrane of thickness x would cause a diffusional flux of solute out of the cell at a rate proportional to the product of the area of membrane through which solute passes and the concentration gradient across the membrane.

$$\text{i.e., } V\frac{dc}{dt} = -DmIIr^2\frac{c}{x}$$

where V is the volume of the cell. Rearranging and simplifying gives

$$dc = -\frac{3r^2Dm}{4b^3x}\cdot c\,dt$$

which has a solution $c = c_0 e^{-t/\tau_f}$ where c is the concentration in the cell at time t, c_0 is its initial concentration and the time constant of the efflux is given by

$$\tau_f = \frac{4b^3x}{3Dmr^2} \tag{4}$$

Hence by measuring the efflux of a solute from a cell of known radius b, and by assuming a free solution diffusion coefficient D (either from the marker itself or a closely related molecule), the number of pores, m, and the membrane thickness x, the apparent radii r of the pores may be calculated. A similar solution is obtained for influx data where the concentration c of the solute in the cell at time t is given by $c = c_o(1 - \bar{e}^{t/\tau_f})$ where c_o is the final concentration and τ_f is as above.

Calcium-EGTA Buffers and Free Calcium Values

200 mM stock Ca EGTA buffers were made of various Ca/EGTA ratios and the pH brought to 6.6 with KOH. Volumetric $CaCl_2$ (BDH) and EGTA (Sigma) were measured to give molar ratios of Ca/EGTA of 0, 0.1 ± 0.002, 0.4 ± 0.003, 0.7 ± 0.008, 0.9 ± 0.005, 0.95 ± 0.003, 0.975 ± 0.003, 0.9875 ± 0.003. The errors in these ratios represent the maximum error in measuring out the $CaCl_2$ and EGTA. These solutions have been made up several times and have given identical physiological responses. The free calcium concentrations of these buffers in glutamate solution of pH 6.6 were measured using a calcium selective electrode (Orion research No. 92-20) and were in agreement with values calculated using equilibrium constants between EGTA and Ca, and EGTA and Mg of $10^{-5.708}$ M and $10^{-1.136}$ M, respectively, and equilibrium constants between ATP and Ca, and ATP and Mg of $10^{-3.47}$ M and $10^{-3.61}$ M, respectively. These constants were calculated from the stability constants listed in Martell and Sillen (1964). In some experiments Ca EGTA buffer was added to a solution containing 0.4 mM EGTA, and in these cases a pH change of less than 0.02 units occurred.

Electrode measurements gave contaminating calcium levels in our basic media (i.e., in the absence of EGTA) of between 1 and 4 μM.

Analytical Methods

Potassium concentrations were measured on a Corning Flame Photometer (model 400); ^{14}C, ^{45}Ca and ^{3}H were counted on a Packard tricarb liquid scintillation counter. Internal standardization was used to check for tritium quenching. This was monitored in over 40 experiments, and the quenching corresponded to only 3.8% (sθ 0.6%) and was, therefore, neglected in our calculations.

Assays

Catecholamine was measured fluorometrically (Von Euler & Floding, 1961) and lactate dehydrogenase (LDH) and Dopamine-β-Hydroxylase (DβH) spectrophotometrically (Bergmeyer, Bernt & Hess, 1965; Aunis, Serck-Hanssen & Helle, 1978). One unit of LDH is defined as the amount of enzyme which converts 1 μmol of NADH to NAD per min at 25 °C. One unit of DβH is defined as the amount of enzyme that produces 1 μmol of octapamine per min at 37 °C. The noradrenaline/adrenaline ratios were determined over a range of experiments investigating both calcium-dependent and calcium-independent release. However, as they were always similar to the ratios found within the cells, i.e., there appeared to be no preferential release, we have expressed our results as total catecholamine. Our experimental conditions did not interfere with catecholamine or LDH assays; however, the DβH assay was inhibited by the major anion, glutamate in Solution B, and also by the EGTA in our solutions. The interfering effect of glutamate (and other anions) on the DβH assay is shown as follows. A gland was perfused retrogradely at 37 °C as described by Banks (1965) with solution A containing 3.6 mM $CaCl_2$. It was stimulated to secrete catecholamine by raising the K level in the perfusion fluid, and aliquots of this perfusate were added to 5 volumes of either 300 mM sucrose 5 mM K Hepes, or into solutions in which the chloride had been replaced by such anions as glutamate, thiocyanate, iodide, or acetate. The DβH activity relative to that based on a chloride solution is shown in Table 2.

Table 2. DβH activity in different media[a]

	Chloride	Sucrose	Acetate	Glutamate	Iodide	Thiocyanate
Relative activity	1	1.08	1.04	0.32	0.21	0.02
Standard deviation		0.12	0.05	0.09	0.02	0.02
(No. of samples)		5	5	5	5	5

[a] Cl replaced isosmotically.

Table 3. DβH activity at different EGTA or Ca-EGTA concentrations relative to that in the absence of EGTA[a]

	DβH mU/ml	EGTA or Ca-EGTA (mM)							
		0	0.2	0.4	0.6	0.8	1.0	8.0	40
EGTA	25	1.0	0.9	0.83	0.8	0.7	0.59		
EGTA	11	1.0				0.73		0.05	0
EGTA	4.8	1.0	0.95	0.83	0.85	0.88	0.73		
Ca-EGTA	4.8	1.0	1.0	0.95	0.82	0.95	0.61		

[a] The first column shows the various concentrations of DβH used

As can be seen, there is no difference between the DβH activities when the sampling solution is made up of either chloride, acetate, or sucrose. However, the solutions containing the anions glutamate, iodide, or thiocyanate interfere with the assay. One might expect that iodide and thiocyanate would interfere with the enzyme as their positions in the lyotropic series (Fridovich, 1963) suggest that they might interact with proteins. The reason for interference from glutamate is, however, not clear at first sight. As will be described later it was necessary for us to quantify the amount of DβH released from our material. The small volume and large number of samples together with the small amount of DβH released made it prohibitive to dialyze the glutamate away or reduce its concentration by dilution. Secretion in a glutamate solution gives us qualitative results of DβH release, but we need to have secretion in either a chloride, acetate, or sucrose solution in order to determine precisely how much is released. As will be seen in the results section (p, 127), both chloride and acetate seem partially to inhibit the secretory process in leaky cells and so a suspending medium based on sucrose (Solutions C, p. 109) was chosen for experiments in which DβH, LDH, and catecholamine were all assayed.

As copper is a co-factor of the enzyme DβH (Mahler & Cordes, 1966) it is not surprising that the chelator EGTA in our solution interferes with the assay. Attempts to overcome this problem by covering the EGTA with copper failed, presumably because we were unable to define the μM levels of free copper necessary for optimum assay conditions (Aunis, Bouclier, Peschelocke & Mandel, 1977). Our attempts to define μM levels by displacing the chelated copper from EGTA by using bismuth or by using copper-glycine buffers were unsuccessful. The affinity of EGTA for copper is calculated to be $10^{-12.5}$ M (Martell & Sillen, 1964) which is over six orders of magnitude higher than that of calcium ($10^{-5.7}$ M). This means that the inhibitory effect of EGTA should be the same as Ca-EGTA. To determine the magnitude of the inhibitory effect the perfusate from a stimulated whole gland was diluted to give a range of DβH concentrations and then incubated with various concentrations of EGTA buffers (0–40 mM). The activities were assayed and the results shown in Table 3. As can be seen, EGTA is inhibitory at concentrations above 1 mM, and indeed at 10 mM the DβH activity is reduced to less than one tenth. There is no significant difference between buffers with or without calcium or over a wide range of DβH concentrations. For these reasons our DβH assays were performed on samples containing not more than than 0.4 mM EGTA.

Experimental Procedure with Leaky Cells

To investigate the effect of calcium on secretion, the cells were either rendered leaky in the presence of 0.4 mM EGTA (no added calcium) and aliquots of these pooled leaky cells challenged with Ca-EGTA buffers or exposed to the intense electric fields in media containing the required test concentrations of Ca. Where it was necessary to quantify the amount of DβH released, the cells were either rendered leaky in the presence of 0.4 mM Ca-EGTA buffers or rendered leaky in the presence of 0.4 mM EGTA and subsequently challenged with 0.6 mM Ca^{2+} (0.4 mM $Ca(OH)_2$ and 0.2 mM $CaCl_2$).

In some experiments the intracellular ionized Ca was raised from 10^{-8} to 10^{-5} M in steps by means of the sequential addition of Ca-EGTA buffers. The final Ca-EGTA concentration did not exceed 12.8 mM. The sequential additions of buffer did not change the pH of the solution by more than 0.01 units. In other experiments it was necessary to challenge the cells repeatedly with the same ionized calcium level. The lowering of the calcium concentration between challenges was achieved by either diluting with large volumes of solution B (p. 109) containing 1 mM EGTA or by repeatedly washing the cells. In both cases Ca electrode measurements confirmed that the ionized calcium levels were reduced to less than 10^{-7} M.

Flux Experiments

Influx experiments were performed with ^{45}Ca-EGTA and $^{3}H_2O$. The cells were incubated with these markers for 10 min, after which time the $^{3}H_2O$ had equilibrated fully with the cell water. The cells were then rendered leaky, aliquots of the suspension layered on Versilube F50 oil (a gift from Alfa Chemicals, Staines, Middlesex, U.K.) and the cells spun through the oil at 10,000 × *g* for 2 min in an Eppendorf centrifuge. The tubes were immersed in a mixture of dry ice and acetone and when frozen, the tube ends containing the pellets cut off. The oil surrounding the pellet was drained off and the pellet solubilized in 0.5 ml of 1% Triton in distilled water. 4 ml of scintillation cocktail was added and the mixture counted. The amount of $^{3}H_2O$ spun down reflected the total volume of cells and extracellular fluid in the pellet. More ^{45}Ca-EGTA was associated with pellets of cells rendered leaky than with pellets of intact cells. This difference is explained by the calcium buffer diffusing into those cells rendered leaky. The accessability of the calcium buffer to the interior of the cell is expressed in terms of the ^{45}Ca-EGTA space as a percentage of the tritium space.

Influx experiments were also performed with the γ emitter ^{51}Cr-EDTA. The procedure here was identical to that with the ^{45}Ca-EGTA and $^{3}H_2O$ markers, except that the pellet was solubilized in 1 ml of 0.1 M HNO_3, counted, diluted with 4 ml of distilled water and the potassium content measured by a flame photometer (Corning Model 400). The amount of K^+ reflects the total volume of the pellet because for cells immersed in solution B there is no loss of cellular potassium even from leaky cells as the potassium content of the external medium is roughly the same as that of the intact cell. More ^{51}Cr-EDTA was associated with leaky cells than with intact cells and the accessability to the

interior of the cell is expressed in terms of the γ emitter space as a percentage of the potassium space.

^{86}Rb and [^{14}C]3-O-methyl D-glucose efflux measurements were performed as follows. Aliquots of the markers were added to the cell suspension during the last 30-min period of enzyme digestion of the isolation procedure. During this time the ^{86}Rb enters the cells by the Na—K exchange pump (Pocock, 1979) and the nonmetabolizable sugar 3-O-methyl D glucose by the glucose transport system. The cells were washed in solution A as usual (thereby reducing the concentration of the extracellular marker) and after rendering them leaky were spun through oil as before and counted.

Electron Microscopy

The method of fixation of adrenal medullary cells for electron microscopy was essentially that described elsewhere (Fenwick, Fajdiga, Howe & Livett, 1978; Baker & Knight, 1981). Cells were diluted into 10 volumes of ice-cold 2% (wt/vol) glutaraldehyde in solution C containing 10 mM EGTA. After 2 hr the cells were washed twice in ice cold buffer and resuspended in a small volume of 2% agar in buffer and post fixed for 2 hr with 2% osmium tetroxide in buffer. After post fixation the matrix of cells was washed in buffer to remove residual osmium, dehydrated in a graded series of ethanols and embedded in Spurr's epoxy resin. Blocks were sectioned on an LKB ultratome. The silver-gold sections were stained with uranyl acetate followed by alkaline lead citrate. The preparation of cells exposed to the extracellular marker horseradish peroxidase (5 mg/ml), was essentially as described by Fried & Blaustein (1978). The buffer used was, as above, solution C containing 10 mM EGTA. Cells exposed to horseradish peroxidase were not stained with uranium and lead salts.

Results

Gaining Access to the Cell Interior

Choice of applied field. Although the conditions required to effect dielectric breakdown can be calculated, it is essential to have a direct means of assessing the increase in permeability. As we are particularly concerned about accessability to Ca-EGTA, its uptake was measured as a function of the duration and magnitude of the applied field. Figure 4 shows that when the time constant of field decay is 5 μsec or less little or no calcium buffer enters the cell even when the field strength is increased to 5 kV cm^{-1}. Fields of 200 μsec time constant, however, do render the membrane permeable to the buffer, the degree of permeability increasing with increasing field strength, threshold appearing to be about 500 V cm^{-1} (corresponding to a *trans*membrane voltage of 0.75 V) and the maximum rate of change occurring between 1 and 2 kV cm^{-1}. Exposure to higher field strengths causes an appreciable temperature rise and a greater risk of disturbing the integrity of the intracellular organelles, and although exposure to higher voltages further increases the permeability we chose to work routinely with a field strength of 2 kV cm^{-1} and a duration of 200 μsec.

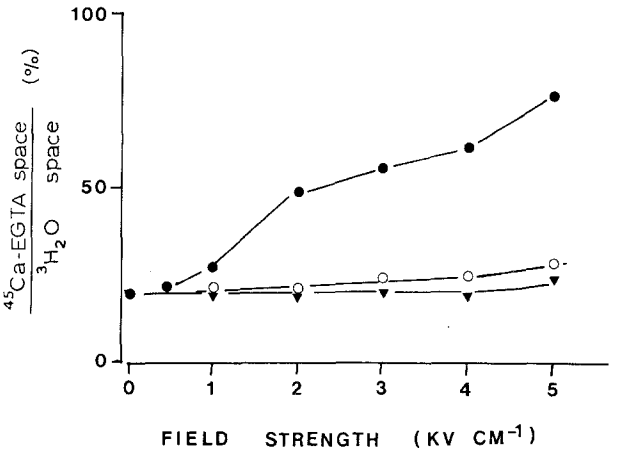


Fig. 4. The amount of ^{45}Ca-EGTA associated with adrenal medullary cells 25 min after exposing them to an electric field (500 to 5000 V cm^{-1}) for durations of 200 μsec (●), 5 μsec (○) and 0.5 μsec (▼)

Effect of cell density. For experiments involving only catecholamine assay, we routinely used cell densities of 10^6/ml, but for a few of our experiments (influxes, DβH assays) it was necessary to work with higher densities (up to 2×10^7 ml^{-1}). Although it is possible that at very high densities all the cells may not experience the full electric field applied, no evidence for this was found up to the highest densities used. At 2×10^7 ml^{-1}, the volume fraction is 0.08 which is theoretically (Cole, 1928; Schwan, 1957) insufficient to perturb the electric field. Direct evidence that the effect of cell density is negligible comes from measurements of catecholamine release in response to a calcium challenge where the percentage of catecholamine secreted is independent of the cell density (up to 2×10^7 ml^{-1}) at which the cells were rendered leaky.

Calculation of effective pore size from permeability data. Figure 5 shows that after subjecting cells to a single discharge of 2 kV cm^{-1}, $\tau = 200$ μsec, the increased efflux of ^{86}Rb or [^{14}C]3-O-Methyl D-Glucose or influx of ^{51}Cr-EDTA are all exponential processes. If it is assumed that the exponentials reflect movement of the marker substances through pores [2/discharge for which there is direct evidence in the much larger sea urchin egg (Baker, Knight & Whitaker, 1980; Knight, 1981)] generated in the cell membrane – as opposed to a general increase in the permeability – it is possible to use the data in Fig. 5 in conjunction with Eq. (4) (p. 111) to calculate the effective pore radius. Application of more shocks should create additional holes with a concomitant reduction in τ_f. Table 4 summarizes the effective pore radii calculated for different experimental protocols.

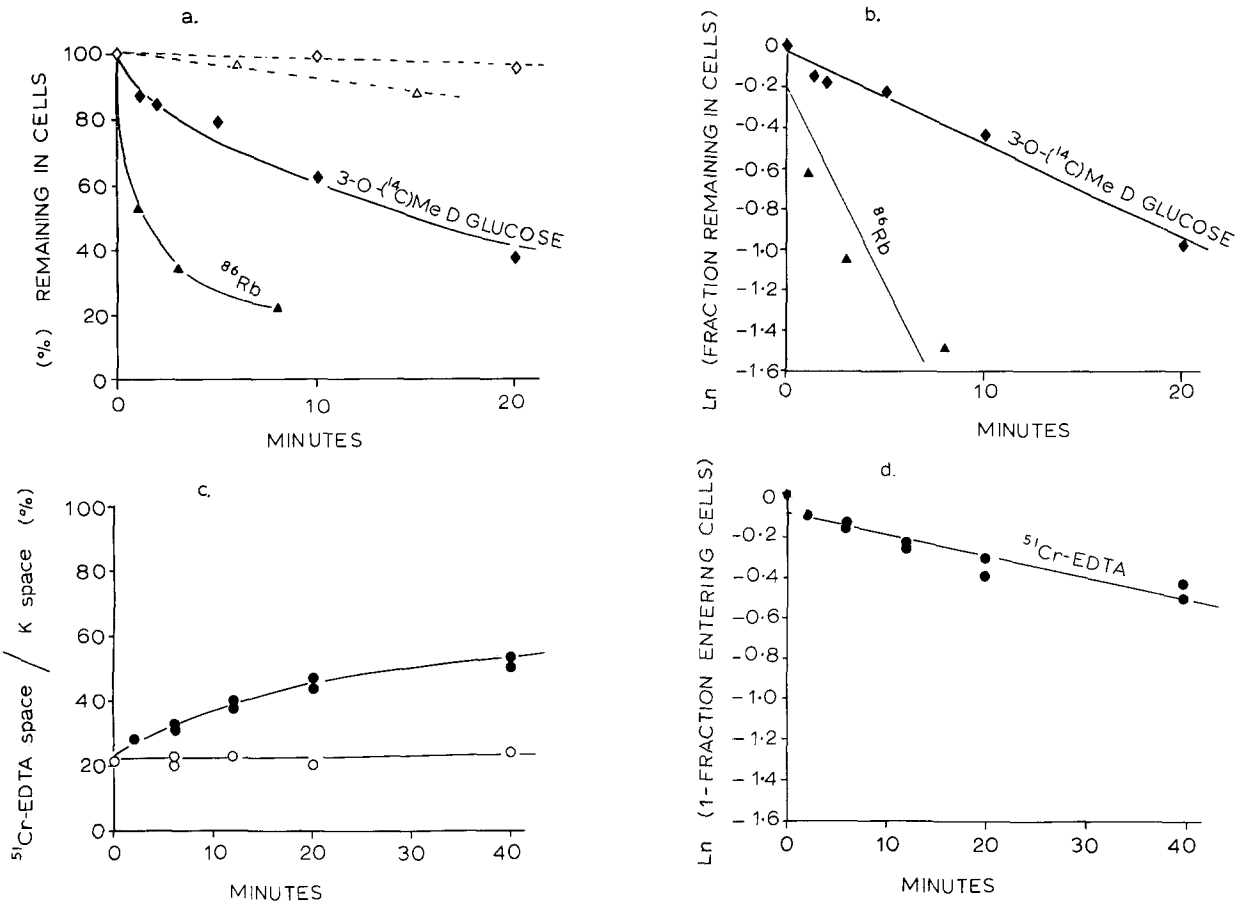


Fig. 5. Use of marker substances to monitor accessibility to cytosol. (*a*): Efflux of marker substances. The percentage of radioactive marker remaining in the cells at times after a single exposure to an electric field of 2000 V cm^{-1}, $\tau \sim 200$ µsec ^{86}Rb, ▲; [^{14}C]3-O- Me-D-glucose, ◆. Control cells not exposed to the electric field, open symbols. Cells were suspended in glutamate medium. Soln. B (in Methods) containing 0.4 mM EGTA. Temp. 20 °C. (*b*): A semi-log plot of the amount of ^{86}Rb (▲) and [^{14}C]3-O-Me-D-glucose (◆) remaining in the cells (from Fig. 5*a*). (*c*): Influx of ^{51}Cr-ECTA. The amount of ^{51}Cr-EDTA associated with K^+ in a pellet of cells obtained at different times after a single exposure of the cell suspension to 2000 V cm^{-1} $\tau = 200$ µsec. The ordinate is expressed as a percentage of that in the supernatant (●). Cells not exposed to the electric field (○). The 20% ^{51}Cr-EDTA associated with the control cells reflects the amount of extracellular fluid associated with the pellet. Medium: solution based on glutamate (Soln. B, Methods) containing 1 mM EDTA and 2 mM Mg^{2+}. Temp. 20 °C. (*d*): A semi-log plot of the amount of ^{51}Cr-EDTA in the cell. (From Fig. 5*c*)

Table 4. Estimation of effective pore size created by exposure to an electric field of 2 kV/cm, τ 200 µsec

Data	Marker	Mol wt	Nos. of shocks	τ_f min-efflux	τ_f min-influx	D (cm^2 sec^{-1}) in free solution	Calculated effective pore radius (nm)
Fig. 5	^{86}Rb	86	1	5.2		$2{\cdot}7\times10^{-5}$	2
Fig. 5	3-O-Methyl D glucose	196	1	21.4		7×10^{-6}	2
Fig. 5	^{51}Cr-EDTA	423	1		89	$\sim4\times10^{-6}$	1.2
Fig. 7	^{45}Ca-EGTA	425	10		3	$\sim4\times10^{-6}$	2.1
Fig. 6	Noradrenaline	288	10		~ 2.9	$\sim5\times10^{-6}$	2.0
Fig. 14*c* and *f*	LDH	~130,000	10	543			1 -0.5
Fig. 15	LDH		10	935		$\sim1\rightarrow5\times10^{-7}$	0.8–0.3
Fig. 17	LDH		10	429			1.1–0.5
Fig. 22*b*[a]	LDH		1–20				1.9–0.8

[a] The effective pore size is calculated from the LDH data of Fig. 21*b*, in the following way: From Eq. (4) in the text the amount of LDH diffused into the extracellular fluid is given by $c = 1 - e^{-t/\tau_f}$ where $\tau_f = 4b^3 x/3Dmr^2$ (symbols as defined in text). When t is small compared with τ_f, the equation approximates to $c = t/\tau_f$, i.e., $c = \frac{3Dr^2 t}{2b^3 x}\left(\frac{m}{2}\right)$. This equation describes that the amount of LDH lost should be proportional to the number of discharges $\left(\frac{m}{2}\right)$ and have a slope of $\frac{3Dr^2 t}{2b^3 x}$. From Fig. 21*b* the slope is measured to be 0.0065 and by knowing the values of D, t, b and x the value of r is calculated.

Table 5. Temperature at which cells were exposed to electric fields and subsequently incubated (15 min)

	4 °C	22 °C	37 °C
$\frac{^{51}\text{Cr-EDTA space}}{K^+ \text{ space}}$ %	72±6(6)[a]	64.5±8(6)	72±7(5)

[a] Mean±SD (number of samples).

The different estimates are remarkably consistent and suggest that the effective pore radius probably lies in the neighborhood of 1 to 2 nm. The extent to which a marker can access the interior of the cell seems largely independent of the temperature at which the pores are formed (Table 5).

Stability of the 'pores' once found. Both at 37 and 0 °C adrenal medullary cells remained permeable to ^{51}Cr-EDTA for up to 60 min – the longest time examined – after exposure to 2 kV cm^{-1}, $\tau = 200$ μsec (*see* Fig. 19*a*).

Accessibility is not dependent on calcium. Measurement of ^{86}Rb efflux showed no obvious differences between cells subjected to high voltage discharges in media of different Ca content; but in view of our interest in catecholamine secretion and our desire to control the ionized Ca in the neighborhood of the exocytotic site by Ca-EGTA buffers we measured the uptake of [^{14}C]-noradrenaline and ^{45}Ca-EGTA into cells that had been exposed to 5 or 10 discharges in media containing approximately 10^{-9} and 10^{-5} M Ca, respectively. Figure 6 shows that the high voltage treatment renders cells permeable to noradrenaline and that there is no obvious difference in this permeability at the two Ca concentrations examined. Figure 7 shows an essentially similar picture for Ca-EGTA except that the Ca-EGTA space is greater in cells exposed to 10^{-5} M Ca than in cells exposed to 10^{-9} M Ca. This difference presumably reflects cytoplasmic binding of Ca at the higher Ca concentration.

Evidence for this comes from the following analysis and data. When a leaky cell is fully equilibrated with the extracellular fluid the number of moles of calcium in the cell will be $Ca_E(V_c - V_{ca} - V_o) + Ca_{org} V_{ca}$, where V_c is the volume of the cell, V_{ca} the total volume of the organelles that sequester calcium to a concentration of Ca_{org}, V_o is total volume of impermeant organelles and Ca_E is the concentration of total calcium in the extracellular space. If there is no sequestering or impermeant compartments in the cell, the number of moles of calcium in the cell will be $Ca_E \cdot V_c$. The calcium space is the calcium in the cell expressed as a fraction of the calcium in an equivalent volume of extracellular fluid (or the tritium space of the cell) is simply the ratio of these volumes,

i.e. $\frac{Ca_E(V_c - V_{ca} - V_o) + Ca_{org} V_{ca}}{Ca_E V_c}$.

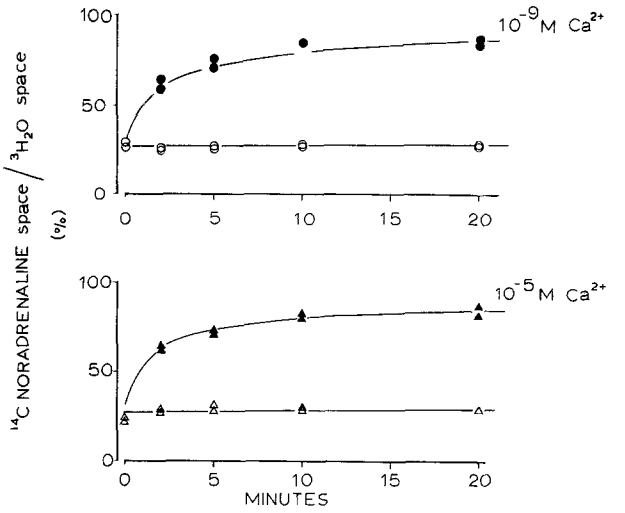


Fig. 6. The amount of [^{14}C]-DL-noradrenaline associated with ^3H_2O in a pellet of cells at different times after subjecting the cell suspension to 10 exposures of 2000 V cm^{-1} $\tau = 200$ μsec (●, ▲). Control cells (○, △) were not exposed to the electric field. The basic medium was solution B (Methods) containing trace amounts of [^{14}C]-DL-noradrenaline, and ^3H_2O, 1 mM cold L-noradrenaline and either 10 mM EGTA corresponding to 10^{-9} M Ca^{2+} (●, ○), or 10 mM Ca-EGTA corresponding to 10^{-5} M Ca^{2+} (▲, △)

The relative Ca space $= 1 - \left(\frac{V_{ca} + V_o}{V_c}\right) + \frac{Ca_{org} \cdot V_{ca}}{V_c} \cdot \frac{1}{Ca_E}$. Now the term $1 - \left(\frac{V_{ca} + V_o}{V_c}\right)$ reflects the volume fraction of the cell that is not impermeant nor sequesters calcium – call it the accessibility of the cell. The term $\frac{Ca_{org} V_{ca}}{V_c}$ is a calcium concentration in the cell due to the sequestering organelles.

Ca_E is the total calcium concentration in the extracellular fluid. At 10^{-5} M levels of free calcium, the Ca/EGTA ratio is 0.9. Therefore at this constant ratio [Ca_E] may be represented by 0.9 × [EGTA]. A plot, therefore, of the Ca space against the reciprocal of the EGTA concentration used should be linear with a slope reflecting the amount of calcium sequestered by the organelles and an intercept reflecting the accessibility. A linear regression on such data (Fig. 8*b*) has a correlation coefficient of 0.96 and a slope of 7.3±std 0.6. This figure corresponds to 6.5 mM (std 0.5) calcium and reflects a high capacity of the cell to bind calcium. The intercept, corresponding to the accessibility of the cell, is 67% of the cellular volume. This value is consistent with the data of Table 5 for the accessibility of ^{51}Cr-EDTA. At EGTA concentrations lower than 0.4 mM the relation between relative Ca space and (EGTA)$^{-1}$ became nonlinear, due presumably to the inadequate buffering capacity of the EGTA diffusing into the cell compared with the sequestering ability of the organelles.

At free calcium concentration of about 10^{-9} M the relative calcium space reflects the accessibility to the cell – rather than any sequestering component (Fig. 8*a* and 7*c*). The affinity of the organelles involved in sequestering calcium appears to be mainly in the micromolar range (Fig. 8*a*).

Effects of Ionized Ca on Catecholamine Release from 'Leaky' Cells

Catecholamine release in 10^{-9} and 10^{-5} M ionized Ca. Figure 7*a* and *b* reveals a marked difference in

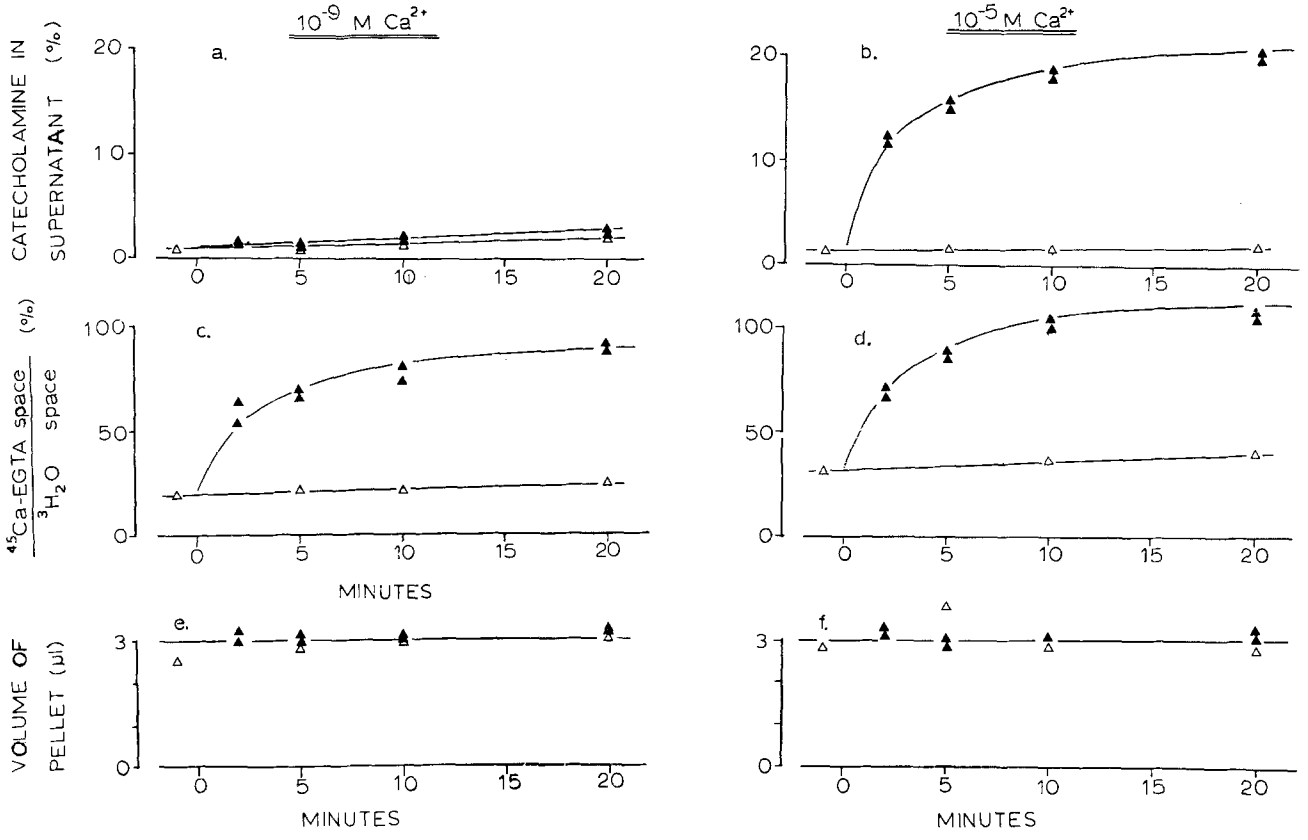


Fig. 7. Comparison of catecholamine release with accessibility to ^{45}Ca-EGTA at different absolute levels of ionized calcium. Cells in glutamate solution (solution B, Methods) containing trace amounts of ^{45}Ca and $^{3}H_2O$, and either 10 mM EGTA, giving calculated ionized Ca of 10^{-9} M (*a*, *c*, *e*), or 10 mM Ca-EGTA, giving calculated ionized Ca of 10^{-5} M (*b*, *d*, *f*). Temp., 37 °C. Closed symbols: cells subjected to 10 exposures of 2000 V cm^{-1}, $\tau = 200$ µsec; open symbols: control cells not exposed to the field. (*a*, *b*): Catecholamine in the supernatant measured at times after applying the electric fields. The ordinate is expressed as a percentage of the total in the suspension. (*c*, *d*): The ^{45}Ca-EGTA space expressed as a % of the $^{3}H_2O$ space in the pelleted cells. The 20% ^{45}Ca space of the $^{3}H_2O$ space in the control cells represents the extracellular fluid in the pellet and therefore the accessibility of the ^{45}Ca-EGTA to the cell interior is reflected by the increment above this value. (*e*, *f*): The volume of the pellets as measured by the $^{3}H_2O$ space at various times after exposure to the electric field

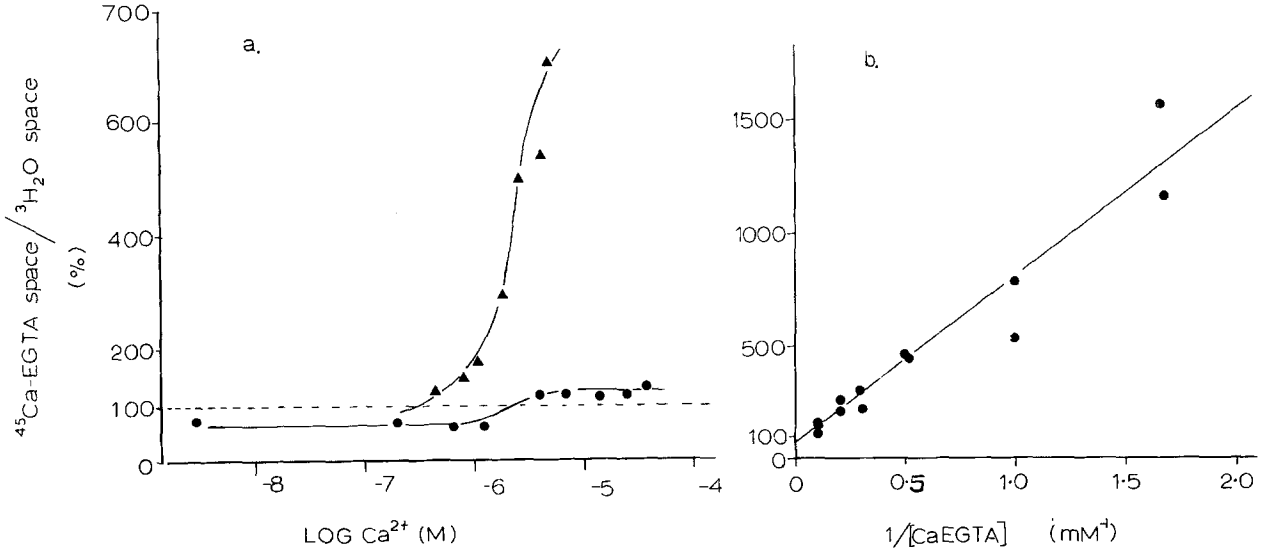


Fig. 8. Evidence for ^{45}Ca-binding. Ordinate: The ^{45}Ca space in the cell pellets expressed as the % of the $^{3}H_2O$ space. (*a*): Cells rendered leaky by 10 exposures of 2000 V cm^{-1}, $\tau = 200$ µsec in glutamate medium (solution B, Methods) containing 0.4 mM EGTA and trace amounts of $^{3}H_2O$. Aliquots were incubated for 10 min with either 0.4 or 10 mM Ca-EGTA buffer of various Ca/EGTA ratios containing trace amounts of ^{45}Ca to give a final buffer concentration of 0.8 mM (▲) and 10.4 mM (●). Temp. 37 °C. (*b*): Cells rendered leaky by 10 exposures 2000 V cm^{-1}, $\tau = 200$ µsec in medium B containing 0.2 mM EGTA and trace amounts of $^{3}H_2O$ followed by 10-min incubation with various additional concentrations of Ca-EGTA buffer (10 to 0.4 mM) containing trace amounts of ^{45}Ca. The Ca in the EGTA buffer were of amounts such that when added to the cell medium containing 0.2 mM EGTA, the calculated free calcium was always approximately 10^{-5} M (range 0.7×10^{-5} M to 1.3×10^{-5} M). Temp. 37 °C. The straight line is a least squares fit and corresponds to a slope of 6.5 mM Ca and an intercept (cell accessibility) of 67%

catecholamine release from cells subjected to high-voltage discharges in solutions containing 10^{-9} and 10^{-5} M ionized calcium. Less than 1% of the total cellular catecholamine is released from cells shocked in the presence of 10^{-9} M Ca, whereas 20% of the total catecholamine is released by the same procedure applied in the presence of 10^{-5} M calcium. As these results seem unlikely to have their origin in calcium-dependent differences in permeability to catecholamine or Ca-EGTA (*see* previous section), two conclusions seem inescapable: (i) that only a very small fraction of the total cellular catecholamine is normally present in the cytosol in a form that can diffuse out of a 'leaky' cell and (ii) up to 20% of the total cellular catecholamine can be rendered diffusable by exposure to 10^{-5} M Ca. Involvement of a voltage-sensitive Ca channel in the action of Ca seems unlikely for two reasons: (i) high voltage discharges are given in a high K medium in which any voltage-sensitive Ca channels should be fully inactivated (Baker & Rink, 1975) and (ii) the catecholamine release from shocked cells is independent of the presence of D 600 (Fig. 9) which blocks Ca channels in intact adrenal medullary cells (Baker & Knight, 1981).

Both in the presence of 10^{-9} and 10^{-5} M Ca, the catecholamine that remains bound within the cells can be released by exposure to distilled water, which suggests that the bulk of this bound catecholamine is located within osmotically-sensitive structures.

The data of Fig. 7*a* and *b* raises an interesting question. Does the 20% release of catecholamine reflect a complete loss of catecholamine from 20% of the cells? Or does it reflect a 20% loss from all of the cells? Figure 7*c* and *d* suggest that over 80% of the cells in the suspension (which contains 84% adrenal medullary cells) have been accessed, a conclusion that is supported by the data of Table 5. It seems most probable that the catecholamine release induced by 10^{-5} M Ca reflects a generalized (20%) loss of catecholamine from all the 'leaky' cells in the preparation. Electron microscopic analysis (*see* p. 125) supports the idea of a generalized loss rather

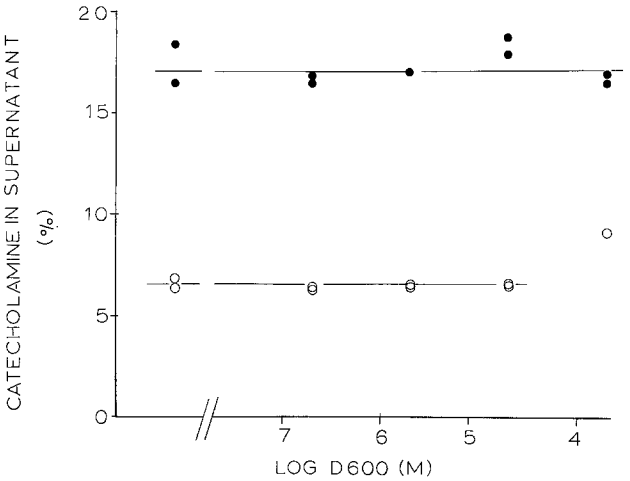


Fig. 9. Catecholamine release from 'leaky' cells is insensitive to D600. Catecholamine in the supernatant after rendering cells leaky by 10 exposures to 2000 V cm^{-1}, $\tau = 200$ µsec, in glutamate medium (solution B, Methods) containing 0.4 mM EGTA followed by incubation of aliquots with various concentrations of D600 for 2 min before challenging for a further 10 min with either 10 mM EGTA ~ 10^{-9} Ca (o) or 10 mM Ca-EGTA ~ 10^{-5} Ca (●)

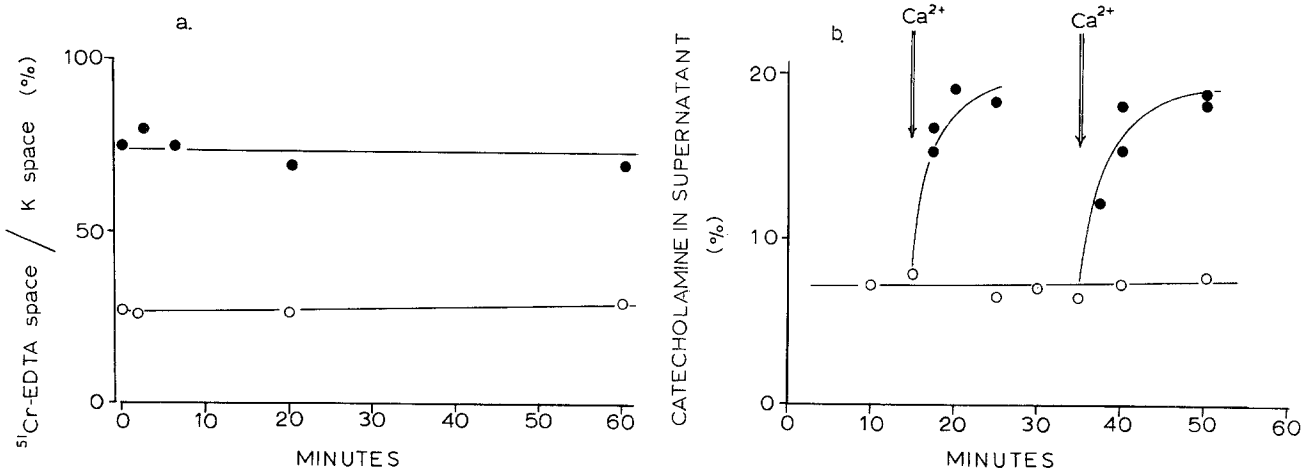


Fig. 10. Failure of the cells to reseal. (*a*): Penetration to the cell interior of ^{51}Cr-EDTA at different times after exposing the cells to brief intense electric fields (10 exposures of 2000 V cm^{-1}, $\tau = 200$ µsec in solution B (Methods) containing 1 mM EDTA). ^{51}Cr-EDTA was added at times up to 60 min after the cells were rendered leaky, and the amount of marker associated with the K^+ space in the cells determined 10 min later. Cells rendered leaky at 0 min (●). Cells not exposed to the electric field (o). Temp. 37 °C. (*b*): Response to a calcium challenge at different times after exposing cells at 0 min to brief intense electric fields. 10 exposures to 2000 V cm^{-1}, $\tau = 200$ µsec in solution B (Methods) containing 0.4 mM EGTA (-10^{-8} M Ca^{2+}). 10 mM Ca-EGTA corresponding to 10^{-5} M Ca^{2+} was added to aliquots of the cell suspension at the times indicated. Catecholamine in the supernatant from cells at 10^{-8} M Ca^{2+} (o) and in 10^{-5} M Ca^{2+} (●).

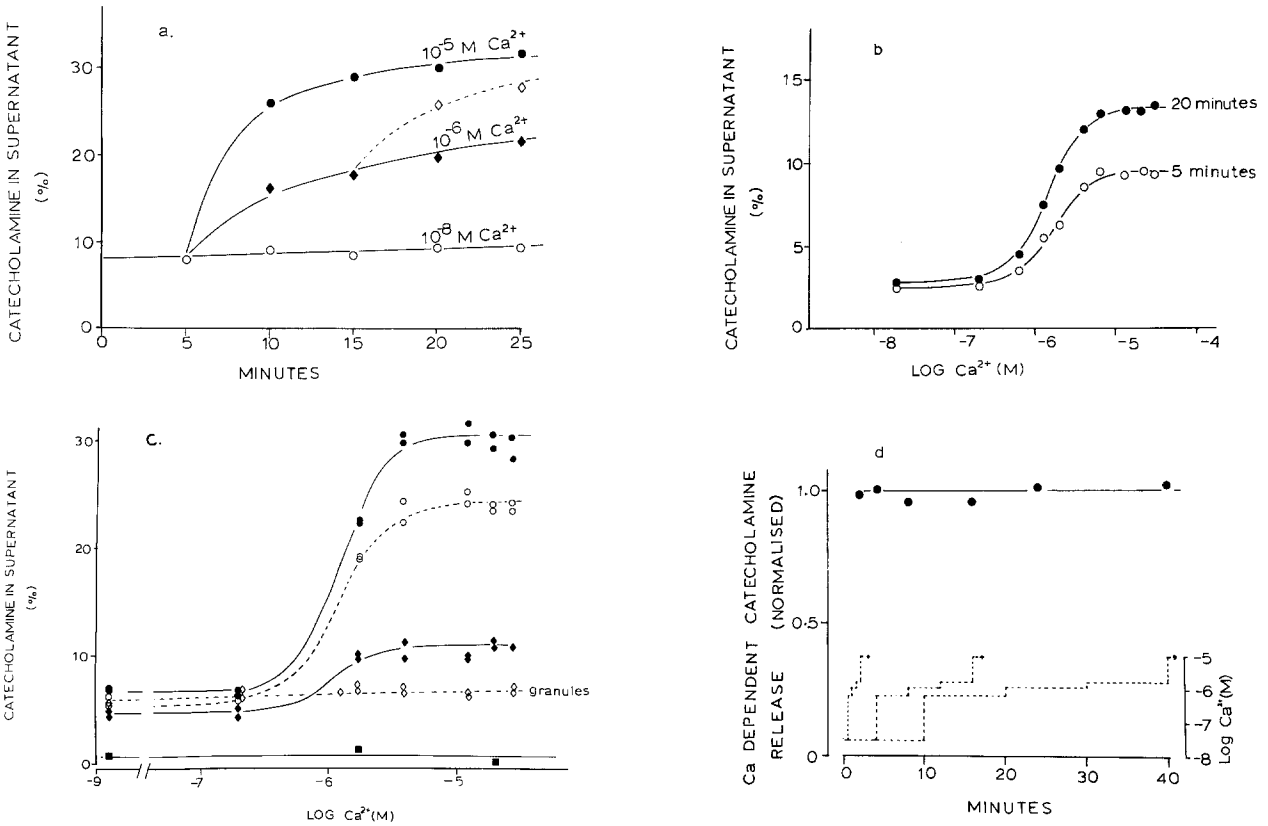


Fig. 11. Catecholamine release in response to a calcium challenge. (*a*): Catecholamine release as a function of time. Cells rendered leaky by 10 exposures to 2000 V cm^{-1}, $\tau = 200\,\mu$sec in solution B (Methods) containing 0.2 mM EGTA. After 5 min the cells were challenged by adding 0.4 mM Ca-EGTA buffer, giving a final calculated free Ca concentration of 10^{-8} M (○), 10^{-6} M (◆) and 10^{-5} M (●). After a further 10 min the calcium level of an aliquot of the 10^{-6} M cells was raised to 10^{-5} M by the addition of 10 mM Ca-EGTA (◇). Temp. 37 °C. (*b*): The calcium-activation curve determined 5 and 20 min after the calcium challenge. Cells were rendered leaky as in *a* above except that solution B contained 0.4 mM EGTA, and subsequently challenged with 10 mM Ca-EGTA buffers. Temp. 37 °C. (*c*): Calcium-activation curves determined after different numbers of exposures to brief intense electric fields of 2000 V cm^{-1}, $\tau = 200\,\mu$sec. Medium: solution B (Methods) containing 0.4 mM EGTA. Catecholamine in the supernatant 10 min after adding 10 mM Ca-EGTA buffer to cells previously subjected to 1 (◆), 5 (○) and 10 (●) exposures, and to chromaffin granules (◇) previously subjected to 10 exposures. Temp. 37 °C. The Figure also shows catecholamine released from intact cells as a result of raising the temperature suddenly from 23 to 37 °C (■) in the presence of different ionized calcium concentrations. (*d*): The effect of the rate of increase of Ca^{2+} on the amount of catecholamine released. Cells in solution B (Methods) containing 0.4 mM EGTA (calculated free Ca^{2+} 2×10^{-8} M) were subjected to 10 exposures of 2 kV cm^{-1}, $\tau = 200\,\mu$sec. The free Ca level was raised to 10^{-5} M through three intermediate steps, i.e., 6×10^{-7} M, 1×10^{-6} M and 2×10^{-6} M, each step being over the same interval of time. Some of the rates at which the Ca was increased are shown by the dotted lines. For the three examples shown the Ca was raised from 10^{-8} to 10^{-5} M in $1\frac{1}{2}$, 12 and 30 min i.e., the intermediate levels were held for $\frac{1}{2}$, 4 and 10 min respectively. The total amount of catecholamine released by the cells 15 min after the extracellular Ca concentration reached 10^{-5} M was measured. The amount released is expressed relative to the amount released by controls in which the Ca level was raised from 10^{-8} to 10^{-5} in one jump at the time indicated.

than total loss from a few cells. Cells rendered 'leaky' in solution B in the presence of 10^{-9} M Ca remain permeable for up to 1 hr (the longest period examined) and during this period they can be caused to release catecholamine simply by raising the ionized Ca concentration (Fig. 10). In general, the amount of catecholamine that can be released decreases the longer the time interval between rendering the cells leaky and challenging them with calcium. This rate of decrease is very variable but is not associated with a decreased permeability to Ca-EGTA. The form of the dependence of catecholamine release on ionized Ca is unaffected by prolonged exposure to 10^{-9} M Ca in solution B.

The relation between ionized Ca and catecholamine release. Figure 11*a* illustrates two of the problems inherent in obtaining the precise relation between ionized Ca and catecholamine release in 'leaky' cells. With high concentrations of Ca, the rate of release seems to be determined by the rate of entry of Ca-EGTA buffer into the 'leaky' cells and not by the kinetics of the release process. With lower Ca concentrations release increases slowly with time

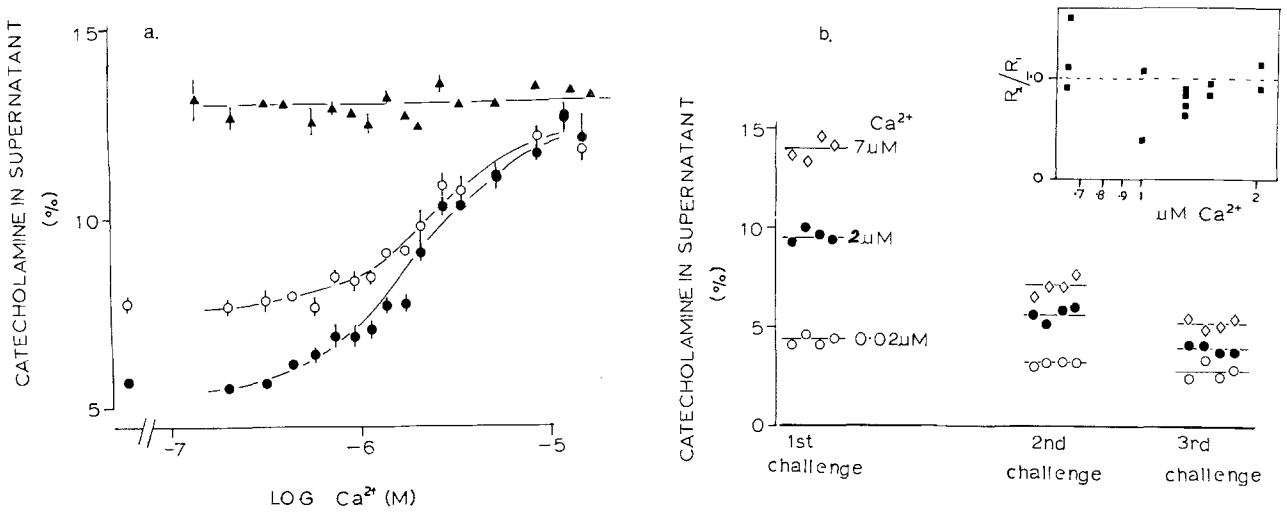


Fig. 12. (*a*): Ca dependence of catecholamine release from cells previously challenged with micromolar levels of calcium. Cells in solution B (Methods) containing 0.4 mM EGTA were rendered leaky by 10 exposures to 2 kV cm^{-1}, $\tau = 200$ μsec, and challenged with 10 mM Ca-EGTA buffers corresponding into 10^{-9}, 10^{-6}, and 10^{-5} M Ca^{2+}. After 13 min the free calcium concentration was lowered by diluting aliquots of cell suspension into 80 volumes of solution B containing 1 mM EGTA, giving calculated free Ca concentration of 10^{-8}, 6×10^{-8} and 10^{-7} M, respectively. Ca electrode measurements confirmed the calcium levels were less than 10^{-7} M. 15 min later the cells were challenged with 10 mM Ca-EGTA buffers and the catecholamine released over the next 15 min was measured. Cells prechallenged with 10^{-9} M Ca (●), 10^{-6} M Ca (○), 10^{-5} M Ca (▲). Each point is the mean of four determinations and the bars show the standard errors. Temp. 37 °C. (*b*): Cells in solution B (Methods) containing 0.4 mM EGTA and a calculated free Ca^{2+} of 2×10^{-8} M were rendered leaky by exposing them to 10 fields of 2 kV cm^{-1}, $\tau = 200$ μsec, challenged for the first time with 10 mM Ca-EGTA buffers corresponding to 2 and 7 μM free Ca, and the catecholamine released over 10 min was measured. The cells in buffer corresponding to 2 μM Ca^{2+} were then washed three times in 10 ml of solution B (0.4 mM EGTA), 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 (●) and 7 μM Ca (◇). Temp. 37 °C. The relative amount released by the first 2-μM Ca challenge is defined as

$$R_1 = \frac{(\text{amount in supernatant at } 2\,\mu\text{M } Ca^{2+} - \text{amount in supernatant at } 0.02\,\mu\text{M Ca})}{(\text{amount in supernatant at } 7\,\mu\text{M } Ca^{2+} - \text{amount in supernatant at } 0.02\,\mu\text{M Ca})}.$$

The relative amount released by the second challenge (R_2) is defined similarly. The ratio of these amounts is R_2/R_1 and represents the response of cells to a particular calcium challenge compared with their response to a similar previous challenge. This ratio is shown in the inset of the Figure over a range of calcium challenges from 0.64 to 2 μM

and appears to level off at an intermediate value, an effect that is often much more marked than that shown in Fig. 11*a*. This is surprising as release might have been expected to reach the 10^{-5} M Ca level but at a slower rate. Failure to achieve this could reflect desensitization to Ca. This seems unlikely because after prolonged exposure to 10^{-6} M Ca the rate of release can be accelerated in an apparently normal fashion by raising the ionized Ca to 10^{-5} M (Fig. 11*a*), and the total amount of catecholamine released is independent of the time period over which the calcium concentration is increased (Fig. 11*d*). Despite these problems, the shape of the curve relating catecholamine release to ionized Ca is very similar when determined 5 and 20 min after transfer from 10^{-8} M Ca to the test Ca concentrations (Fig. 11*b*). The shape of the Ca-activation curve is also essentially the same in cells subjected to 1, 5 or 10 discharges (Fig. 11*c*).

Figure 11*c* also shows two other important points: (i) the same shocking regime that renders cells sensitive to micromolar concentrations of calcium has no effect on isolated chromaffin granules and (ii) the temperature jump that is always associated with exposure to brief intense electric fields (*see* p. 110) does not by itself confer sensitivity to micromolar levels of calcium.

One possible explanation of the apparent intermediate extents of catecholamine release might be that granules differ in their sensitivity to calcium. As the release of the contents of individual granules is likely to be an all or none process, a 1-μM Ca^{2+} challenge might only bring about release from those granules whose calcium thresholds are sub-micromolar. It follows, therefore, that if there is no alteration in the calcium thresholds of the remaining granules, a second challenge with 1 μM Ca^{2+} should be subthreshold and elicit no catecholamine release. Figure 12*a* shows that this is not so. Although the data is scattered it does suggest that the calcium sensitivity of catecholamine release from cells prechallenged with μM levels of calcium appears to be the same as the control cells which had not been prechallenged. A more conclusive experiment is shown in Fig. 12*b*.

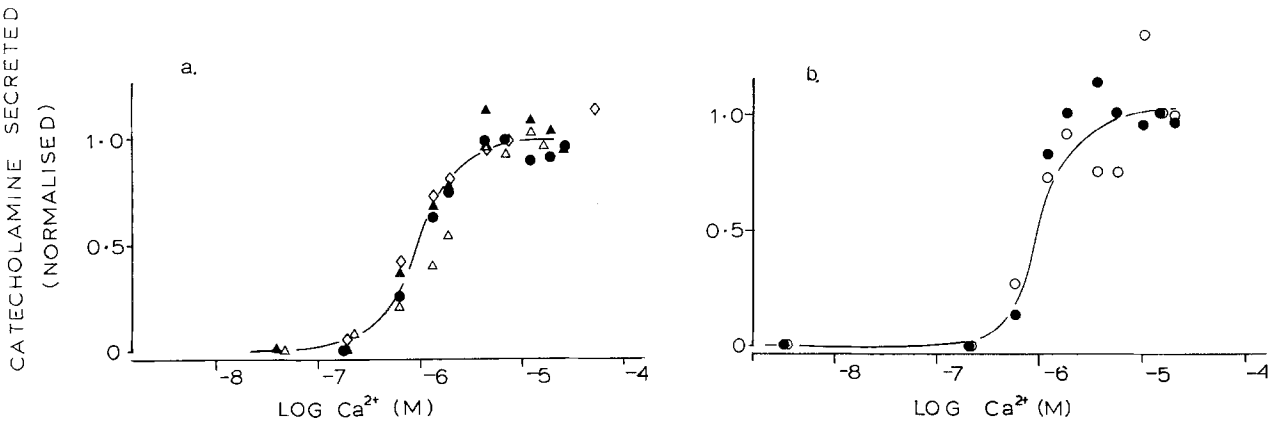


Fig. 13. The Ca-activation curve is independent of EGTA buffer concentrations and sequence of adding reactants. The ordinate is the amount of secreted catecholamine normalized relative to the at 10^{-5} M Ca^{2+}. (*a*): Buffer concentration. Catecholamine secreted from leaky cells 10 min after being challenged with calcium. Cells were rendered leaky by 10 exposures to 2000 V cm^{-1}, $\tau = 200$ μsec, in glutamate medium (solution B, Methods) containing 0.4 mM Ca-EGTA buffer corresponding to a range of free calcium (△). Cells were rendered leaky in a similar fashion but in the presence of 0.4 mM EGTA and subsequently challenged with 10 mM (▲, ●) and 50 mM (◊) Ca-EGTA buffer. The 10 mM Ca-EGTA buffer was made up with either calcium acetate (▲) or calcium sulphate (●) unlike the other buffers which were made up with calcium chloride. The data (▲, ●) therefore reflect secretion in a medium in which there is nominally no chloride. Temp. 37 °C. (*b*): Catecholamine secretion initiated by a temperature jump or by ATP addition. Cells were rendered leaky at 22 °C by 10 exposures to 2000 V cm^{-1}, $\tau = 200$ μsec, in a medium (soln. B, Methods) containing 0.4 mM EGTA. The cells were cooled to 4 °C over 5 min and 10 mM Ca-EGTA buffers were added to aliquots of the suspension. 15 min later half of the buffered solutions were warmed to 37 °C, and the catecholamine in the supernatants was measured after a further 10 min. The catecholamine released on raising the temperature is plotted (○). Cells were rendered leaky in a similar fashion in solution B (Methods), containing 0.4 mM EGTA but in the absence of Mg-ATP. 10 min later 10 mM Ca-EGTA buffers were added to aliquots of the suspension, and a further 13 min later 5 mM Mg-ATP was added back to half of them. 10 min later the catecholamine in the supernatant of both the ATP and non-ATP-containing aliquots was measured, and the differences corresponding to a particular Ca buffer were plotted (●). Temp. 37 °C.

Cells which have been previously challenged with 2 μM calcium are washed repeatedly in EGTA and then rechallenged with 2 μM Ca. The response due to the second 2 μM calcium challenge expressed relative to the response to a higher Ca concentration is the same as before. The cells challenged with 2 μM calcium can be rewashed with EGTA and challenged again for a third time. The inset to Fig. 12*b* shows that over the calcium range 0.64 to 2 μM the sensitivity to calcium is not dependent on the history of calcium challenges and thus does not support the idea that within a population of granules, individual granules may have different and fixed thresholds for calcium.

Another interesting feature shown in both Fig. 12*a* and *b* is the appearent limit to the amount of catecholamine that is available for release. It suggests that there is a limited number of granules primed ready for release and that under our experimental conditions no more are made available even though the existing pool can be partially or wholly depleted by previous calcium challenges.

The data presented so far show that after exposure of isolated cells to brief intense electric fields, Ca-EGTA buffers have ready access to the cell interior and that catecholamine release can be activated half-maximally by buffers that stabilize an ionized Ca close to micromolar. One cannot conclude directly from this, however, that the release mechanism has an affinity for Ca in the micromolar range as a much lower affinity might be satisfied if micromolar concentrations of Ca were able to liberate much higher concentrations from intracellular stores located close to the sites of release. There are a number of very strong arguments against this possibility.

1) Essentially the same Ca-activation curve is obtained over two orders of magnitude of Ca-EGTA buffer concentration from 0.4 to 50 mM (Fig. 13*a*).

2) The slope of the Ca-activation curve is the same after 1 and 10 discharges (Fig. 11*c*) where the degree of leakiness of the cells is markedly different, and one would expect much less effective buffering in cells subjected to only one discharge.

3) Ca-dependent release of catecholamine is inhibited at 4 °C (Fig. 19*f*) so it is possible to allow 'leaky' cells to equilibrate fully with any particular buffer and then to initiate Ca-dependent release by raising the temperature to 37 °C. This procedure reduces the uncertainty about the buffering capacity in the neighborhood of the release sites. Figure 13*b* shows that in cells fully equilibrated with 10 mM EGTA buffers at 4 °C and then raised to 37 °C the calcium-activation curve is half maximal at a calcium concentration close to 1 μM.

4) Ca-dependent release of catecholamine also has an absolute requirement for Mg-ATP (*see* p. 126). In

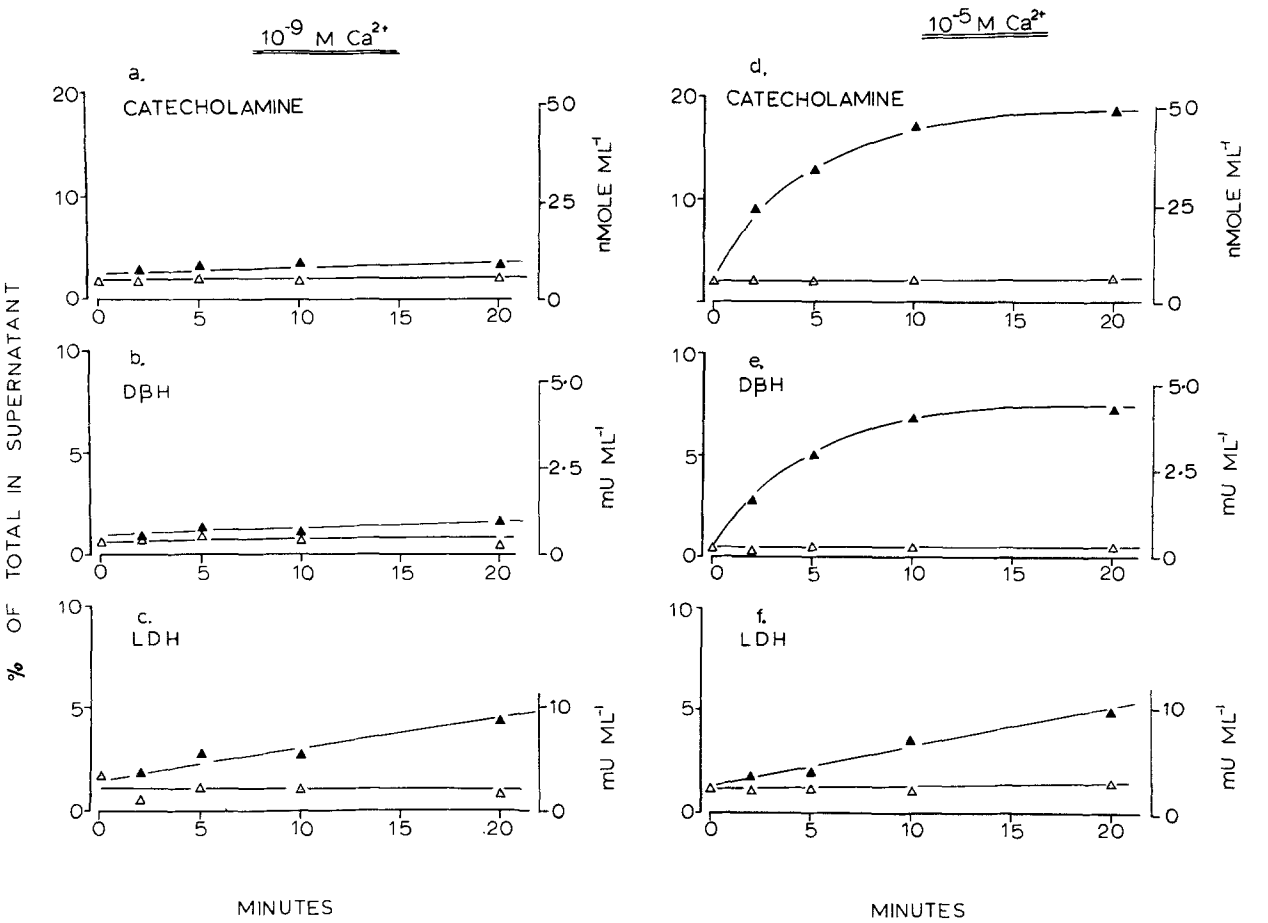


Fig. 14. The release of catecholamine, dopamine-β-hydroxylase (DβH), and lactate dehydrogenase (LDH) from a population of cells rendered leaky by 10 exposures of 2000 V cm^{-1}, $\tau = 200\,\mu sec$, in which the solution bathing the cells (medium C, Methods) contained either 0.4 mM EGTA buffer, corresponding to 10^{-8} M Ca^{2+} (*a, b, c*), or 0.4 mM Ca-EGTA, corresponding to 10^{-5} M Ca^{2+} (*d, e, f*). Cells exposed to the electric field, ▲. Control cells, i.e., those not exposed to the electric field, △. Temp. 37 °C. Ordinates: Amount of catecholamine, DβH, and LDH in the supernatant expressed in units of concentration and also as a precentage of the total cellular content

the nominal absence of Mg-ATP leaky cells can be equilibrated with different Ca-EGTA buffers without evoking any catecholamine release. Release is only initiated when Mg-ATP is re-introduced. The Ca-activation curve determined in this way is also half maximal at a calcium concentration close to 1 μM (Fig. 13*b*).

These four experiments provide strong support for the view that the catecholamine release mechanism is half-maximally activated at a calcium concentration close to 1 μM.

The Ca-EGTA buffers were normally made up using $CaCl_2$. As essentially similar results were obtained when calcium acetate or calcium sulphate was used in place of $CaCl_2$ (Fig. 13*a*), the possibility that small changes in chloride concentration may affect the Ca-activation curve seems unlikely. This is an important point because we show later (p. 127) that higher concentrations of chloride can exert an appreciable effect.

The calcium curves presented so far have shown that the extent of catecholamine release saturates at about 10^{-5} M Ca^{2+}. One would expect that at higher concentrations the extent should remain the same. However, this is not so, and at millimolar levels of calcium less catecholamine is released (Fig. 21*d*). We have not investigated this phenomenon in detail but it is not affected by the presence of a protease inhibitor Na-P tosyl-L-lysine chlormethyl ketone (TLCK, p. 26).

Evidence that Calcium-Dependent Release of Catecholamine Occurs by Exocytosis

Parallel measurements of dopamine-β-hydroxylase and lactate dehydrogenase. We must now consider whether the catecholamine release that has been described so far occurs by exocytosis. Fortunately, in the adrenal medulla there are excellent biochemical criteria for exocytosis because catecholamine is stored in the chromaffin granules along with ATP, special storage proteins, the chromogranins, and the enzyme dopamine-β-hydroxylase (DβH). The selec-

tive release of catecholamine along with DβH and/or chromogranin under conditions where typical cytosolic proteins, such as lactate dehydrogenase, are not released is usually taken as strong evidence for release of catecholamine by exocytosis.

Figure 14*a*, *b* and *c* shows that when cells are rendered leaky in the presence of enough EGTA to buffer the ionized Ca concentration close to 10^{-9} M, less than 1% of the total catecholamine and DβH are released. Lactate dehydrogenase is, however, lost at a steady rate equivalent to 4% of the cellular lactate dehydrogenase every 20 min, consistent with an effective pore radius of about 1 nm. Cells rendered permeable in the presence of 10^{-5} M calcium continue to lose lactate dehydrogenase at about the same rate, but the rates of release of both catecholamine and DβH are markedly increased. Catecholamine and DβH are released in parallel and in a proportion of 0.1 mU DβH/nmol catecholamine. When the release data is expressed as a percentage of the total in the suspension, roughly 7.5% of the

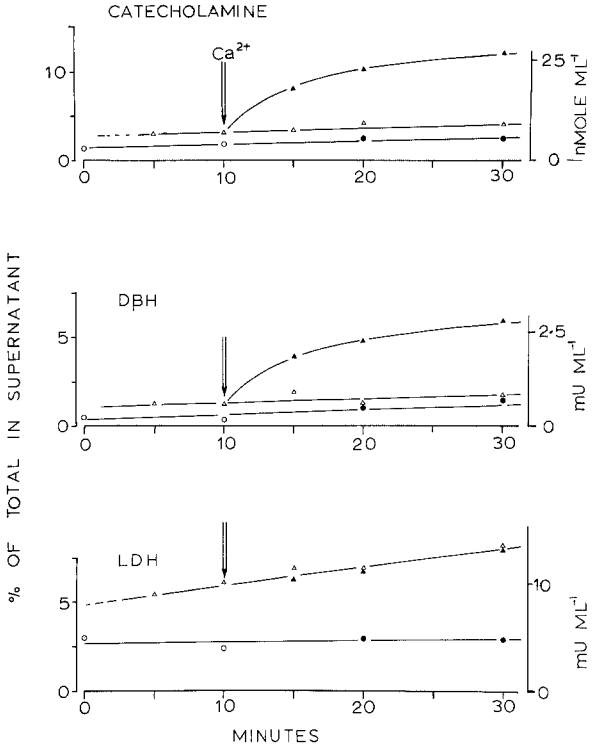


Fig. 15. The time course of release of catecholamine, DβH, and LDH in response to a Ca challenge. Cells in medium C (Methods) containing 0.4 mM EGTA were subjected to 10 exposures of 2000 V cm^{-1}, $\tau = 200$ μsec (△). 10 min after rendering them leaky, 0.4 mM $Ca(OH)_2$ was added to the cell suspension (▲). Cells from the same population not rendered leaky (○) also had $Ca(OH)_2$ added at the same time (●). Temp. 37 °C. Ordinates: Amounts of catecholamine, DβH, and LDH in the supernatant expressed in units of concentration and also as a percentage of their respective total cellular contents

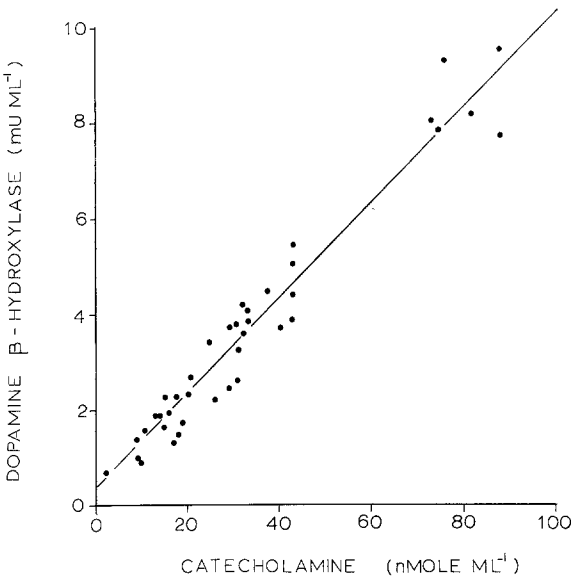


Fig. 16. The relative amounts of catecholamine and DβH released at different times as a result of either rendering the cells leaky in the presence of various Ca-EGTA buffers, or by adding Ca back to leaky cells held in EGTA solution. The straight line is a least squares fit and corresponds to a slope of 0.100 mU DβH/nmol catecholamine. SD 0.004 ($n = 41$)

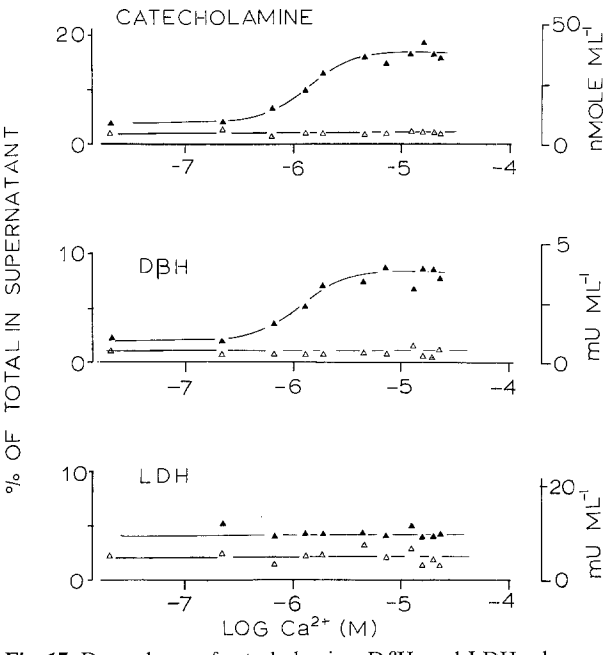


Fig. 17. Dependence of catecholamine, DβH, and LDH release on ionized calcium. Cells in medium C (Methods) containing 0.4 mM Ca-EGTA, corresponding to a range of free Ca concentrations, were subjected to 10 exposures of 2000 V cm^{-1}, $\tau = 200$ μsec, and the amount of catecholamine, DβH, and LDH in the supernatant was measured 10 min later (▲). Cells not exposed to the electric field, △. Temp. 37 °C. *Ordinate:* the amounts of catecholamine, DβH, and LDH in the supernatant expressed in units of concentration and also as a percentage of their respective total cellular contents

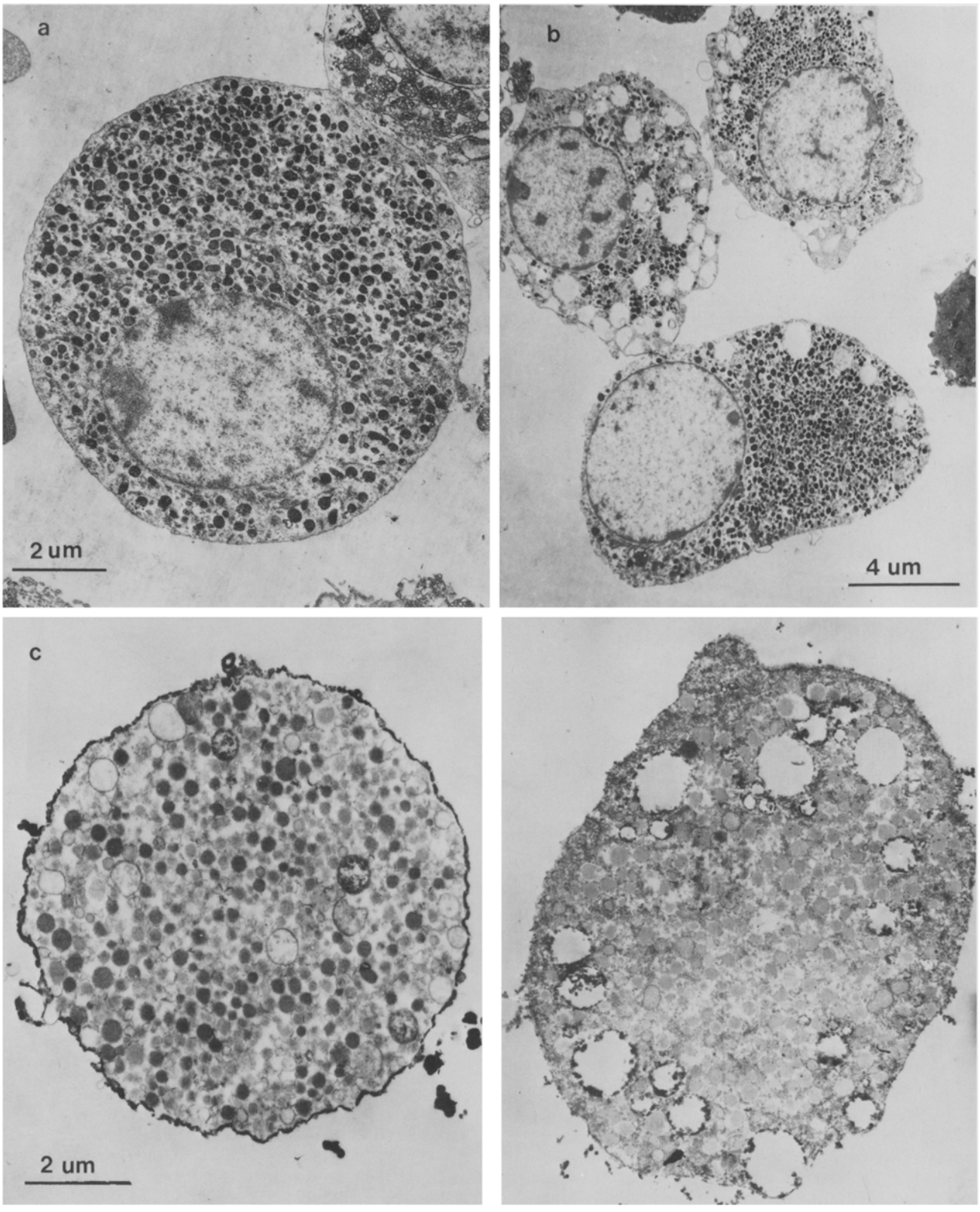
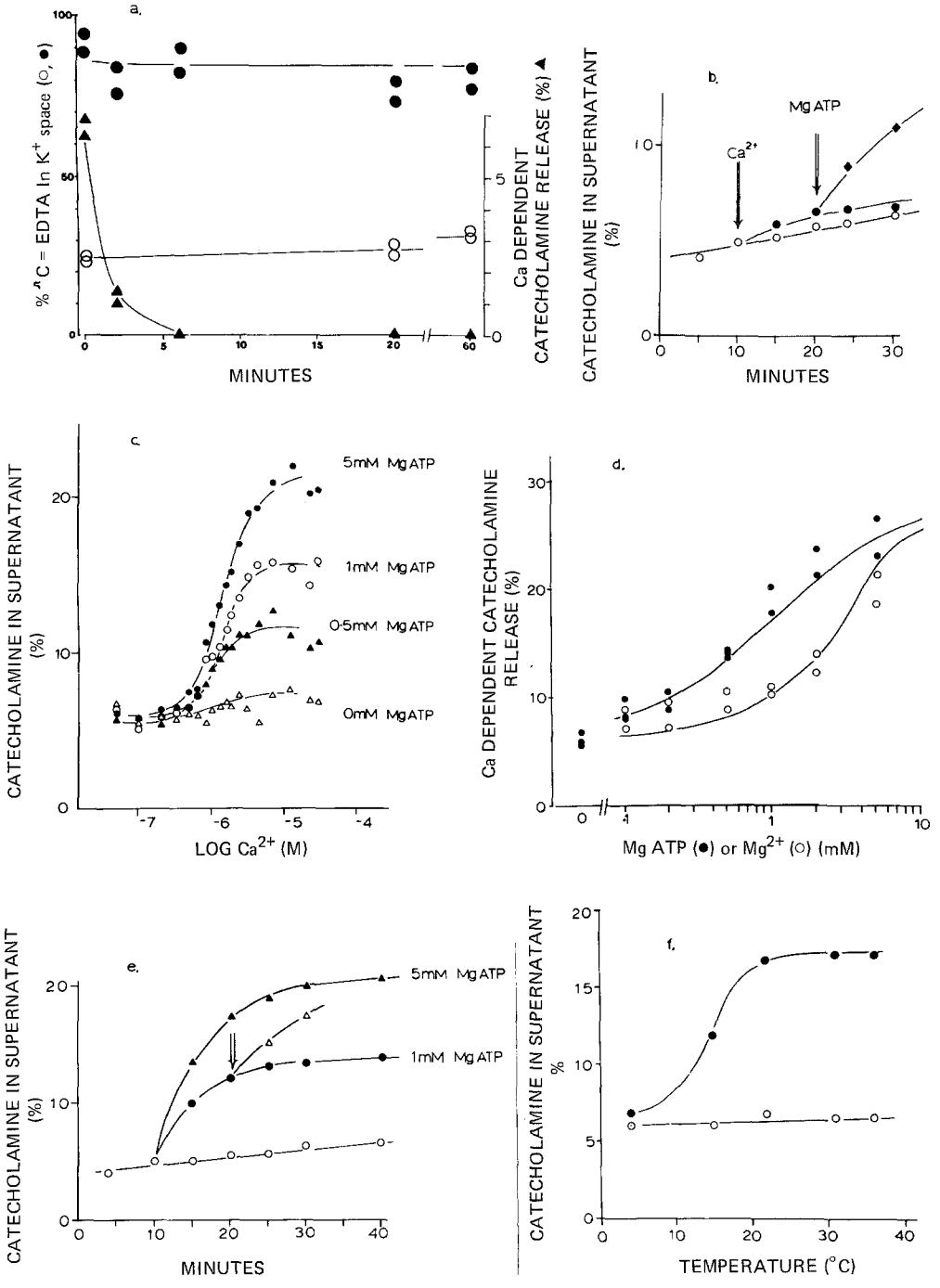


Fig. 18. Transmission electron micrographs of adrenal medullary cells subjected to intense electric fields of brief duration. Cells were subjected to 10 exposures of 2 kV cm^{-1}, $\tau = 200\,\mu sec$. (*a*, *b*): Taken from the same population of chromaffin cells exposed to intense electric fields in solution C containing 0.4 mM EGTA and subsequently challenged with either 10 mM EGTA, ionized calcium in the region of 10^{-8} M (*a*) or in 10 mM EGTA, ionized calcium in the region of 10^{-5} M (*b*) and fixed 20 min later. The 10^{-5} M Ca^{2+} evoked a release of 25% of the catecholamine in the bulk of the cells. Note the appearance of apparently empty vacuoles within the cytoplasm (*b*). (*c*, *d*): Cells treated as in *a* and *b* except that the cells were challenged in solution C containing horseradish peroxidase. (*c*): Ca^{2+} $\sim 10^{-8}$ M; (*d*): Ca^{2+} $\sim 10^{-5}$ M. The sections are unstained



total DβH is released in association with 15% of the total catecholamine. Precisely the same result is found in intact cells where there is strong evidence that only half the total cellular DβH is releasable, the other half being bound (*see* Viveros, Arqueros & Kirshner, 1968, and Baker & Knight, 1981).

Figure 14 illustrates two other important points:

1) When release is expressed as a percentage of the total in the suspension, in the presence of 10^{-5} M calcium, the percentage release of DβH exceeds that of lactate dehydrogenase providing further evidence that these two molecules do not come from the same compartment - for instance, from a few damaged cells.

2) The time course of appearance in the external medium of both catecholamine and DβH is identical. This is only consistent with release by exocytosis because had Ca caused granules to lyse within the cell, the relative rates of appearance in the external medium of DβH and catecholamine should be de-

Fig. 19 (facing page). Dependence of catecholamine release on ATP. (*a*): Loss of Ca-sensitivity in the absence of Mg-ATP. Cells in solution B lacking Mg-ATP (and containing 0.5 mM EDTA in place of EGTA to give a calculated ionized Ca of $\sim 10^{-7}$ M Ca^{2+}) were exposed ten times to 2 kV cm^{-1}, $\tau = 200$ μsec, and challenged with 1 mM Ca^{2+} containing trace amounts of ^{51}Cr-EDTA. Ten minutes later the catecholamine secreted and the ^{51}Cr-EDTA space were determined. Temp. 37 °C. ^{51}Cr-EDTA space: shocked cells (●), unshocked cells (○): catecholamine released (▲). (*b*): Restoration of Ca-sensitivity by Mg-ATP. Cells in solution B lacking Mg-ATP were rendered leaky by 10 discharges of 2 kV cm^{-1}, $\tau = 200$ μsec (○). 10 min later 10 mM Ca-EGTA (corresponding to 10^{-5} M Ca^{2+}) was added (●), and after a further 10 min 5 mM Mg-ATP (◆). Temp. 37 °C. (*c*): Ca-activation curves at different Mg-ATP concentrations. Cells in solution B lacking Mg-ATP were rendered leaky by 10 discharges of 2 kV cm^{-1}, $\tau = 200$ μsec, 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. Temp. 37 °C. (*d*): Requirement for Mg-ATP as opposed to free ATP. Cells in solution B containing various concentrations of Mg-ATP were subjected to 10 discharges of 2 kV cm^{-1}, $\tau = 200$ μsec. After 10 min they were challenged with 10^{-5} M Ca^{2+} (10 mM Ca-EGTA), and the catecholamine released into the supernatant was determined 10 min later (●). ATP^{2-} cannot substitute the role of Mg-ATP. Cells in solution B containing 5 mM ATP and concentrations of magnesium indicated were rendered leaky, challenged at 37 °C, and the secreted catecholamine determined as before (○). In this experiment the total ATP concentration was kept constant (5 mM), but the relative amounts of ATP^{2-} and Mg-ATP altered by varying the magnesium level: e.g., with 1 mM Mg, the solution contained 0.6 mM Mg^{2+}, 4.06 mM ATP^{2-} and 0.94 mM Mg-ATP, and with 5 mM Mg the solution contained approximately 1 mM Mg^{2+}, 1 mM ATP^{2-} and 4 mM Mg-ATP. The Mg-ATP dependent lines are based on the model that in the presence of Ca^{2+}, Mg-ATP stimulates secretion ($K_{Mg\text{-}ATP} = 1$ mM) and is inhibited by ATP^{2-} competing for the same site ($K_{ATP^{2-}} = 1$ mM). Calculations suggest that the various Mg and ATP concentrations used in this experiment should not alter the levels of ionized Ca^{2+} significantly. In the nominally calcium-free state, the calculated extreme values are 1.3 and 1.4×10^{-8} M, and the Ca^{2+} due to the Ca-EGTA challenge varies from 1.1 to 1.5×10^{-5} M. (*e*): Catecholamine release in response to increasing the Mg-ATP levels. Cells in Mg-ATP free solution B were rendered leaky by 10 exposures of 2 kV cm^{-1}, $\tau = 200$ μsec, diluted into similar solutions containing 5 or 1 mM Mg-ATP and after 10 min were challenged with 10^{-5} M Ca^{2+} (5 mM Mg-ATP, ▲; 1 mM Mg-ATP, ●). After a further 10 min the 1 mM Mg-ATP level of the Ca-stimulated cells was raised to 5 mM (△). Cells in 1 mM Mg-ATP not challenged with Ca^{2+} (○). (*f*): The effect of temperature on the response to a calcium challenge. Cells were rendered leaky at 22 °C by subjecting them to 10 exposures of 2000 V cm^{-1}, $\tau = 200$ μsec, in solution B (Methods) containing 0.4 mM EGTA and then allowed to equilibrate at the different temperatures indicated for 5 min before adding 10 mM Ca-EGTA. The catecholamine in the supernatant was measured 20 min later: cells challenged with 10^{-8} M Ca^{2+}, ○; 10^{-5} M Ca^{2+}, ●. The effect of temperature on the calculated free calcium levels was insignificant, as over the range from 5 to 37 °C the free calcium concentration decreases by less than a factor of 2

termined by their respective molecular masses. As the molecular mass of DβH is 290,000 daltons and that of adrenaline 330 daltons, if diffusion is proportional to the square root of the molecular mass, the rate of appearance of catecholamine should be 30 times faster than that of DβH. In practice, a greater difference seems likely because DβH is a larger molecule than lactate dehydrogenase and its rate of escape from the cell may be limited by the size of pore.

Another way of examining the question is shown in Fig. 15. Cells were rendered 'leaky' in the presence of EGTA and subsequently challenged with calcium. Calcium had no effect on the rate of release of lactate dehydrogenase but increased that of DβH and catecholamine with an identical time course. Figure 16 summarizes data on the relative amounts of DβH and catecholamine released in a number of experiments of this type. The correlation coefficient between the amount of DβH and catecholamine secreted is 0.97, and a regression line has a slope of 0.100 U DβH/μmol catecholamine (SD 0.004; $n = 41$). This figure compares well with the relative amounts secreted by intact cells (0.1 ± 0.01, $n = 23$) and the perfused gland (0.09 ± 0.01, $n = 15$).

Figure 17 shows that this proportionality persists over a wide range of calcium concentrations. The release of both catecholamine and DβH from 'leaky' cells is half maximal at a calcium concentration close to 1 μM.

Ultrastructural studies. If exocytosis is taking place it should be possible to find some ultrastructural correlate. Figure 18*a* and *b* show a marked difference in ultrastructure between 'leaky' cells in EGTA ($Ca^{2+} \sim 10^{-8}$ M) and 'leaky' cells from the same population which have subsequently been exposed to 10 μM Ca. In low Ca the ultrastructure of 'leaky' cells closely resembles that of intact resting cells, whereas 2–20 min after exposure of 'leaky' cells to 10 μM Ca^{2+}, large membrane bounded vacuoles are prominent in the cytoplasm. Similar structures are visible in intact cells after exposure to carbamylcholine or veratridine (Baker & Knight, 1981). Increased vacuolation is seen in nearly all the 'leaky' cells rather than in only a few percent. The extent of vacuolation appears to be related to the amount of catecholamine secreted.

These observations suggest that the 'leaky cells' may be capable of responding to a calcium challenge with both exocytosis and endocytosis. Although exocytotic profiles are rarely seen, this interpretation finds some support in the observation that horseradish peroxidase can be found within the vacuoles provided it is present in the medium before exposure of the 'leaky' cells to calcium (Fig. 18*d*).

Other Requirements for Exocytosis

General. The experiments described so far all relate mainly to the use of solution B (p. 109). In this

Table 6. The catecholamine and DβH contents of granules isolated from cells able to secrete in response to a calcium challenge (control) or unable to secrete either as a result of the absence of Mg-ATP or high concentration of chloride[a]

	Catecholamine secreted (%) by cells due to 10^{-5} M Ca^{2+}	Isolated granules		
		Catecholamine in the granule pellet (μmol)	DβH in the granule pellet (units)	DβH/Catecholamine (units/μmol)
Cells in Solution B				
5 mM Mg ATP (control)	13	0.17	0.039	0.23
0 mM Mg ATP	4	0.15	0.033	0.22
Cells in glutamate, Solution B (control)	10	0.15	0.03	0.2
Solution B with 140 mM glutamate replaced by 140 mM Cl^-	1	0.19	0.034	0.18

[a] In each case granules were isolated from $\sim 4 \times 10^7$ cells.

section we outline some of the experimental evidence on which the choice of this solution is based.

The role of Mg-ATP. Removal of Mg-ATP from solutions B or C rapidly produces a preparation that is refractory to calcium (Fig. 19*a*). This does not reflect any alteration in the accessibility of the cell interior to calcium because the ^{45}Ca-EGTA or ^{51}Cr-EDTA spaces remain unaltered (Fig. 19*a*). Furthermore, the apparent nonresponsiveness does not reflect a loss of catecholamine from the storage granules for the following reasons:

(i) a depletion of catecholamine from the granules due to unloading into the cytosol should, if the catecholamine was diffusible, be detected as a rapid rise in the basal (calcium independent) release from the leaky cells. This is not observed (Fig. 19*b*).

(ii) granules isolated from refractory cells (Table 6) still contain their normal complement of catecholamine and DβH.

(iii) secretion of DβH is inhibited in parallel with that of catecholamine.

These points provide strong support for the view that Mg-ATP is required for Ca-dependent exocytosis.

Sensitivity to calcium is restored following readmission of Mg-ATP. It follows that cells equilibrated with high levels of calcium but in the absence of Mg-ATP can subsequently be triggered to secrete by addition of Mg-ATP (Fig. 19*b* and 13*b*). The nucleotide affects the extent of calcium-dependent exocytosis and not the apparent affinity for calcium (Fig. 19*c*). The actual substrate seems to be Mg-ATP rather than ATP (Fig. 19*d*) because ATP is unable to support calcium-dependent exocytosis in the complete absence of magnesium. Indeed the data shown in Fig. 19*d* suggests that ATP apparently inhibits activation by Mg-ATP. The limited extent of secretion seen in the presence of sub-maximal levels of Mg-ATP is similar to the extent effect seen in the presence of sub-maximal doses of calcium (Fig. 11*a*). It does not appear to be due to densensitization to Mg-ATP as the rate of release is accelerated by raising the Mg-ATP level (Fig. 19*e*).

Of a variety of nucleotides tested, Mg-ATP is the most effective in supporting calcium-dependent exocytosis (Table 7*a*). Some analogues, of ATP, e.g.,

Table 7. Nucleotide-dependence of Ca-dependent catecholamine release[a]

	a	*b*
Adenosine 5′ triphosphate	1.0	1.0
0 mM Adenosine 5′ triphosphate	0.07 ±0.06 (6)	0.09±0.09 (6)
Adenylylimidodiphosphate (AMP PNP)	0.10 ±0.03 (6)	0.97±0.05 (4)
S Adenosyl L methionine	0.005±0.003 (4)	0.79±0.06 (4)
Guanosine 5′ triphosphate	0.18 ±0.03 (6)	1.5 ±0.1 (4)
Inosine 5′ triphosphate	0.22 ±0.08 (6)	1.3 ±0.1 (4)
Uridine 5′ triphosphate	0.19 ±0.07 (6)	1.8 ±0.1 (4)
Cytidine 5′ triphosphate	0.19 ±0.05 (6)	1.6 ±0.1 (4)
Adenosine 5′ diphosphate	0.5 ±0.04 (6)	1.27±0.07 (4)
Adenosine 5′ diphosphate +1 mM diadenosine pentaphosphate	0.1 ±0.06 (3)	-
Adenosine 5′ monophosphate	0.03 ±0.01 (3)	0.81±0.08 (4)
Adenosine 3′:5′ cyclic monophosphate	0.05 ±0.06 (6)	0.82±0.08 (4)
Guanosine 3′:5′ cyclic monophosphate	0.03 ±0.04 (6)	0.84±0.07 (4)

[a] Cells were rendered leaky in glutamate solution at 10^{-8} M Ca (0 Mg-ATP) and subsequently diluted to give a final concentration of (a) 4 mM of the Mg nucleotide indicated or (b) 0.8 mM Mg-ATP and 0.6 mM of the Mg nucleotide indicated. After 10 min the cells were challenged with 10 mM Ca-EGTA buffer corresponding to 10^{-5} M Ca^{2+}. There was no significant difference in the calcium-independent basal release over the range of the nucleotides. Results are expressed relative to the response seen in (a) 4 mM Mg-ATP and (b) 0.8 mM Mg-ATP, and are of the form mean ± SD (no. of determinations).

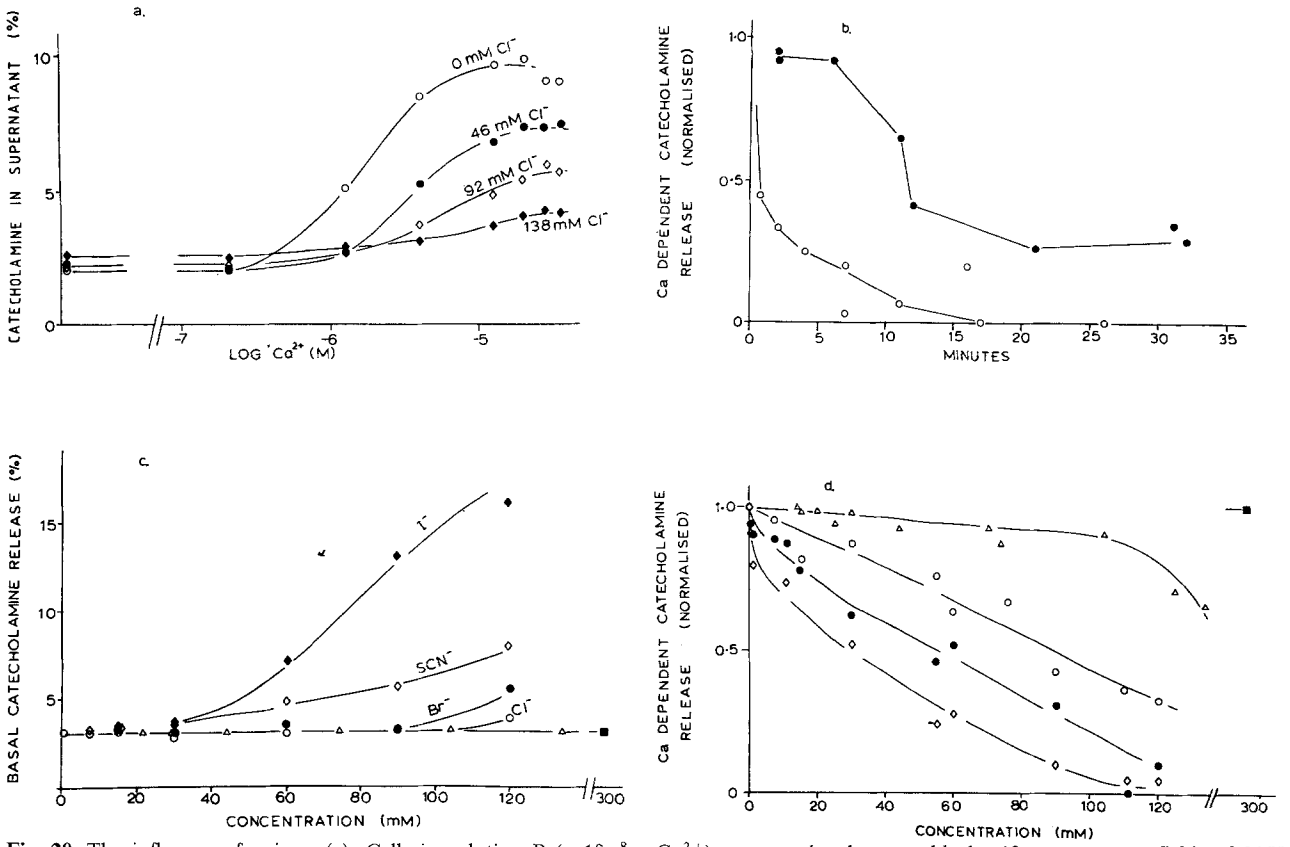


Fig. 20. The influence of anions. (*a*): Cells in solution B ($\sim 10^{-8}$ M Ca^{2+}) were rendered permeable by 10 exposures to fields of 2 kV cm^{-1}, $\tau = 200$ μsec, and then injected into similar solutions in which the glutamate had been wholly or partially replaced by chloride. After 5 min the cells were challenged with 10 mM Ca-EGTA (10^{-5} M Ca^{2+}), and the catecholamine in the supernatant was determined 10 min later. Temp. 37 °C. (*b*): Leaky cells were diluted into a solution to give a final concentration of 127 mM Cl^-, and at the times shown were either challenged with 10^{-5} M Ca^{2+} for 5 min (the catecholamine secreted over this period is expressed relative to the amount secreted by cells of the same population not exposed to chloride (○)), or the chloride levels were reduced from 127 to 21 mM by further diluting the cells into glutamate solution, and after 5-min incubation were challenged with 10^{-5} M Ca^{2+}. The catecholamine secreted over 5 min relative to the amounts secreted from cells of the same population not exposed to chloride is shown (●). Temp. 37 °C. (*c, d*): Cells in solution B were rendered leaky by 10 exposures to fields of 2 kV cm^{-1}, $\tau = 200$ μsec, and then diluted into similar solutions in which the glutamate had been wholly or partially replaced by one of several anions. After 3 min the cells were challenged with 10^{-5} M Ca^{2+} for 15 min or kept at 10^{-8} M Ca^{2+}. The catecholamine in the supernatant at 10^{-8} M Ca^{2+} is shown in *c* and reflects the basal release rate at the various ion concentrations. The amount of catecholamine secreted due to the 10^{-5} M Ca^{2+} challenge (i.e., secretion in 10^{-5} M Ca minus that in 10^{-8} M Ca) is shown in *d*, the ordinate being normalized to that secreted in the presence of glutamate alone. Iodide, ◆; thiocyanate, ◊; bromide, ●; chloride, ○; acetate, △; sucrose, ■. Temp. 37 °C

GTP, CTP, UTP, ITP, are about 20% as effective whilst a nonhydrolyzable form of ATP (AMP·PNP), s-adenosyl-L-methionine and the cyclic nucleotides are virtually without effect. On a molar basis ADP is 50% as effective as ATP (Table 7*a*). The mechanism by which ADP protects the calcium sensitivity appears to be by formation of ATP as inclusion of diadenosine pentaphosphate, which blocks the synthesis of ATP from ADP by myokinase (Leinhard & Secemsky, 1973) eliminates the Ca-dependent exocytosis in the presence of ADP alone (Table 7*a*). When submaximal levels of Mg-ATP are used, there is an enhanced effect on secretion by the addition of GTP, UTP, CTP, ITP or ADP but no significant enhancement or inhibition by the addition of the other nucleotides. None of the nucleotides (or mixtures) affected the calcium independent basal level of catecholamine release.

The major anion. The major anion in solution B is glutamate. This was chosen because the extent of the Ca-dependent exocytosis proved to be very sensitive to the anionic composition of the medium. When glutamate is replaced by the physiologically important anion chloride, the basal rate of release increases slightly (Fig. 20*a*, *c* and Table 8), and the response to calcium is reduced (Fig. 20*a, d*). These nonresponsive cells are still accessable, and there is no evidence for unloading of catecholamine from granules because (i) the decrease in the amount of secreted catecholamine is very much larger than the

Table 8. The effect of chloride on catecholamine release in the presence of 10^{-8} M Ca^{2+}. (*see* text for details)

Cl^- (mM)	(Regression slope) release %/minute	(Standard deviation)	Intercept. catecholamine in supernatant immediately after diluting the cells (%)
0	0.046	(0.008)	2.7
30	0.052	(0.007)	2.8
70	0.059	(0.005)	2.8
120	0.070	(0.005)	2.9
160	0.106	(0.003)	3.1

Cells were rendered leaky (10 exposures of 2 kV cm^{-1}, τ = 200 μsec) in solution B (except 160 mM K glutamate and 1 mM EGTA were used) and then diluted into large volumes of similar solutions in which the glutamate had been wholly or partially replaced by chloride. The cells were incubated over a period of 60 min at 37 °C, the catecholamine in the supernatant determined at 5-min intervals, and a regression line fitted to the data.

increased basal release (Figs. 20*a*, *c* and *d*), (ii) secretion of DβH is inhibited in parallel with catecholamine, and (iii) granules isolated from refractory cells contain a normal complement of catecholamine and DβH (Table 6). Figure 20*a* examines the effect on the Ca-activation curve of replacing glutamate by chloride. It shows clearly (i) the small progressive increase in the Ca-independent release of catecholamine, (ii) a progressive decrease in the apparent affinity for calcium, and (iii) the extent of exocytosis is reduced eventually almost to zero. The inhibitory effect of chloride is not a result of the removal of glutamate, as this can be replaced completely by sucrose without altering the Ca-activation curve (Figs. 20*d* and 21*a*). Fig. 20*b* shows that the inhibitory effect occurs within minutes of incubating the leaky cells in chloride and that the responsiveness of the cells may recover if they are returned to a glutamate solution after a very short time in chloride.

The inhibitory effect of chloride is not unique. When glutamate is replaced by a variety of other anions, their order of effectiveness at inhibiting calcium-dependent catecholamine release is $SCN^- > Br^- > Cl^-$ > acetate > glutamate (Fig. 20*d*), the typical concentrations necessary to effect half-maximal inhibition being (mM) 30, 60, 90 and >140, respectively. Other anions such as iodide cause a large increase in Ca-independent basal release (Fig. 20*c*), which makes subsequent analysis of the inhibitory step difficult.

The release of catecholamine from intact cells in response to carbamyl choline and potassium challenges is independent of the major extracellular anion and so the conclusion from these studies on leaky cells seems inescapable – that the nature of the *intracellular* anion is of crucial importance in exocytosis.

The major cation. Solution B contains potassium because our original intention was to depolarize cells before exposure to the electric field. In practice no significant difference can be detected in the Ca-activation curve when cells are shocked in either K, Na or sucrose and subsequently exposed to calcium (Fig. 21*a*) or shocked in any one of them and transferred to the other before exposure to calcium. Other cation substitutes, e.g., choline and lithium, have not been examined.

pH. Measurement of Ca-dependent catecholamine release at a variety of different pH values (Fig. 21*c*) revealed a rather flat pH dependence. Fig. 21*d* shows the Ca-activation curves measured at various pH values, and although the calciums necessary to elicit a half-maximal response are not comparable (because a maximal response is not achieved with the Ca-EGTA buffers at pH 6.8 and above), the threshold for secretion appears to be the same, i.e., about 10^{-7} M Ca. Because of the much greater ease of stabilizing an accurately known range of ionized calciums up to 10 μM at pH on the acid side of physiological, a pH of 6.6 was chosen for solution B; but all our results were checked at pH 7.3 using a less well buffered Ca-concentration of about 10^{-4} M.

Magnesium. The magnesium content of solution B is chosen to provide a free Mg^{2+} concentration of 2 mM in addition to 5 mM of Mg-ATP. Raising the free Mg^{2+} concentration has two effects (Fig. 21*b*).

1) it reduces the apparent affinity for calcium and
2) it reduces the maximum extent of exocytosis.

The changes seem to be brought about by magnesium because essentially similar results are obtained with both $MgCl_2$ and magnesium acetate. Similar inhibitory results are obtained when the Mg^{2+} levels are raised such as to keep the Mg-ATP/Mg^{2+} ratio constant. This indicates that the changes are not a result of the Mg^{2+} competing with the Mg-ATP sites. As with the nucleotide and anion effects, the inhibition by Mg does not stem from an inaccessibility to the interior of the cell, nor to an unloading of granules.

The Extent of Exocytosis

A particularly interesting aspect of our results is that we have never been able to effect a Ca-dependent release of more than 30% of the catecholamine in 'leaky' cells. Application of multiple dis-

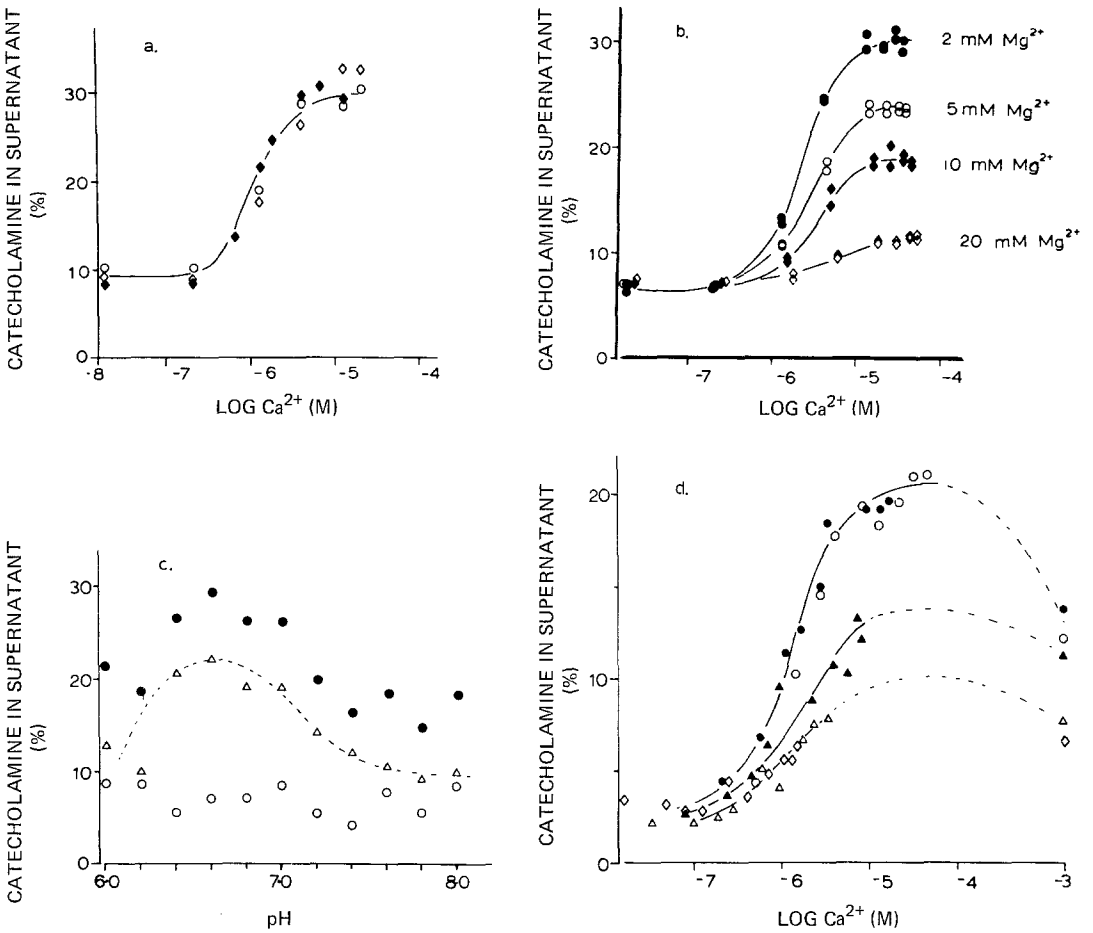


Fig. 21. Effect of Na^+, K^+, Mg^{2+} low ionic strength and H^+ on Ca-sensitive release of catecholamine. (*a*): Replacement of K glutamate by Na glutamate or sucrose. Cells in solution B were rendered leaky by 10 exposures to 2 kV cm^{-1}, $\tau = 200\,\mu$sec, and immediately diluted into either an 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 (●), and after 3 min were challenged with 10 mM Ca-EGTA buffer. The catecholamine in the supernatant was determined 15 min later. Temp. 37 °C. (*b*): Addition of Mg acetate. Cells in solution B were rendered leaky by 10 exposures to 2 kV cm^{-1}, $\tau = 200\,\mu$sec, 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. Temp. 37 °C. (*c*): Alterations in pH. Cells in solution B containing 0.5 mM K-Pipes pH 6.6 were rendered leaky by 10 exposures of 2 kV cm^{-1}, $\tau = 200\,\mu$sec, and diluted into 25 volumes of solution B containing 20 mM Hepes +20 mM Pipes at various pH and immediately challenged with 1 mM Ca^{2+}. The catecholamine in the supernatant was determined 15 min later. Cells in 1 mM Ca^{2+}, ●; cells in 0.4 mM EGTA, ○; difference, △. (*d*): Cells in solution B containing 20 mM Pipes +20 mM Hepes at various pH were rendered leaky by 5 exposures of 2 kV cm^{-1}, $\tau = 200\,\mu$sec, immediately challenged with Ca-EGTA buffers, and the catecholamine in the supernatant was determined 15 min later. pH 6.4 (○), 6.6 (●), 6.8 (▲), 7.0 (△), and 7.2 (◊). Ionized Ca was calculated for each pH. Temp. 37 °C.

charges that render the cells progressively more permeable to lactate dehydrogenase do not increase catecholamine release beyond 30% (Fig. 22), and the data presented on page 116 leaves little doubt that a very much larger percentage of the cells are accessible to calcium. It follows that our failure to effect the release of all the bound catecholamine must imply either some deficiency in our solutions or heterogeneity of the granule population, only part of which is capable of responding to a calcium challenge. The possibility that our solutions lack a crucial co-factor is an attractive one; but if this were the case, we would expect a much larger catecholamine release from cells rendered 'leaky' in the presence of calcium than from cells rendered 'leaky' in the absence of Ca and only challenged with Ca minutes later. Very little difference is seen and certainly not enough to account for a drop from 100 to 30%. In view of our finding that ATP, anions and Mg can all modify the extent of exocytosis in the 'leaky' cell, it is possible that the failure to release 100% of the catecholamine may reflect a direct inhibitory effect of some component of solution B, but, so far, we have been unable to find any evidence for

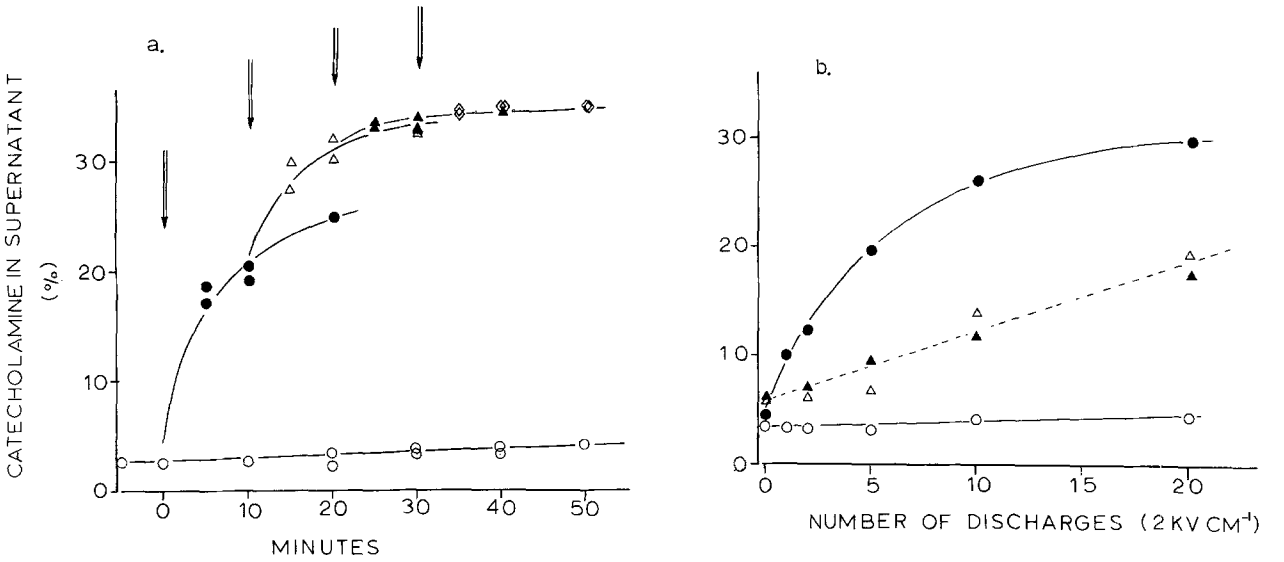


Fig. 22. Evidence that not all the catecholamine in the cells can be released. (*a*): Catecholamine in the supernatant from cells exposed to a series of fields in the presence of 10^{-5} M free Ca. At the times indicated by the arrows, the cell suspension was subjected to 5 exposures of 2000 V cm^{-1}, $\tau = 200$ μsec. After 30 min, therefore, the cell suspension had received 20 discharges. Cells not subject to the electric field, ○. Medium solution B (Methods) containing 10 mM Ca-EGTA. Temp. 37 °C. (*b*): Catecholamine (●, ○) and LDH (▲, △) in the supernatant 10 min after exposing a cell suspension to a number of discharges applied in less than 1 min from a capacitor. 2000 V cm^{-1}, $\tau = 200$ μsec, in the presence of 10^{-5} M Ca^{2+} (●, ▲), and 10^{-9} M Ca^{2+} (○, △). Medium solution B (Methods) containing 10 mM Ca-EGTA buffer. Temp. 37 °C.

this possibility. The apparent limit of secretion reflected in Figs. 7, 11 and 22 suggests that only part of the population of granules is available for secretion.

Effects of Some Potential Inhibitors of Exocytosis

Table 9 and Fig. 23 summarize the effects of a variety of potential inhibitory agents on Ca-dependent exocytosis in 'leaky' cells. Essentially similar results were obtained at pH 6.6 and pH 7.3. The agents examined can be subdivided in terms of their known actions.

i) *Agonists and antagonists of the acetylcholine receptor in medullary cells.* Acetylcholine (5×10^{-5} M), carbamylcholine (5×10^{-4} M), hexamethonium (10^{-4} M), and atropine (10^{-4} M) were without significant effect on both the Ca-dependent and Ca-independent release of catecholamine.

ii) *Peptides.* Met-enkephalin (10^{-4} M), leu-enkephalin (10^{-4} M), substance *P* (10^{-4} M), and somatostatin (10^{-6} M) were all without effect on the Ca-dependent and Ca-independent release of catecholamine.

iii) *Agents that bind to tubulin.* Colchicine only affected Ca-dependent release of catecholamine at the highest concentrations examined (10^{-3} M), and even at these concentrations inhibition was only partial. Vinblastine was without effect at 10^{-4} M. Higher concentrations caused large calcium-independent release which subsequently made inhibition studies impracticable.

iv) *Interference with known cell motility mechanisms.* Cytocholasins B and D (10^{-3} M), phalloidin (10^{-3} M), and vanadate (10^{-3} M) were all without effect on the Ca-dependent release of catecholamine (Table 9).

v) *Known antagonists of calmodulin.* Ca-dependent exocytosis is inhibited by about 10^{-5} M levels of the antipsychotic agent trifluoperazine (Fig. 23*c*). Higher concentrations cause a large Ca-independent release of catecholamine. Two other antipsychotics diazepam and pimozide show no inhibition at doses of up to 10^{-4} and 10^{-5} M, respectively. Above these concentrations the Ca-independent release is so large that inhibition studies are inconclusive.

vi) *Inhibitors of anion permeability.* Neither 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) or 4,4′-diisothiocyano-2,2′-disulfonic acid (DIDS) (up to 10^{-4} M) had any effect on Ca-dependent release of catecholamine (Fig. 23*a*) although there was a small increase in the Ca-independent release at high doses.

vii) *Hydrogen ionophores.* Carbonylcyanid-*p*-trifluormethoxy phenylhydrazon (FCCP) at concentrations up to 10^{-5} M reduced the extent of Ca-dependent release of catecholamine whilst having little effect on the Ca-independent release (Fig. 23*b*). At higher doses, however, this Ca-independent release increases significantly. 10^{-5} M of 2,4-dinitrophenol (DNP) had

Table 9. The effects of some agents on catecholamine release

		pH 6.6		pH 7.3	
		a $\sim 10^{-8}$ M Ca	*b* $\Delta 10^{-5}$ M Ca	*a* $\sim 10^{-9}$ M Ca	*b* $\Delta 6 \times 10^{-4}$ M Ca
Acetyl choline	5×10^{-5} M	1.05±0.16	0.96±0.12		
Carbamyl choline	5×10^{-4} M	1.05±0.11	0.99±0.15		
Hexamethonium	10^{-4} M	1.05±0.11	0.87±0.14		
Atropine	10^{-4} M	1.13±0.14	0.77±0.15		
Metenkephalin	10^{-4} M	1.0	1.07±0.12		
Leuenkephalin	10^{-4} M	1.02±0.08	1.2 ±0.2		
Colchicine	10^{-4} M	1.15±0.07	0.88±0.10		
	10^{-3} M	1.06±0.08	0.58±0.10	1.07±0.11	0.68±0.11
Vinblastine[a]	10^{-4} M	0.88±0.02	1.09±0.12		
Cytochalasin B	10^{-4} M	1.0 ±0.08	0.85±0.13		
	10^{-3} M	1.0 ±0.03	0.89±0.10	1.18±0.09	1.1 ±0.1
Cytochalasin D	10^{-3} M	1.02±0.05	1.02±0.11		
Phalloidin	10^{-3} M	0.92±0.02	0.97±0.01		
TLCK	10^{-3} M	1.07±0.06	0.85±0.16	1.22±0.09	1.4 ±0.21
Trifluoperazine[a]	10^{-5} M	1.05±0.05	0.6 ±0.05		
Pimozide[a]	10^{-5} M	1.3 ±0.06	0.92±0.09		
Diazepam[a]	10^{-4} M	1.12±0.07	0.79±0.16		
SITS[a]	10^{-4} M	1.5	1.0		
DIDS[a]	10^{-4} M	1.5	1.0		
FCCP[a]	10^{-5} M	1.35±0.15	0.48±0.1		
DNP	10^{-5} M	1.03±0.13	0.94±0.01		
Trimethyl tin*	10^{-3} M	1.1 ±0.2	0.74±0.26		
Tetracaine[a]	10^{-4} M	1.05±0.1	0.98±0.10		
Urethane	3×10^{-3} M	0.93±0.03	1.03±0.05		
	10^{-2} M	1.0 ±0.04	0.75±0.16		
Procaine	10^{-3} M	1.03	1.0 ±0.03		
NH_4*	3×10^{-2} M	1.3 ±0.2	0.82±0.12		
NADPH	10^{-3} M	1.2	0.87±0.07		
NADH	10^{-3} M	1.09±0.06	0.92±0.11		
NADP	10^{-3} M	1.17±0.1	1.04±0.14		
NAD	10^{-3} M	1.16±0.04	1.2 ±0.2		
NEM	10^{-3} M	1.4 ±0.1	0.05		

Cells were incubated for about 5 min at 37 °C or 20 °C* in solution B containing the various agents listed before being challenged with calcium for a further 15 min. The results are expressed as (i) the amount of catecholamine in the supernatant at $\sim 10^{-8}$ M Ca relative to that in control cells, and (ii) the amount of catecholamine released on raising the free calcium concentration, relative to the amount released in control cells. At pH 6.6 the calcium was raised from $\sim 10^{-8}$ M to $\sim 10^{-5}$ M using Ca-EGTA buffers, and at pH 7.3 the calcium was raised to 0.6 mM by covering the EGTA with $CaCl_2$. The errors represent the range of between 2 and 4 determinations.

[a] Higher concentrations of these agents resulted in a large calcium-independent release of catecholamine.

little effect on the Ca-dependent release. Higher concentrations were not used due to interference with the catecholamine assay.

viii) *Dissipation of the chromaffin granule pH gradient.* The interior of the chromaffin granule is more acid than the cytosol, and this pH gradient can be neutralized by including ammonium chloride in the medium (*see* Salama, Johnson & Scarpa, 1980). Ammonium chloride (30 mM) has no effect on the Ca-dependent release of catecholamine.

ix) *The chromaffin granule membrane potential.* The interior of the chromaffin granule is maintained positive with respect to its external (cytosolic) face. This positive internal potential seems to be generated by an ATPase which pumps H^+ ions into the granule (*see* Salama et al., 1980). This ATPase can be inhibited by trimethyltin and the potential can be reversed by exposure to the ionophore FCCP. Both agents have no effect on Ca-dependent release of catecholamine at low concentrations but cause some inhibition at high concentrations. It is therefore of some importance to determine whether inhibition is associated with alterations in potential or whether it occurs in a different range of concentrations. One way to monitor the granule potential in leaky cells is to use trace amounts of [^{14}C]-SCN which should be accumulated within any positively

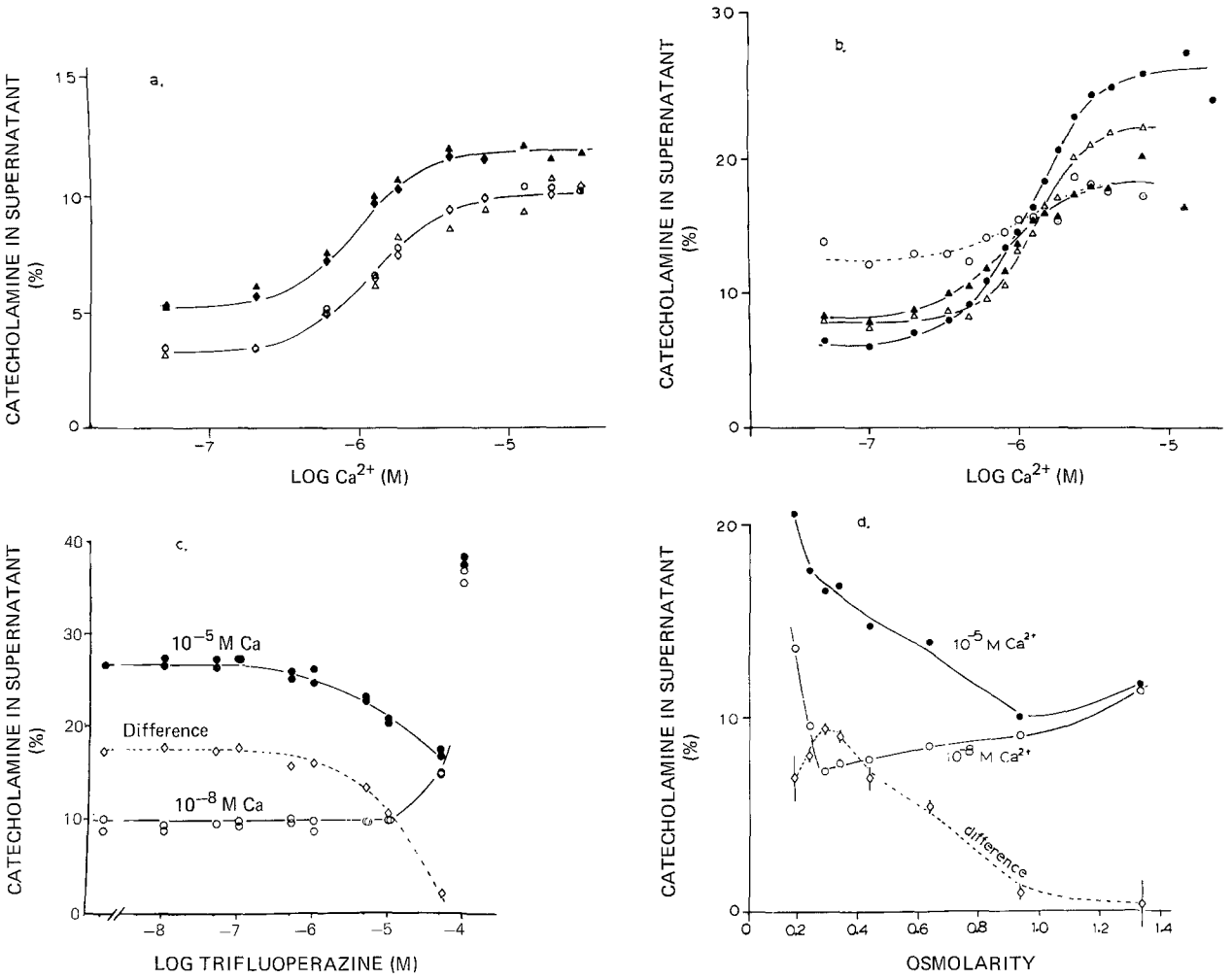


Fig. 23. Effects of some potential inhibitors on Ca-sensitive release of catecholamine. (*a, b*): Cells in solution B were rendered leaky and incubated in various media for 5 min before being challenged with Ca-EGTA buffers. The catecholamine in the supernatant was determined 10 min later. (*a*): SITS. 10^{-4} M (♦), 10^{-6} M (◊); DIDS 10^{-4} M (▲), 10^{-6} M (△), control (○). (*b*): FCCP. 10^{-4} M (○), 10^{-5} M (▲), 10^{-6} M (△), control (●). (*c*): Leaky cells in solution B were incubated with various concentrations of trifluoperazine for 5 min before being challenged with 10^{-5} M Ca^{2+} and the catecholamine in the supernatant determined 10 min later. Cells challenged with 10^{-5} M Ca^{2+} (●). Cells held at 10^{-8} M Ca^{2+} (○). Calcium dependent release (◊). (*d*): Cells in solution C (corresponding to 340 mosmolar) were rendered leaky and diluted into solutions of various osmolarities. (Sucrose concentration varied.) After 5 min the cells were challenged with 10^{-5} M Ca^{2+} and the catecholamine in the supernatant determined 20 min later (●). Cells not challenged (○), calcium dependent release (◊). Error bars represent standard error of 4 determinations

charged structures. As Fig. 24 shows, the [^{14}C]-SCN space exceeds the $^{3}H_2O$ space by a factor of about 4. This effect is reduced in the absence of ATP and can be abolished by trimethyltin or FCCP. However, the concentrations of these inhibitors that reduce SCN accumulation to half its control value are about two orders of magnitude lower than the concentration at which these same agents bring about half-maximal inhibition of Ca-dependent release of catecholamine.

One tentative conclusion is that, although we cannot rule out the possibility that the vesicle potential plays a small part in exocytosis, the establishment and maintenance of a vesicle potential is not essential for Ca-dependent exocytosis.

x) *Protease inhibitors.* The protease inhibitor N-α-*p* tosyl-L-lysine chlormethyl ketone (TLCK) had no effect on Ca-dependent release of catecholamine. The highest dose used was 10^{-3} M (Table 9).

xi) *Sulphydryl reagents.* Catecholamine release due to 10 μM Ca is strongly inhibited by N-ethylmaleimide (10^{-3} M).

xii) *Anesthetics.* Tetracaine (10^{-4} M) and procaine (10^{-3} M) had no significant effect on the Ca-dependent exocytosis. Higher concentrations, however, increased the Ca-independent release. 10^{-2} M urethane (largest dose tested) had no inhibitory effect.

xiii) *Nicotinamide coenzymes.* NAD, NADH, NADP and NADPH (up to 10^{-3} M – the highest con-

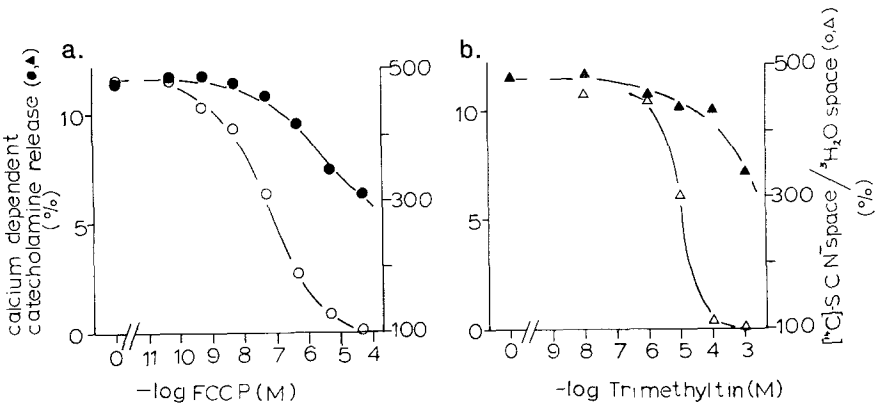


Fig. 24. Comparison of the effects of trimethyltin and FCCP on Ca-dependent release of catecholamine and the $[^{14}C]$-SCN/^3H_2O spaces. Measurements were made on the same batch of cells at 20 °C. Cells in solution B (Methods) containing 0.4 mM EGTA and trace amounts of ^3H_2O and $[^{14}C]$-SCN were rendered leaky by 10 exposures of 2000 V cm^{-1}, $\tau = 200\,\mu sec$, incubated for 15 min in various concentrations of (*a*) FCCP or (*b*) trimethyltin, and then aliquots were challenged for a further 15 min with 10 mM Ca-EGTA. The catecholamine released as a result of this 10^{-5} M Ca^{2+} challenge is shown (●, ▲). The amount is expressed as a % of the total cellular content. At the same time the unchallenged cells were spun through oil and the $[^{14}C]$-SCN^- space in the cell pellets expressed as a % of the total ^3H_2O space (○, △). Accumulation of the anion within organelles is reflected by relative spaces exceeding 100%. Temp. 20 °C

centrations tested) had no effect on the Ca-dependent exocytosis nor on the Ca-independent release.

xiv) *Temperature.* The temperature dependence of Ca-dependent catecholamine release is shown in Fig. 19*f*. From 37 to 23 °C the amount of the catecholamine released and the calcium sensitivity seems independent of temperature. Lowering the temperature below 23 °C inhibits the release.

xv) *Osmolarity.* Fig. 23*d* shows the dependence of Ca-dependent catecholamine release on osmolarity. There is a progressive inhibition as the osmolarity is increased above 0.3 osmolar. Increasing the osmolarity reduces the extent of calcium-dependent secretion rather than the apparent affinity for calcium.

Discussion

The High Voltage Technique

Exposure of adrenal medullary cells to intense electric fields of brief duration permits the plasma membrane barrier to be breached without impairing the capacity of the cells to undergo exocytosis. After such treatments the plasma membrane is freely permeable to substances of relatively low molecular mass and exocytosis proves to be very sensitive to the major intracellular anion, has a requirement for Mg-ATP, and is activated by buffered concentrations of ionized calcium in the micromolar range.

Brief exposure to an intense electric field provides a simple, reliable and clean method for rendering cell membranes permeable to molecules of low molecular mass. It has the great advantage that large numbers of cells can be treated simultaneously which greatly facilitates subsequent chemical analysis, and by suitable choice of voltage it is a relatively simple matter to increase the permeability of the plasma membrane without affecting that of intracellular membrane-bound organelles. In the adrenal medullary cell the increase in permeability persists for at least one hour after exposure to the electric field.

It is not clear at first sight why adrenal medullary cells fail to reseal after exposure to a high electric field, whereas red blood cells seemingly have the capacity to do so (Kinosita & Tsong, 1977; 1978). This discrepancy may not be so marked, however, as there are conditions in which red blood cells do not completely reseal. Our experiments show that when red cells are exposed to intense electric fields so as to allow rapid and complete access to the cytosol by substances of molecular mass in the region of 400, further incubation at 37 °C does not allow complete resealing. The membranes may recover to the extent that the permeability to the 400 mol wt substances is much reduced, but when the dextran (10,000 mol wt) which is used to offset the colloidal osmotic pressure is removed, the cells eventually lyse. This indicates that the cells are still permeable to small molecular weight chemicals, e.g., Na^+ and K^+. The degree of permeability of the adrenal cells appears to depend on the duration of the electric field. It is not clear, however, whether the very short duration fields are insufficient to cause membrane breakdown or that the membrane reseals so quickly that no flux movement is measureable. There is evidence that such a resealing process might occur within 10 μsec (Benz et al., 1979). What is certain is that when the membrane is rendered permeable to the extent that radioactive tracer movements are measurable, the membrane does not reseal fully. Our studies on platelets, cultured cells, and sea urchin eggs are consistent with the adrenal finding, i.e., when cells are exposed to intense electric fields so as to allow rapid and complete access to the cytosol by substances of molecular mass 400, they do not reseal fully.

A proper understanding of the resealing process necessitates a more detailed knowledge of the nature of the 'pores' created by the electric field. If we assume the generation of two pores per exposure, all our data is quantitatively consistent with the formation of pores of average diameter 4 nm; but the experimental

evidence does not permit us to exclude the possibility that each exposure produces many pores of much smaller diameter. Perhaps the strongest arguments against the last point are (i) that the permeability increase after one, two three or more discharges to a range of molecules of widely differing molecular mass is fully consistent with that predicted theoretically on the basis of two pores per discharge (p. 114) and (ii) the appearance of shocked cells. When large cells such as the sea urchin egg are exposed to electric fields two effects may be seen (Knight, 1981): (i) a morphological change associated with an increased permeability occurring at two opposite points on the cell surface and (ii) cytoplasmic material leaving the cell through pores. If the interpretation of two pores per field exposure is correct we need to explain why pores of about 2 to 4 nm diameter can remain stable for up to 1 hr whereas many cells recover very rapidly after withdrawal of a microelectrode which must leave a hole at least 10^3 nm in diameter. One possibility is that, once the intense electric field has created a pore, the current driven through this small hole may produce enough local heating to denature a ring of protein which serves to stabilize the pore. Whatever the explanation, the production of stable pores is a great help in the present work.

Bound vs. Free Catecholamine

One striking finding is that despite the fact that the holes are of a size to permit ready equilibration of the cell water with ions and physiologically interesting molecules such as catecholamines, ATP and EGTA, in the presence of an ionized Ca of 10^{-8} M less than 1% of the total cellular catecholamine is lost from these 'leaky' cells. The implication must be that the bulk of the cellular catecholamine is tightly bound presumably within the catecholamine storage granules. The high voltage technique would seem to provide a rather simple way to sample the cytosolic compartment of cells and, coupled with suitable isotopes, could provide direct information on the intracellular origin of substances released into the external medium.

The rate of loss of catecholamine from leaky cells incubated at 37 °C in EGTA is on average about 5% of the total cellular catecholamine content per hour. This is not dissimilar to the rate of loss from intact cells (*see* Baker & Knight, 1981). Although we have not made a detailed investigation, the rates of loss of catecholamine from granules *in situ* seem far slower than for granules isolated on sucrose gradients (*see* Hillarp, 1958), strongly suggesting that there may be cytosolic factors that are important for retention of granule contents.

The Requirements for Exocytosis

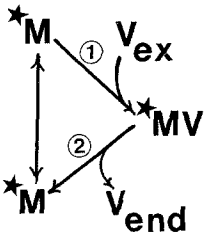
In order to elicit a substantial release of catecholamine, the medium with which the leaky cells are equilibrated must resemble the interior of the cell in containing Mg-ATP and a rather low concentration of chloride and chloride-like anions and, in addition, the ionized Ca must be raised into the micromolar range. Under these conditions dopamine-β-hydroxylase is released in parallel with catecholamine in a manner closely resembling that seen in intact cells. That these solutes of widely different molecular mass are released from the leaky cell at the same rate and in stoichiometric amounts whereas the intermediate-sized molecule lactate dehydrogenase is released at a far slower rate strongly suggests that the mechanism by which catecholamine and DβH are released is exocytosis. We have not found any conditions that favor the release of noradrenalin over adrenalin, the relative amounts secreted being in the same proportion as that found in the cells.

Ultrastructure: Evidence for Endocytosis

Preliminary examination of 'leaky' cells by transmission electron microscopy shows very little difference between control cells and cells rendered 'leaky' in the presence of an ionized calcium concentration $\ll 10^{-7}$ M; but 'leaky' cells exposed to 10 μM Ca^{2+} show a number of differences, the most pronounced being the appearance of large membrane-bounded cisternae (about 1 μm diameter) similar to those seen in intact cells that have been stimulated to secrete massively. As horse-radish peroxidase is taken up into these cisternae both in intact and 'leaky' cells, their function seems most likely to be membrane retrieval by endocytosis. If this interpretation is correct, it follows that challenging a leaky cell with micromolar levels of calcium sets in motion both exocytosis and endocytosis – a sequence of events that might explain the rarity of omega profiles in transmission electron micrographs of this tissue! In this respect the 'leaky' cell is behaving very like an intact cell, confirming its usefulness as a model of the secretory process.

Interpretation of the Data

The finding that Ca triggers a cycle which involves both exocytosis and endocytosis greatly complicates the interpretation of our experimental results. For instance, if exocytosis takes place at a relatively limited number of sites located at the inner face of the plasma membrane, the capacity to undergo exocytosis might be lost or at least altered markedly once all these sites are filled. A possible simplified reaction scheme is set out below:



where $*M$ is a 'receptor' region of the plasma membrane specialized for exocytosis and endocytosis, V_{ex} are vesicles poised to participate in exocytosis and V_{end} membrane that has undergone exocytosis and been retrieved as endocytotic vesicles. $*MV$ is the vesicle-plasma membrane complex that is perhaps visualized as an Ω-profile. According to this view the rate and extent of Ca-dependent secretion (reaction 1) will depend not only on the number of vesicles available to react but also both on the number of unoccupied plasma membrane 'vesicle receptor' sites ($*M$) and on the rate at which these sites are cleared by endocytosis (reaction 2) after a cycle of exocytosis and are thus made ready to react again. Once these possibilities are realized, it becomes extremely difficult to assign our experimental findings unequivocally to reaction 1 or reaction 2.

Take, for instance, activation by calcium. In the presence of free $*M$, Ca could increase the rate of reaction 1; but it could be argued that perhaps $*MV$ builds up at low ionized Ca-concentrations and that Ca-generates free $*M$ by activating reaction 2 – perhaps in a reaction involving Mg-ATP – and that once $*M$ is available reaction 1 is spontaneous. Arguing solely from our data, a strict differentiation between these two hypotheses is not yet possible; but widening the arena of analysis suggests strongly that Ca activates reaction 1. There are three major arguments:

1) In some cells exocytosis is not immediately followed by endocytosis. The cortical granule reaction in the sea urchin egg is one such example and Baker et al. (1980) have shown in this preparation that exocytosis is activated by ionized Ca concentrations in the same range as those required to promote catecholamine release in bovine adrenal medullary cells.

2) If membrane retrieval *preceded* exocytosis in a stoichiometric fashion, the initiation of exocytosis should be associated with a decrease in membrane area. Measurements of the capacitance of both sea urchin eggs (Cole, 1935; Jaffe, Hagiwara & Kado, 1978) and the presynaptic terminal squid giant synapse (Gillespie, 1979) shows an increase in capacitance immediately preceding secretion, findings that are most easily explained by the formation of $*MV$.

3) If $*MV$ were the predominant form in the resting condition ($Ca < 10^{-7}$ M), Ω-profiles should be more frequent in transmission electron micrographs and freeze-fracture studies of resting cells whereas they are most often seen under conditions where secretion has been activated (Normann, 1976; Heuser & Reese, 1973). This argument is a difficult one in the particular case of bovine adrenal medullary cells because the frequency of Ω-profiles is very low under all experimental conditions.

While a convincing case can be made for the participation of Ca in reaction 1 without in any way ruling out its participation in reaction 2 as well, the same cannot be said for the requirement for Mg-ATP and inhibition by Mg and chloride-like anions, all of which might act on reaction 2. In each case it might be argued that if their site of action is reaction 2, Ca ought to promote some catecholamine release before inhibition becomes apparent. Unfortunately, the size of this release might be very small if there are only a few $*M$ sites. The limit of detection of a significant change in catecholamine release under our experimental conditions is about 1% of the total in the cell. If we assume that the catecholamine content of each granule is of the order of 5×10^{-18} moles, and the total catecholamine in the cell is 3×10^{-14} moles release of 1% of the total catecholamine is equivalent to exocytosis of the contents of about 60 granules/cell. In the absence of other information about the number of $*M$ sites it is not possible to say whether our methods have the sensitivity to follow a single round of exocytosis.

Some other interesting and somewhat puzzling features of our results are:

1) That raised Mg and Cl decrease both the apparent affinity for Ca as well as the maximum catecholamine release attainable. While the former of these effects could relate to reaction 1, the latter is hard to explain, and one attractive possibility is that the reduction in maximum secretion attainable may reflect inhibition of reaction 2. More experiments of a different kind are clearly required to answer these points.

2) That even under optimum conditions we have never succeeded in releasing more than 30% of the cellular catecholamine in a Ca-dependent manner. Over long periods of time, intact cells can be caused to release a similar percentage of their catecholamine — for instance when exposed to veratridine — but under normal conditions cells rarely release more than 6% of their catecholamine when challenged with acetylcholine, carbamylcholine, or potassium. In the absence of electron microscopic evidence, the failure to release more than 30% of the cellular catecholamine might have been attributed to failure of endocytosis; but as there is experimental evidence for membrane retrieval, the 30% ceiling might reflect either that at any particular time only 30% of the granule population is in a releasable form or the necessity for some form of intracellular transport of secretory vesicles by a process that is blocked in our 'leaky' cells.

Whatever the precise site of action of Ca, our data permits two important conclusions: (i) that exocytosis can be activated by a buffered concentration of ionized calcium in the micromolar range. Our data (*see* p. 120) seems to rule out the possibility that activation of exocytosis requires localized pools of calcium at much higher concentrations and (ii) although our data provides no direct kinetic information because the response of our preparation is

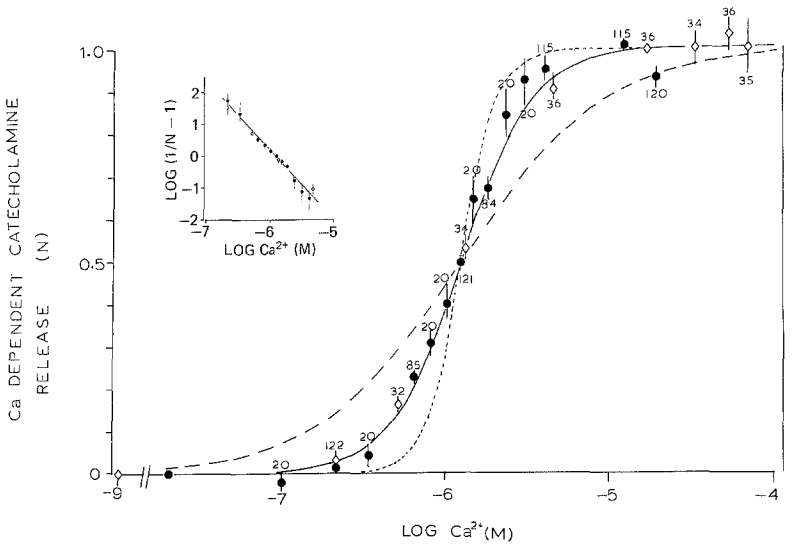


Fig. 25. Catecholamine released from leaky cells as a result of raising the Ca^{2+} concentration from 10^{-9} M (◊) or 2×10^{-8} M (●). The ordinate (N) is the release expressed relative to the amount released at 10^{-5} M Ca^{2+}. Cells were rendered leaky in the presence of Ca-EGTA buffers (◊). Cells were rendered leaky in the presence of 0.4 mM EGTA ($\sim2\times10^{-8}$ M Ca^{2+}) and subsequently challenged with calcium buffer (●). The numbers represent the number of observations and the error bars the SEM. The solid line is a theroetical curve based on 2 calcium ions involved in the secretory process, the dotted curve based on 4 calciums, and the dashed curve on 1 calcium (in each case the binding sites are assumed to be independent, each with an apparent affinity for calcium of 1.2×10^{-6} M). The inset is a Hill plot. The correlation coefficient of the 15 points is 0.99, and the regression line has a slope of 2.2, sθ 0.1

limited by the diffusion of calcium into the 'leaky' cells, nevertheless — after 10 min — the extent of exocytosis is markedly dependent on the ionized calcium concentration, and Fig. 25 shows that under these conditions the relation between catecholamine release and ionized Ca is best fitted by a relation in which activation requires two Ca ions. More precise data must clearly await the development of an accurate method for monitoring the true kinetics of secretion.

10^{-3} M levels of calcium usually promote less secretion than 10^{-5} M. This effect seems unlikely to result from activation of proteases by the higher concentrations of calcium as the protease inhibitor TLCK does not modify the effect. One possibility is that at these high levels of calcium Ca-ATP is formed and perhaps competes with Mg-ATP.

Activation of exocytosis by micromolar concentrations of calcium is physiologically reasonable because there is now a large body of evidence that the resting level of ionized calcium inside cells is close to 0.1 μM (*see* Baker, 1972; Baker & Knight, 1981) and that quite small fluxes of calcium would be required to raise the ionized calcium into the micromolar range.

The Effect of Sub-Maximal Concentrations of Ca and Mg-ATP

Although simultaneous exposure to saturating concentrations of Ca and Mg-ATP triggers exocytosis of about 30% of the total cellular catecholamine, even prolonged exposure to less than saturating concentrations of these activators fails to achieve the same final level of exocytosis. This is an observation of considerable interest. At the level of each individual granule, exocytosis appears to be an all-or-none process with a finite probability. If, in the presence of either excess Mg-ATP or excess Ca, raising the concentration of the other activator to an intermediate level increases the probability of exocytosis for part of the granule population, it is necessary to explain why the remaining granules fail to react at all, yet can be caused to release their contents by a further increase in the activator concentration. Although this latter observation would seem to rule out any simple form of inactivation of the release process, it might conceivably be consistent with models in which Ca and Mg-ATP are involved in both exocytosis (reaction 1) and endocytosis (reaction 2). While the latter possibility cannot at present be excluded especially in view of recent evidence that endocytosis may have a requirement for Ca (Ceccarelli & Hurlbut, 1980; Salisbury, Cordeelis & Satir, 1980; Taylor, Blinks & Reynolds, 1980), it seems worth exploring other possible explanations.

One such possibility that we have examined is that within the population of vesicles individual vesicles may have fixed and somewhat different sensitivities (thresholds) to calcium. It follows that as the

Ca concentration is raised, in a series of steps, the vesicles with the highest sensitivity (lowest threshold) to Ca will be the first to react. Once these vesicles have reacted, the rest should remain stable until the calcium concentration is raised further. The clear prediction from this hypothesis is that after exposure to say, 2 μM Ca, washing the cells with EGTA and re-exposing these same cells to 2 μM Ca should bring about no further secretion. The experiments described on p. 119 show this is not the case and provide convincing evidence against a population of vesicles of fixed but differing sensitivities to calcium.

One explanation that is, however, entirely consistent with our findings is as follows. If in the presence of Ca and Mg-ATP a site concerned with exocytosis is altered – perhaps phosphorylated – to a new steady level ($E \rightarrow E^*$, f {[Ca], [Mg-ATP]}), the probability of a vesicle participating in exocytosis may be proportional to the rate at which this alteration occurs and not to the final steady level. According to this hypothesis exocytosis will only take place *whilst* the level of the unknown intermediate (E^*) is changing and will cease once a new steady level has been achieved – i.e., the rate of exocytosis will be proportional to $\frac{dE^*}{dt}$ and the amount secreted on $\int \frac{dE^*}{dt} \cdot \delta t$. In view of the fact that secretion only occurs in response to an increase in the levels of Ca and ATP, it is also necessary to assume that a similar asymmetry exists in the relation between exocytosis and alterations in the level of the unknown intermediate E^*, i.e., the probability of exocytosis is increased only when E^* is increasing and not when it is decreasing. The finding that Mg-ATP is essential for Ca-dependent exocytosis coupled with the evidence for membrane phosphorylation at the time of exocytosis (De Lorenzo et al., 1979) makes phosphorylation an obvious candidate for the unknown reaction, but this possibility has not yet been examined quantitatively in our experimental system.

The finding that exocytosis only occurs whilst the Ca concentration is changing provides an alternative explanation for the well-known transient response of many secretory systems to depolarization. Baker and Rink (1975) suggested this might reflect inactivation of the channel through which Ca enters the cell – but the present data suggest an alternative explanation in that exocytosis will be transient even when exposed to an increased level of ionized calcium.

Inhibitors and Potential Inhibitors

It might be expected that precise control over the Ca-dependent step in secretion, such as is provided in cells subjected to intense electric fields, would help define the chemical nature of the cycle of exocytosis and endocytosis. Surprisingly, the bulk of compounds tested were either completely without effect (TLCK, SITS, DIDS, cytochalasins, vinblastine) or only brought about partial inhibition and that at very high concentrations (colchicine, FCCP). Apart from the absence of Mg-ATP, the only agents that produced more than 50% inhibition of the Ca-dependent reaction were Mg, hypertonicity, trifluoperazine, and a variety of anions. Trifluoperazine is known to inhibit phosphodiesterase and other calcium-dependent processes by binding to the calcium form of calmodulin (Weiss & Levin, 1978), and it is possible that its action on exocytosis might also involve a calmodulin. But the possibility needs to be treated with extreme caution because trifluoperazine has calcium-independent effects on catecholamine release in a very similar concentration range to its inhibitory effects on secretion, and also other drugs, for example, pimozide, that are known to bind to calmodulins have no clear cut effects on Ca-dependent exocytosis in our preparation.

At constant ionic strength the order of effectiveness of a variety of anions at inhibiting Ca-dependent exocytosis follows the Hofmeister lyotropic series. This is a sequence of anions arranged according to their effectiveness at interacting with proteins (Alexander, 1937; Fridovich, 1963; Tasaki, Singer & Takenaka, 1965; Rubinson & Baker, 1979). It is possible, therefore, that these anions inhibit secretion as a result of interaction with a specific enzyme or by perturbing a protein matrix necessary for secretion. The data suggests that if the cytosolic chloride concentration of intact cells is raised from its low level to concentrations approaching extracellular levels the secretory response will be impaired, and after a few minutes the effect might not be readily reversible.

Over the years, a host of working hypotheses for the mechanism of exocytosis and its control by Ca have been advanced, and it is worth examining some of the more important of these in the light of our experimental results:

1) *Change in viscosity.* One rather simple idea is based on the observation that squid axoplasm is liquified by calcium (Hodgkin & Katz, 1949). The concept of a gel-sol transition controlled by calcium has been applied to secretory cells where it is assumed that the secretory vesicles may be trapped within the gel and unable to make contact with the inner face of the plasma membrane. By liquifying the gel, calcium may facilitate vesicle movement and greatly increase the chances of exocytosis. Although this cannot be tested directly with 'leaky' adrenal medullary cells, in its simplest form it seems unlikely to be a complete explanation for the following reasons: (i) millimolar concentrations of Ca are needed to liquify squid axoplasm (Rubinson & Baker, 1979), whereas exocytosis in bovine adrenal medullary cells is fully activated by micromolar concentrations of Ca; (ii) the

liquifying action of Ca in squid axoplasm involves activation of a protease the action of which can be completely blocked by the protease inhibitor TLCK (Rubinson & Baker, 1979). This same inhibitor has no effect on Ca-dependent exocytosis from bovine adrenal medullary cells; (iii) squid axoplasm is liquified by a variety of anions. These same anions *inhibit* rather than promote Ca-dependent exocytosis in leaky adrenal cells. Exposure to high concentrations of certain anions – for example chloride – elicit some Ca-independent release of catecholamine but this might simply reflect direct leakage of catecholamine out of the intact granules into the cytosol.

2) *Charge neutralization.* If this plays any part in the initiation of exocytosis (Blioch, Glagoleva, Liberman & Nenashev, 1968; Matthews, 1970) the present results confirm that the process of charge neutralization must be highly *selective* for Ca as micromolar concentrations of ionized Ca can promote exocytosis in the presence of millimolar levels of Mg. At Ca levels below 10^{-8} M, up to 100 mM ionized Mg has no effect whatsoever on the rate of release of catecholamine.

3) *Role of microtubules and microfilaments.* As high concentrations of cytochalasin B and D have no effect whatsoever on Ca-dependent exocytosis, the present data provides no support at all for a role of microfilaments in exocytosis. The same conclusions seem likely to apply to microtubules. Very high concentrations of colchicine (10^{-3} M) do give some inhibition, but this inhibitory effect is not seen with vinblastine, and may be nonspecific. It is, however, always possible that exocytosis may make use of some very stable microtubules that are resistant to colchicine and vinblastine. Secretion from intact cells may be inhibited by colchicine, vinblastine, and cytocholasin B (Schneider, Herz & Rosenheck, 1977; Douglas & Sorimachi, 1972); but the site of inhibition seems not to be the site of exocytosis. The failure of both phalloidin and vanadate to inhibit Ca-dependent release of catecholamine does not support a role for either actin or dynein in exocytosis.

4) *Phosphorylation.* The present results provide clear evidence that Mg-ATP is essential for Ca-dependent exocytosis. A number of other workers have shown changes to phosphorylation associated with secretion of catecholamine (De Lorenzo, Freidman, Yohe & Maurer, 1979; Amy & Kirshner, 1981), but as yet, it is not possible to say with any certainty for what reaction this Mg-ATP is needed, nor whether phosphorylation *is* an obligatory step in exocytosis.

5) *Phospholipase activation.* It has been suggested that Ca may activate a phospholipase and that the resulting alteration in phospholipids may play a part in the exocytotic process (*see* Michell, 1975). Our data provides no information on this possibility, although the leaky cell preparation may provide a good system in which to examine this hypothesis.

6) *Methylation.* Levels of methylation change during secretion (Viveros, Diliberto & Axelrod, 1977), and it seemed essential to examine whether s-adenosyl-L-methionine could replace ATP or in any way modify the response to Ca in the presence of ATP. In all cases the results were essentially negative, suggesting that methylation does not play a major role in the mechanism of Ca-dependent exocytosis in adrenal medullary cells, although, under physiological conditions, methylation may, of course, be involved in steps that we are not monitoring in 'leaky' cells – for instance calcium entry, regulation of cytosolic Ca, or turnover of the vesicle population.

7) *Anion transport and permeability.* Pollard et al. (1977) have shown that catecholamine is released from isolated granules in the presence of ATP and chloride. While our data confirms that high concentrations of chloride can promote catecholamine release in the absence of calcium, the main effect of chloride is to inhibit Ca-dependent exocytosis. Our data is not consistent with the specific suggestion that entry of chloride into the granules promotes exocytosis as (i) the anion-channel blockers SITS and DIDS that inhibit the Cl^--dependent release from isolated granules have no effect on exocytosis from leaky cells, and (ii) exocytosis can occur in the complete absence of chloride ions or even when the main component of the medium (solution C methods) is non-ionic.

8) *The role of the granule pH gradient and potential.* Our data provide no evidence for an important role of the chromaffin granule pH gradient or potential in Ca-dependent release of catecholamine.

9) *Osmotic effects.* Osmotic instability of the granule contents has been implicated in the mechanism of secretion (Pollard et al., 1977; Zimmerberg, Cohen & Finkelstein, 1980). Our results are not inconsistent with this possibility as calcium-dependent secretion seems dependent on the osmolarity of the medium.

At present the mechanism by which Ca ions act to promote exocytosis is very obscure, but 'leaky cells' offer an experimental preparation that may provide answers to at least some aspects of this difficult problem. There have been a number of reports that Ca-dependent exocytosis can take place in cell-free systems (Davis & Lazarus, 1976; Baker, 1977; Gratzl, Dahl, Russell & Thorn, 1977; Konings & de Potter, 1981), where the reported affinity for Ca is in the same range that we find in 'leaky' cells. Unfortunately, the cell-free systems described to date are rather capricious and have not yet been shown to meet all the strict criteria for exocytosis. The 'leaky' cell seems to offer a very convenient preparation intermediate between the intact cell where the exo/endocytotic apparatus is inaccessible and subcellular systems where there is a very real possibility that only part of the system is isolated.

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