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The Chromaffin Granule Proton Pump and Calcium-Dependent Exocytosis in Bovine Adrenal Medullary Cells

D.E. Knight and P.F. Baker
Department of Physiology, King's College, London WC2R 2LS, England

Summary. Calcium-dependent exocytosis in 'leaky' bovine adrenal medullary cells has a requirement for Mg-ATP. One possibility is that exocytosis depends in some way on the operation of the ATP-dependent proton pump that serves to maintain the core of the secretory vesicles both acid and at a positive potential with respect to the cytosol. This possibility has been tested in 'leaky' cells by monitoring exocytosis under conditions where the secretory vesicle pH and potential gradients are measured *in situ*. The results show rather clearly that exocytosis can persist, with unchanged Ca-activation kinetics, in the virtual absence both of a difference in pH between the cytosol and secretory vesicle core and also of a difference in potential across the vesicle membrane. The results do not, however, exclude a small modulating effect of vesicle pH or potential on exocytosis and shed no light on whether or not the plasma membrane potential, which is maintained close to zero in these experiments, influences exocytosis.

Key Words Exocytosis · proton pump · calcium · secretion · adrenal medulla

Introduction

The immediate source of energy for both exocytosis and membrane retrieval by endocytosis is still uncertain. The observation that catecholamine secretion from the adult adrenal medulla can be blocked by exposure to metabolic inhibitors (Kirshner & Smith, 1969; Rubin, 1970) is consistent with a role for ATP, a conclusion that is strengthened by the finding that, in 'leaky' bovine adrenal medullary cells, exocytosis is dependent both on Mg-ATP and calcium (Baker & Knight, 1978*a,b*, 1981). The requirement for Mg-ATP is very specific, but its mode of action is obscure. One possibility is that exocytosis may derive energy, in some way, from the operation of the ATP-dependent proton pump, which both serves to acidify the vesicle core and maintain the interior of the secretory vesicle at a positive potential with respect to the cytosol (*see* Bashford, Casey, Radda & Ritchie, 1975; Johnson & Scarpa, 1976*a,b*, 1979; Toll & Howard, 1980; Winkler & Carmichael, 1982). This idea can be tested rather directly in permeable medullary cells (Baker & Knight, 1978*a,b*) where it is possible to monitor both Ca-dependent exocytosis and also, by use of suitable probes, the internal pH and potential of the secretory granules *in situ*.

Although our results cannot exclude a small role for the granular potential and pH gradient in exocytosis, there seems little doubt that exocytosis can take place in their virtual absence.

Some aspects of this work have been described in preliminary form in earlier papers (Baker & Knight, 1981, 1982; Knight & Baker, 1982).

Materials and Methods

Preparation of 'Leaky' Adrenal Medullary Cells

Cells were isolated and rendered 'leaky' as described in detail in a previous paper (Knight & Baker, 1982). The medium in which cells were normally made leaky was (mM): K glutamate, 150; PIPES (pH 6.6), 20; EGTA, 0.4; Mg ATP, 5; Mg acetate or sulphate, 2; and glucose, 5.

Assays

These were as described previously (Baker & Knight, 1978*b*).

Source of Chemicals

Radiochemicals were obtained from Amersham International, and most other chemicals were from Sigma except trimethyl tin, which was a gift from Dr. R.G. Johnson.

Measurement of Vesicular pH and Potential *in Situ*

Compartments of acid internal pH in the 'leaky' cells were monitored by use of trace amount of the membrane-permeable base methylamine. The accumulation of [^{14}C]-methylamine relative to

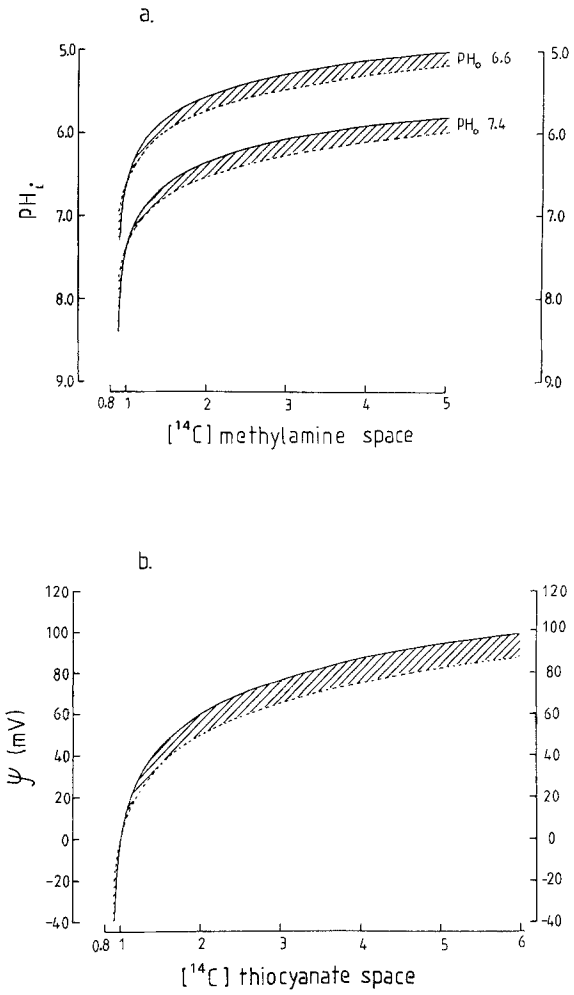

Fig. 1. Relationship between marker spaces and calculated intragranular pH and membrane potential. (*a*): Values of pH_i for various methylamine spaces were calculated as described in Materials and Methods for two cytosolic pH's, 6.6 and 7.4, and for compartment volumes of 10% (smooth line) and 16% (dotted line). The uncertainty in the calculated values of pH_i as a result of the uncertainty of the measured compartment volume (13% std 3%—*see* Fig. 2*b*) is shown by the hatched areas. (*b*): Values of granule membrane potential (ψ) against thiocyanate space are shown for the cases where the intracellular compartments occupy 10% of the cellular volume (smooth line) and 16% (dotted line), respectively. The uncertainty of membrane potential for the measured compartment volume of 13% is, as described in *a*, shown by the hatched areas

that of [^{3}H]-H_2O provides a convenient measure of the extent of such compartments. The probe is, of course, not specific for secretory granules (for instance, it will also enter lysosomes), but there seems little doubt that the secretory granules will contribute a large part of the signal detected. An analogous approach was adopted to measure compartments with a positive internal potential except here the membrane-permeable anion [^{14}C]-SCN was used in trace amounts as the probe. In both cases, the labeled cells were separated from the bulk of the medium by spinning through a suitable oil (versilube F50, a gift from Alfa Chemicals, Staines, Middlesex, U.K.) and the extent of secretion was determined from the catecholamine content of the supernatant.

The methylamine and thiocyanate spaces can either be used as a qualitative measure of the extent of a particular type of compartment within the 'leaky' cell or, assuming (i) that the probe is only entering the secretory vesicle compartment and (ii) that the internal pH or potential within this compartment is uniform, to obtain an absolute measure of the vesicular pH or potential. For this latter purpose it is necessary to know the fraction of the volume of the cell occupied by secretory vesicles. Quantitative measurements of electron micrographs of our cell preparation give values in the range 10–15% of the cell volume. This variability between cells, coupled with the fact that 10–30% of the cell preparation is cortical in origin (Knight & Baker, 1982) leaves uncertainties in the absolute values obtained for the pH and potential of chromaffin granules *in situ;* but it does not seriously affect the use of [^{14}C]-methylamine and [^{14}C]-SCN^- spaces as respective measures of the relative magnitudes of vesicular pH or potential in the course of an experiment.

Calculation of Intragranular pH

Suppose the cell (volume V_c) contains organelles (total volume V_i) which have a pH (pH_i) that is different from that of the cytosol (pH_o) which, in turn, in a 'leaky' cell is assumed to be set by the pH buffer in the external medium; the total methylamine in the cell (following Johnson and Scarpa (1976*a*) and Holz, Senter and Sharp (1983)) will be the methylamine in the cytosol together with the methylamine in the organelles, i.e., $(V_c - V_i)[Me_c^- + Me_c] + V_i[Me_i^+ + Me_i]$ where the suffixes c and i refer to the cytosol and organelles, respectively, and Me^+ and Me to the acid and base (uncharged) forms of methylamine. Now the methylamine associated with an equivalent cellular volume of water is $V_c[Me_c^+ + Me_c]$, and hence the methylamine "space" (S) in the cells is thus

$$\{(V_c - V_i)[Me_c^+ + Me_c] + V_i[Me_i^+ + Me_i]\}/\{V_c[Me_c^+ + Me_c]\}. \quad (1)$$

Considering $pH = pK_a + \log(\text{base/acid})$ and assuming the uncharged form of methylamine to be membrane permeant, i.e., $Me_c = Me_i$, then Eq. (1) simplifies to:

$$S = V(1 + 10^{pK_a - pH_i})/(1 + 10^{pK_a - pH_o}) + (1 - V)$$

where $V = V_i/V_c$, i.e., the fraction of the cellular volume occupied by the organelles and $pK_a = 10.62$ (Mahler & Cordes, 1971). Hence, knowing V and pH_o, a value for pH_i can be calculated from the measured methylamine space (*see* Fig. 1*a*).

Calculation of Membrane Potential of Intracellular Organelles

Following Pollard, Zinder, Hoffman and Nikodejevic (1976) and Johnson, Pfister, Carty and Scarpa (1979) and assuming thiocyanate distributes itself passively across the granule membrane, the relative concentrations inside and out should follow the Nernst equation. Using the same analysis and notation as above, it follows that the thiocyanate space is equal to $V \cdot (SCN_i^-/SCN_o^-) + (1 - V)$, i.e., the space measured at 20°C is equal to $V \cdot 10^{\psi_m/58} + (1 - V)$ where ψ_m is the membrane potential of the intracellular organelles. Hence, knowing V, a value for ψ_m can be calculated from the measured thiocyanate space (*see* Fig. 1*b*).

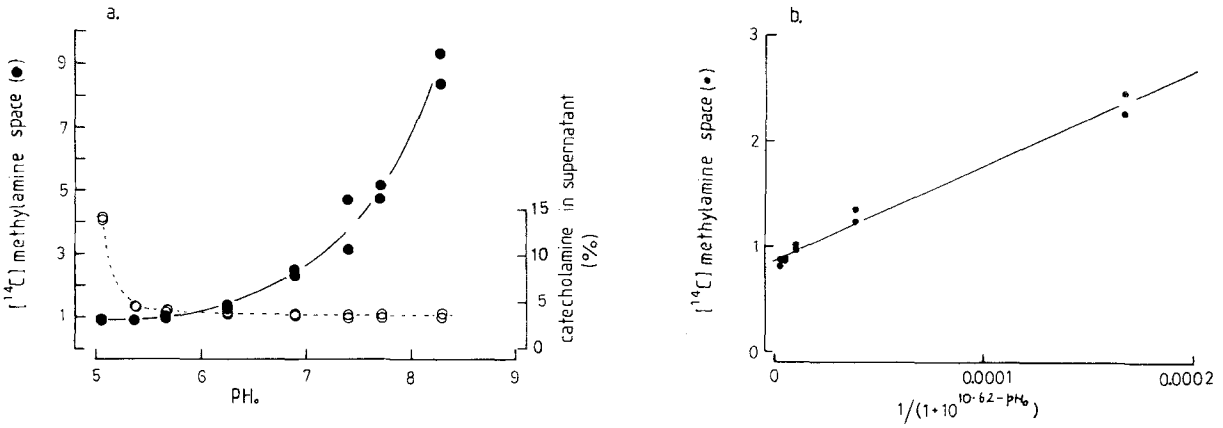

Fig. 2. Methylamine accumulation as a function of pH. (*a*): Cells were suspended in 4 ml of (mM): K glutamate, 150; glucose, 5; K-PIPES, 10; K-HEPES, 10; MgATP, 2; Mg^{2+}, 2; EGTA, 1; pH 6.6, and subjected to 10 exposures of 2 kV cm^{-1} $\tau = 200$ μsec. Aliquots (0.1 ml) were diluted into 2 ml aliquots of the suspending fluid at various pH's each containing 25 μCi ^3H_2O and 5 μCi [^{14}C]-methylamine. After 10 min incubation the cells were spun through oil (Versilube F50—gift from Alfa Chemicals, Staines, Middlesex, UK) and counted as described by Knight and Baker (1982). The methylamine associated with the pellet is expressed as a ratio of the ^{3}H water space associated with the pellet and is shown in the ordinate (●). The catecholamine in the supernatant corresponding to these cells at different pH's is also shown in the ordinate (○) and is expressed as a percentage of the total catecholamine in the suspension. (*b*): Analysis of the data in *a*—Calculation of pH_i and fraction of cell occupied by acid compartments. The methylamine space is plotted against $1/(1 + 10^{10.62-pH_o})$ over 5 different pH values. The correlation coefficient is 0.99. The intercept on the ordinate is 0.87 (SD 0.03). From the analysis as described in the text, the average organelle pH is calculated from the slope and intercept to be 5.8 (SD 0.1)

Results

Measurement of the 'Average' Internal pH of the Secretory Granules *in Situ*

'Leaky' cells accumulate [^{14}C]-methylamine. The extent of this accumulation depends on the pH at which the measurements are made (Fig. 2); but at all pH values the methylamine space reaches a steady value in about 15 min. The [^{14}C]-methylamine/[^{3}H]-H_2O space falls monotonically from a value of about 9 at a medium pH of 8.4 to a value of 1.0 at a pH of about 5.6, which suggests that this pH is close to that of the secretory granule interior. The extent of the accumulation of [^{14}C]-methylamine at pH values more alkaline than 5.6 will depend on the volume of the acidic compartments and their average internal pH. The data in Fig. 2 can be used to obtain values for both of these parameters.

According to the simple analysis presented in Materials and Methods, if the intra-organelle pH is maintained constant in the face of alterations in cytosolic pH, the observed methylamine space should be linearly related to $1/(1 + 10^{10.62-pH_o})$. Figure 2*b* shows that such a linear relation does hold up to a cytosolic pH close to 7. The intercept indicates that 13% (standard deviation 3%) of the cellular volume is occupied by compartments more acid than the cytosol and the gradient of the graph reflects an average intra-organelle pH of 5.8. The standard deviation of the estimate of pH_i is 0.1 and is calculated from both the standard deviation of the gradient and of the intercept (*see* analysis in Materials and Methods). At pH_o values above 7.0 the relation between methylamine space and $1/(1 + 10^{10.62-pH_o})$ becomes non-linear, which may be attributed either to an increased pH_i of the acid compartments or to an inadequacy of the large methylamine space to reflect a pH_i of 5.8. The value of 13% ($V = 0.13$) has been used in all calculations where spaces are converted into values for the average pH or membrane potential ψ_m of the intracellular compartments. Although the calculated values are dependent on the compartments' volume, Fig. 1 shows that the errors in calculated pH_i and ψ_m introduced by the uncertainty in V are quite small.

An average internal pH of 5.8 is close to that reported by a number of workers for isolated chromaffin granules and granule ghosts (Johnson & Scarpa, 1976*b*; Casey, Njus, Radda & Sehr, 1977; Phillips & Allison, 1978; Salama, Johnson & Scarpa, 1980; Holz et al., 1983) and an acidic compartment occupying 13% of the cell volume is within the range found in electron micrographs where secretory vesicles occupy 10–15% of the cell volume. Both findings support the view that a major component of the [^{14}C]-methylamine space is uptake into secretory vesicles.

Table 1 summarizes the effects of various treatments on the accumulation of methylamine. Accumulation is greatly reduced by the presence of ammonium ions or monensin but unaffected by the proton pump blocker trimethyl tin. Ammonium would be expected to generate NH_3, which will enter any acidic compartments and neutralize the acidic pH of their interiors, and monensin will effect exchange of trapped H^+ for cations thereby also

Table 1. (A) Methylamine spaces determined under different experimental conditions, and (B) pH_i derived from these spaces[a]

(A) [^{14}C]-methylamine/^3H_2O	pH_o 6.6	pH_o 7.4
Control	1.9 ± 0.1 (21)	3.6 ± 0.4 (12)
30 mM NH_4^+	1.29 ± 0.06 (14)	1.28 ± 0.03 (4)
10^{-5} M monensin	—	1.2 ± 0.3 (4)
2×10^{-4} M trimethyl tin	1.54 ± 0.08 (9)	2.95 ± 0.35 (4)
10^{-5} M FCCP	1.84 ± 0.11 (7)	2.43 ± 0.13 (4)
(B) pH_i (calculated)	**pH_o 6.6**	**pH_o 7.4**
Control	5.72 ± 0.03 (21)	6.11 ± 0.05 (12)
30 mM NH_4^+	6.2 ± 0.1 (14)	6.9 ± 0.03 (4)
10^{-5} M monensin	—	7.0 ± 0.4 (4)
2×10^{-4} M trimethyl tin	5.9 ± 0.05 (9)	6.21 ± 0.06 (4)
10^{-5} M FCCP	5.7 ± 0.05 (7)	6.33 ± 0.03 (4)

[a] Data expressed as a mean ± SEM; number of determinations in parentheses. Temperature, 20°C.

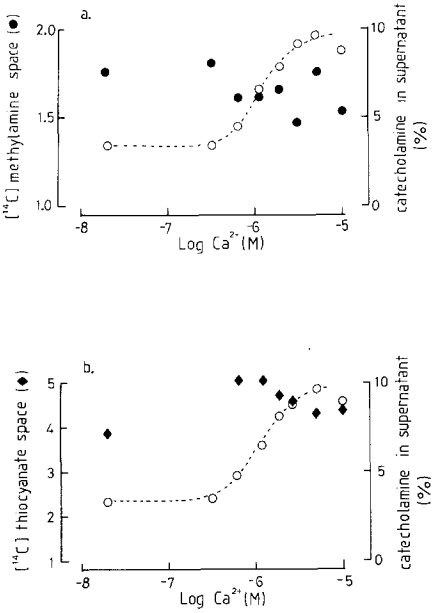

Fig. 3. Ca dependence of methylamine and thiocyanate marker spaces and catecholamine release. Cells in K glutamate medium containing 0.4 mM EGTA, 5 mM MgATP, pH 6.6, were subjected to 10 exposures of 2 kV cm^{-1}, τ = 200 μsec, and incubated with either (*a*) [^{14}C]-methylamine (5 μCi ml^{-1}) and ^3H_2O (25 μCi ml^{-1}) or with (*b*) [^{14}C]-SCN^- (5 μCi ml^{-1}) and ^3H_2O (25 μCi ml^{-1}) for 15 min before challenging aliquots of the cell suspensions with 10 mM of various Ca-EGTA buffers ($CaSO_4$ used to make up buffers) for a further 15 min. The calculated final Ca^{2+} levels are shown in the abscissa. Cells were spun through oil, the pellets counted, and the supernatant was analyzed for radiolabel and catecholamine content. The [^{14}C]-methylamine spaces (●) and [^{14}C] SCN spaces (◆) of the pellets are expressed as a ratio of the water space, and the catecholamine in the supernatant (○) as a percentage of the total in the suspension. Temperature throughout, 20°C

promoting alkalinization. The finding that blocking the proton pump has little effect on [^{14}C]-methylamine accumulation over a 30-min period implies that the acidic compartments must be very impermeable to H^+ ions. The pH gradient even persists in the presence of the H ionophore FCCP presumably because of the absence of an endogenous pathway for exchange of cations with H^+. All these findings are consistent with reported properties of isolated chromaffin granules.

Of particular interest is the finding illustrated in Fig. 3 that methylamine accumulation is not affected by calcium over the range of free calcium concentrations that activate exocytosis. In other experiments, however, in which Ca^{2+} activates a large release of cellular catecholamine (up to 30% of the cellular content), a small but significant decrease in the methylamine space of the cell is observed which is consistent with uptake of methylamine into secretory granules.

Measurement of the Mean Membrane Potential of the Secretory Granules *in Situ*

'Leaky' cells accumulate [^{14}C]-SCN and, after exposure to [^{14}C]-SCN in trace amounts, the thiocyanate space reaches a stable value in about 15 min. These results imply the existence within 'leaky' cells of a compartment, or compartments, with a positive internal potential. Table 2*A* summarizes the spaces observed under a variety of conditions. The [^{14}C]-SCN space is much the same at pH 6.6 and 7.2, is reduced in the nominal absence of ATP, is reduced in the presence of trimethyl tin or FCCP, and is increased following exposure to ammonium or monensin. These spaces have been converted to potentials in Table 2*B* on the assumption that the compartment into which SCN^- penetrates is 13% of the cell volume. The values obtained are, of course, means and may obscure large variations both between cells and between compartments within an individual cell. They are within the range reported for isolated chromaffin granules and granule ghosts (Pollard et al., 1976; Phillips & Allison, 1978; Johnson et al., 1979; Salama et al., 1980) and the ATP-dependence and trimethyl tin sensitivity of the process generating the potential is consistent with the known properties of the chromaffin granule proton pump. FCCP would be expected to collapse or even reverse the membrane potential, and failure to achieve complete collapse in many experiments may either reflect technical limitations in the method used or indicate that the thiocyanate space includes a small contribution from structures other

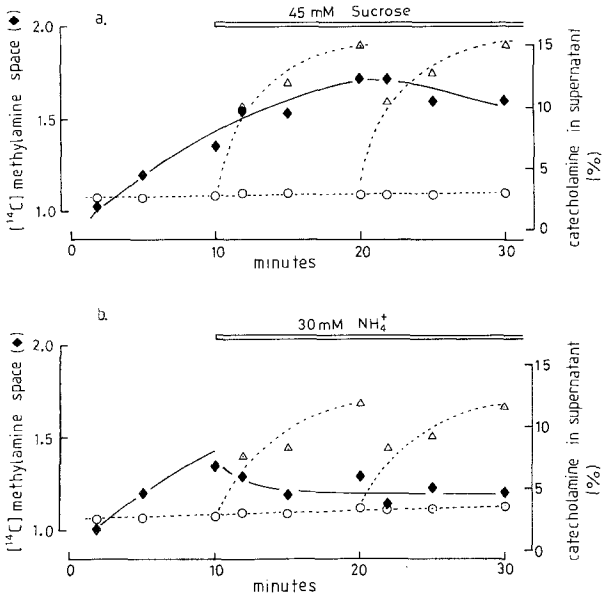

Fig. 4. Effect of NH_4^+ on catecholamine secretion and on methylamine space. Cells in K glutamate solution mM containing 2 MgATP, 1 mM EGTA, pH 6.6, were rendered leaky by 10 exposures of 2 kV cm^{-1}, τ = 200 μsec, and incubated with ^3H_2O (10 μCi ml^{-1}) and [^{14}C]-methylamine (2 μCi ml^{-1}). After 10 min half of the cells (*b*) were incubated with an addition of 30 mequiv. of NH_4^+ (sulphate salt) and the other half (*a*) with an osmotically equivalent addition of sucrose. Aliquots of the cell suspensions were challenged with 10 mM Ca-EGTA buffer (10^{-5} M Ca^{2+}) at this time and also 10 min later. The methylamine spaces (◆) corresponding to the cells in EGTA are expressed as ratios of the cell water space. The catecholamine in the supernatant from the cells in EGTA (○) and from the aliquots challenged with Ca^{2+} (◇) is expressed as a percentage of the total in suspension. Temperature, 19°C

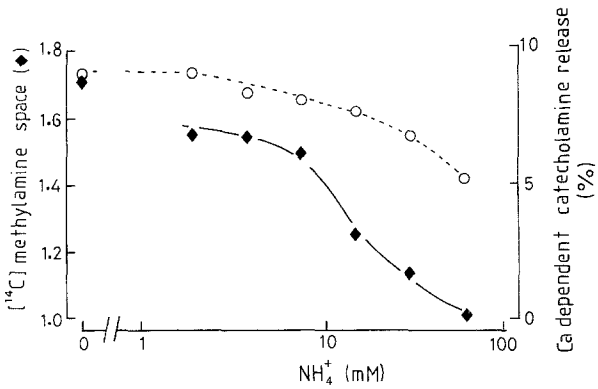

Fig. 5. NH_4^+ dependence of methylamine space and evoked catecholamine release. Cells in K glutamate medium containing 5 mM MgATP, 0.8 mM EGTA, pH 6.6, were rendered leaky by 10 exposures of 2 kV cm^{-1}, τ = 200 μsec, incubated with ^3H_2O (20 μCi ml^{-1}) and ^{14}C-methylamine (4 μCi ml^{-1}) for a few minutes before aliquots were diluted into equal volumes of buffer containing various concentrations of $(NH_4)_2SO_4$. After 15 min incubation, aliquots were challenged with 10 mM Ca-EGTA buffer (calculated 10^{-5} M Ca^{2+}) and centrifuged 5 min later. The catecholamine released as a result of raising the Ca^{2+} to 10^{-5} M is shown in the ordinate (○) and is expressed as a percentage of the total cellular content. The methylamine space of the cells in the EGTA medium (◆) is expressed as a ratio of the water space. Temperature, 19°C

than chromaffin granules. The increase in thiocyanate space and potential in the presence of ammonium or monensin probably results from alkalinization of the granule core leading to an increased inward rate of proton pumping.

Figure 3*b* shows that the thiocyanate space is little affected by alterations in free Ca^{2+} over the range that activates the exocytotic release of catecholamines but, as in the case of the methylamine space, a significant reduction occurs when large amounts of catecholamine are released. These data are consistent with thiocyanate being accumulated in the chromaffin granules.

Calcium-Dependent Exocytosis and Chromaffin Granule pH

Experiments at 20°C

Exposure of 'leaky' cells to concentrations of ammonium that strongly alkalinize the vesicle core fails to block Ca-dependent exocytosis (Fig. 4). The result of an experiment involving different concentrations of ammonium is shown in Fig. 5. It reveals a small reduction in Ca-dependent exocytosis at concentrations of ammonium that greatly reduce the pH gradient between chromaffin granule core and cytosol. An alternative protocol is to lower cy-

Table 2. (A) Thiocyanate spaces determined under different experimental conditions, and (B) membrane potential derived from these spaces[a]

(A) [^{14}C]-SCN/^3H_2O space

	pH_o 6.6	pH_o 7.4
Control	4.0 ± 0.2 (41)	2.8 ± 0.2 (10)
'0' ATP	1.6 ± 0.1 (9)	—
30 mM NH_4^+	—	8.3 ± 0.9 (4)
10^{-5} M monensin	—	8.3 ± 0.3 (4)
2×10^{-4} M trimethyl tin	1.2 ± 0.05 (18)	1.4 ± 0.1 (4)
10^{-5} M FCCP	1.26 ± 0.05 (23)	1.23 ± 0.04 (4)

(B) Calculated vesicle membrane potential relative to the cytosol (mV)

	pH_o 6.6	pH_o 7.4
Control	78 ± 2 (41)	68 ± 2 (10)
'0' ATP	42 ± 4 (9)	—
30 mM NH_4^+	—	101 ± 3 (4)
10^{-5} M monensin	—	102 ± 1 (4)
2×10^{-4} M trimethyl tin	23 ± 3 (17)	31 ± 5 (10)
10^{-5} M FCCP	16 ± 5 (23)	25 ± 3 (4)

[a] Data expressed as mean ± SEM; number of determinations in parentheses. Temperature, 20°C.

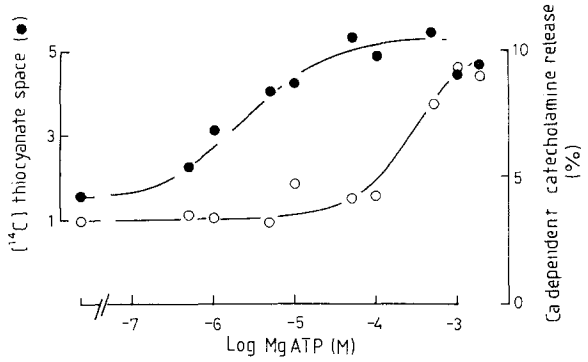

Fig. 6. ATP dependence of thiocyanate space and evoked secretion. Cells in K glutamate medium containing 0.4 mM EGTA, pH 6.6 (calculated Ca^{2+} 5 × 10^{-8} M) but with the omission of MgATP, were subjected to 10 exposures of 2 kV cm^{-1}, τ = 200 μsec, washed twice and resuspended in a similar medium containing [^{14}C]-SCN (10 μCi ml^{-1}) and ^3H_2O (40 μCi ml^{-1}). Aliquots of the cell suspension were immediately diluted into equal volumes of buffer to give final (calculated) Mg ATP levels as shown in the abscissa. After 10 min aliquots of these suspensions were challenged with 10 mM Ca-EGTA (calculated final 10^{-5} M Ca^{2+}) and 5 min later centrifuged. The catecholamine released as a result of raising the Ca^{2+} from 5 × 10^{-8} M to 10^{-5} M is expressed (○) as a percentage of the total cellular content, and the thiocyanate space of the cells held at 5 × 10^{-8} M Ca^{2+} (●) expressed as a ratio of the water space. Temperature throughout, 37°C

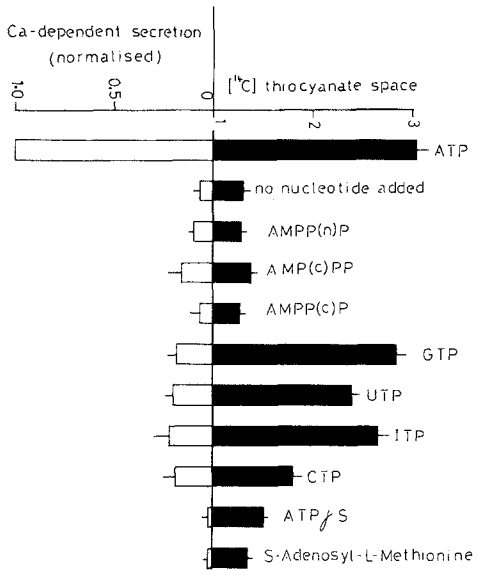

Fig. 7. Comparison of the nucleotide specificity of secretion and vesicular proton pumping in 'leaky' adrenal cells. *Proton pump:* Cells were rendered 'leaky' in the K glutamate based medium (*see* Materials and Methods) containing approximately 5 × 10^{-8} M Ca^{2+}, 1 mM KCN, 1 mM 2 deoxyglucose in place of the 5 mM glucose, and no MgATP. After repeated washes the cells were diluted into a similar medium to give 3 mM of the Mg nucleotide indicated. Proton pumping was measured by the thiocyanate space in the cells after 25 min incubation at 37°C (mean SEM of 4 determinations). *Secretion:* Cells were rendered 'leaky' in the glutamate based medium (*see* Materials and Methods) containing approximately 5 × 10^{-8} M Ca^{2+} and no MgATP. They were diluted into a similar medium to give 4 mM of the Mg nucleotide indicated and incubated for 10 min at 37°C before being challenged with 10^{-5} M Ca^{2+}. The Ca-dependent catecholamine release is expressed relative to the response seen in the presence of Mg ATP (mean std of between 4 and 6 determinations). The figure was partly drawn from data of Table 7, Knight & Baker, 1982

tosolic pH until it equals that of the vesicle core. This occurs at about pH_o 5.8, and at this pH Ca-dependent exocytosis can still be demonstrated although its extent is much reduced.

The conclusion from this series of experiments is that Ca-dependent exocytosis is little affected by the absolute value of the pH of the vesicle core or by the magnitude of the H^+ ion gradient across the limiting membrane of the secretory vesicle (*see also* Fig. 11*a*). Under conditions where the vesicle pH gradient is largely collapsed, there is no detectable alteration in the shape of the Ca-activation curve of exocytosis (Fig. 10*b*).

Experiments at 37°C. Exposure of 'leaky' cells to ammonium at 37°C results in a large (up to 20%) Ca-independent release of catecholamine. Addition of calcium evokes further catecholamine release, but the large Ca-independent release makes quantitative analysis rather difficult. We have not established the nature, whether exocytosis or not, of the two components of catecholamine release observed under these conditions.

Calcium-Dependent Exocytosis and Chromaffin Granule Membrane Potential Experiments at 20°C

Dependence on ATP and Other Nucleotides

Both Ca-dependent exocytosis and the accumulation of [^{14}C]-SCN depend on Mg-ATP, but they differ markedly in their sensitivity to added nucleotides (Fig. 6). [^{14}C]-SCN accumulation seems to require only micromolar amounts of ATP, whereas millimolar concentrations are needed for exocytosis. There are also differences in the nucleotide specificity of the two processes (Fig. 7). Calcium-dependent exocytosis is very specific for Mg-ATP, whereas ATP, GTP, UTP and ITP are almost as effective at activating [^{14}C]-SCN accumulation.

Inhibition of the Granule Proton Pump

Thiocyanate accumulation is strongly inhibited by trimethyl tin which inhibits proton pumping in isolated chromaffin vesicles and collapses the vesicle membrane potential. Figure 8 shows that Ca-dependent exocytosis persists under conditions where the proton pump is inhibited, although high concentrations of trimethyl tin do bring about a small reduc-

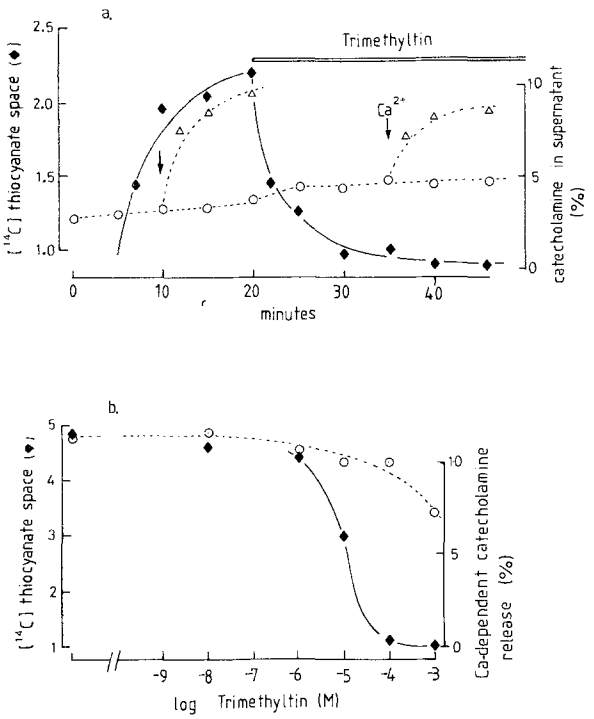

Fig. 8. Effect of trimethyl tin on Ca-dependent release of catecholamine and the [^{14}C] SCN^- space. (*a*): Cells in K glutamate medium (Materials and Methods) containing 5 mM MgATP, pH 6.6, 0.4 mM EGTA (calculated Ca^{2+} or 5×10^{-8} M) were subjected to 10 exposures of 2 kV cm^{-1}, τ = 200 μsec, and 5 min later trace amounts of [^{14}C]-SCN^- and ^3H_2O were added. After a further 5 min an aliquot of the cell suspension was challenged with 10 mM Ca-EGTA corresponding to 10^{-5} M Ca^{2+}. 15 min after the radiolabels were added, the cells were incubated in 2×10^{-4} M trimethyl tin and 15 min later aliquots of the cell suspension were similarly challenged with 10^{-5} M Ca^{2+}. The catecholamine released in response to these challenges are shown (△), as are the catecholamine levels in the supernatant of unstimulated cells (○) and the corresponding thiocyanate spaces (♦) of these unstimulated cells. Temperature, 20°C. (*b*): Cells in K glutamate and containing trace amounts of [^{14}C]-SCN^- and ^3H_2O, as described in *a* above, were rendered leaky and incubated in various concentrations of trimethyl tin for 15 min before being challenged with Ca-EGTA buffers corresponding to 10^{-5} M Ca^{2+}. The catecholamine released over 15 min in response to these Ca^{2+} challenges are shown (○) and are expressed as a percentage of the total cellular content. The [^{14}C]-SCN space (♦) of the unstimulated cells was measured at the same time. Temperature, 20°C

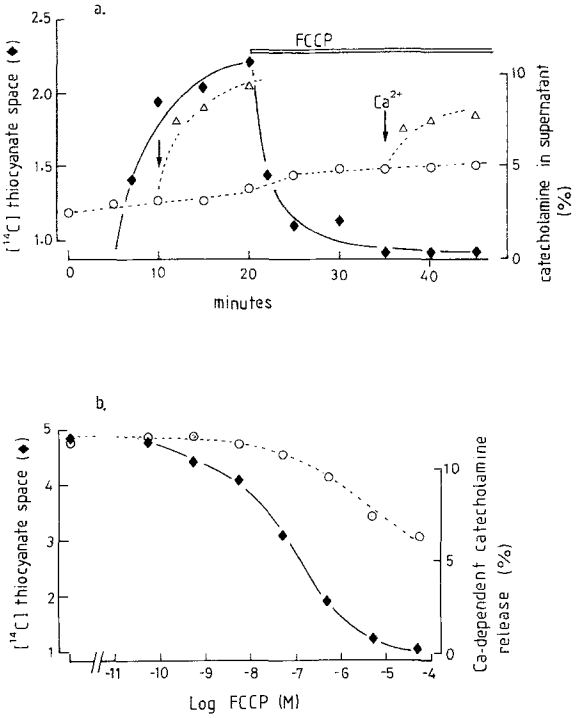

Fig. 9. Effect of FCCP on Ca-dependent catecholamine release and thiocyanate spaces. (*a*): Cells in K glutamate medium containing 5 mM MgATP, 0.4 mM EGTA (calculated Ca^{2+} less than 5×10^{-8} M) pH 6.6, were subjected to 10 exposures of 2 kV cm^{-1}, τ = 200 μsec, and 5 min later trace amounts of [^{14}C] SCN^- and ^3H_2O were added. After a further 5 min an aliquot of the cell suspension was challenged with 10 mM Ca-EGTA (calculated 10^{-5} M Ca^{2+}). 15 min after the radio label was added the cells were incubated in 2×10^{-4} M FCCP, and 15 min into this incubation aliquots of the cell suspensions challenged with 10 mM Ca-EGTA (calculated ~10^{-5} M Ca^{2+}). The catecholamine in the supernatant of the cell suspension containing EGTA (○), and the [^{14}C] SCN^- spaces (♦) corresponding to these same cells are shown. The catecholamine released as a result of the Ca^{2+} challenges (△) are also shown. Temperature, 20°C. (*b*): Cells in K glutamate medium and containing [^{14}C]-SCN and ^3H_2O as described in *a* were rendered leaky and incubated for 15 min in various concentrations of FCCP, before being challenged with 10 mM Ca-EGTA corresponding to 10^{-5} M Ca^{2+} for a further 15 min. The catecholamine released as a result of this Ca^{2+} challenge is shown (○) and is expressed as a percentage of the cellular content. The [^{14}C]-SCN^- spaces of the unchallenged cells were measured at the same time (♦). Temperature, 20°C

tion in Ca-dependent exocytosis. The trimethyl tin concentration required for half-maximal inhibition of [^{14}C]-SCN accumulation in 'leaky' cells is close to 10^{-5} M.

Effect of the H-Ionophore FCCP

Exposure to the H-ionophore FCCP should collapse or even reverse the chromaffin granule membrane potential. Figure 9 shows that Ca-dependent exocytosis persists in the presence of FCCP concentrations that collapse the potential. As with trimethyl tin, very high concentrations of FCCP also bring about a small reduction in Ca-dependent exocytosis, but it is not clear whether this results solely from collapse of the membrane potential or from some other, nonspecific, action of FCCP.

The Calcium Activation Curve for Exocytosis of Depolarized Vesicles

Figure 10*a* summarizes experiments in which the Ca-dependence of exocytosis in 'leaky' cells was

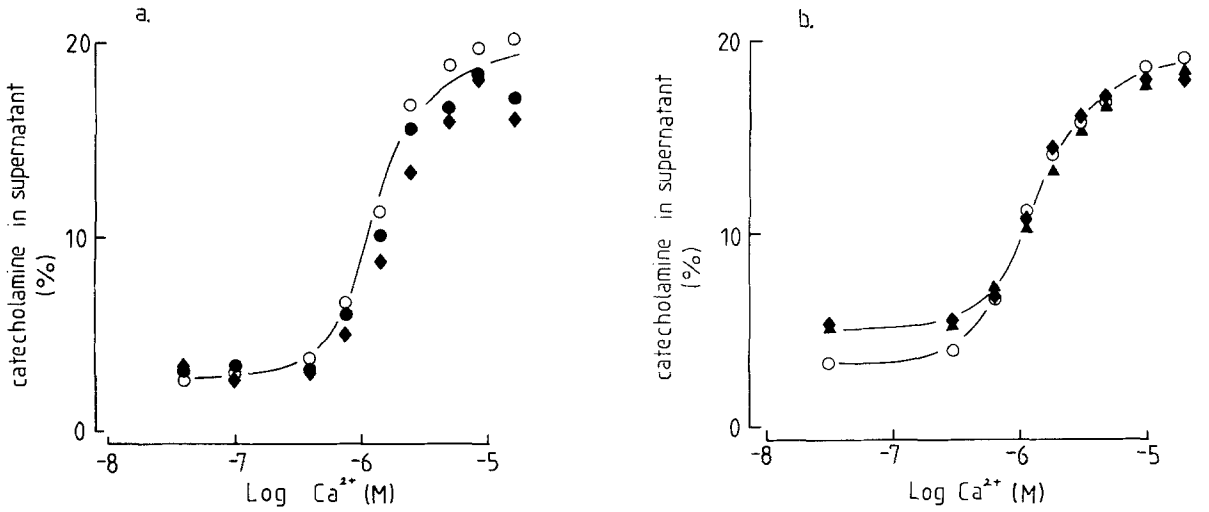

Fig. 10. Ca-dependence of catecholamine secretion under conditions that dissipate granular membrane potentials, and/or pH gradients. (*a*): Cells in K glutamate medium (*see* Materials and Methods) containing 0.5 mM EGTA and 5 mM MgATP at pH 6.6 (calculated $Ca^{2+} \sim 5 \times 10^{-8}$ M) were subjected to 10 exposures of 2 kV cm^{-1}, $\tau = 200$ μsec, and incubated with either 8 μM FCCP (●), or 8 μM FCCP together with 80 μM trimethyl tin and 24 mM NH_4^+ (SO_4^{2-} salt) (◆), for 7 min before being challenged with Ca-EGTA buffers. After a further 15 min the catecholamine in the supernatant was measured. Cells with no additives (○). Temperature, 20°C. (*b*): Cells in K glutamate medium (*see* Materials and Methods) and containing 0.5 mM EGTA, 5 mM MgATP, pH 6.6 ($Ca^{2+} \sim 5 \times 10^{-8}$ M) were rendered leaky as described in *a* above and incubated with 30 mM NH_4^+ for 5 min (▲, Cl^- salt; ◆, sulphate salt) before being challenged with Ca-EGTA buffers for a further 15 min. Cells without additions (○). Temperature, 18°C

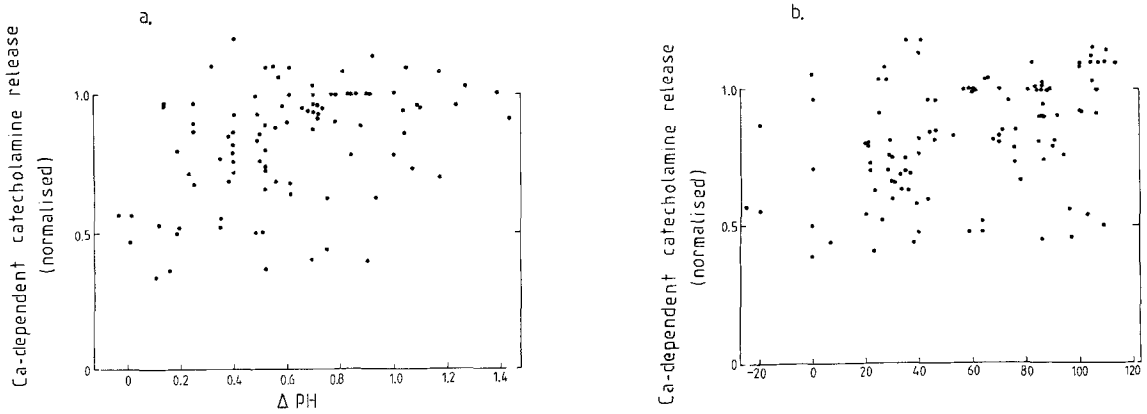

Fig. 11. Summary of the dependence of exocytosis on pH and potential gradient across the granule membrane. Cells in K glutamate medium (*see* Materials and Methods) containing 2–5 mM MgATP and trace amounts of ^3H_2O and (*a*) [^{14}C] methylamine or (*b*) [^{14}C] thiocyanate were rendered leaky and incubated in various agents (*see* text and other figures) that alter the methylamine or thiocyanate spaces. The catecholamine release from these cells in response to an optimal Ca^{2+} challenge is expressed as a ratio to that released from cells of the same batch not incubated with additives. The methylamine and thiocyanate spaces of the cells not challenged with Ca^{2+} were measured and the intragranular pH and potential calculated as described in Methods. (*a*): The difference between the cytosolic pH and intragranular pH (ΔpH) is plotted against the secretory response. The correlation coefficient between these variables is 0.43 (n = 97). (*b*): The granule membrane potential (ψ_m) is plotted against the secretory response. The correlation coefficient is 0.34 (n = 106)

determined in the presence of agents that collapse or reverse the granule membrane potential. The calcium-activation curve is not altered significantly by these agents.

Conclusion

These experiments show that calcium-dependent exocytosis is not obviously dependent on the magnitude or direction of the chromaffin granule *trans* membrane potential (*see also* Fig. 11*b*).

Experiments at 37°C. At 37°C the thiocyanate space is also collapsed by FCCP and trimethyl tin, but at this temperature these agents tend to increase the Ca-independent release of catecholamine much as is seen with ammonium. A Ca-dependent release of catecholamine is, however, still apparent under these conditions of elevated basal release.

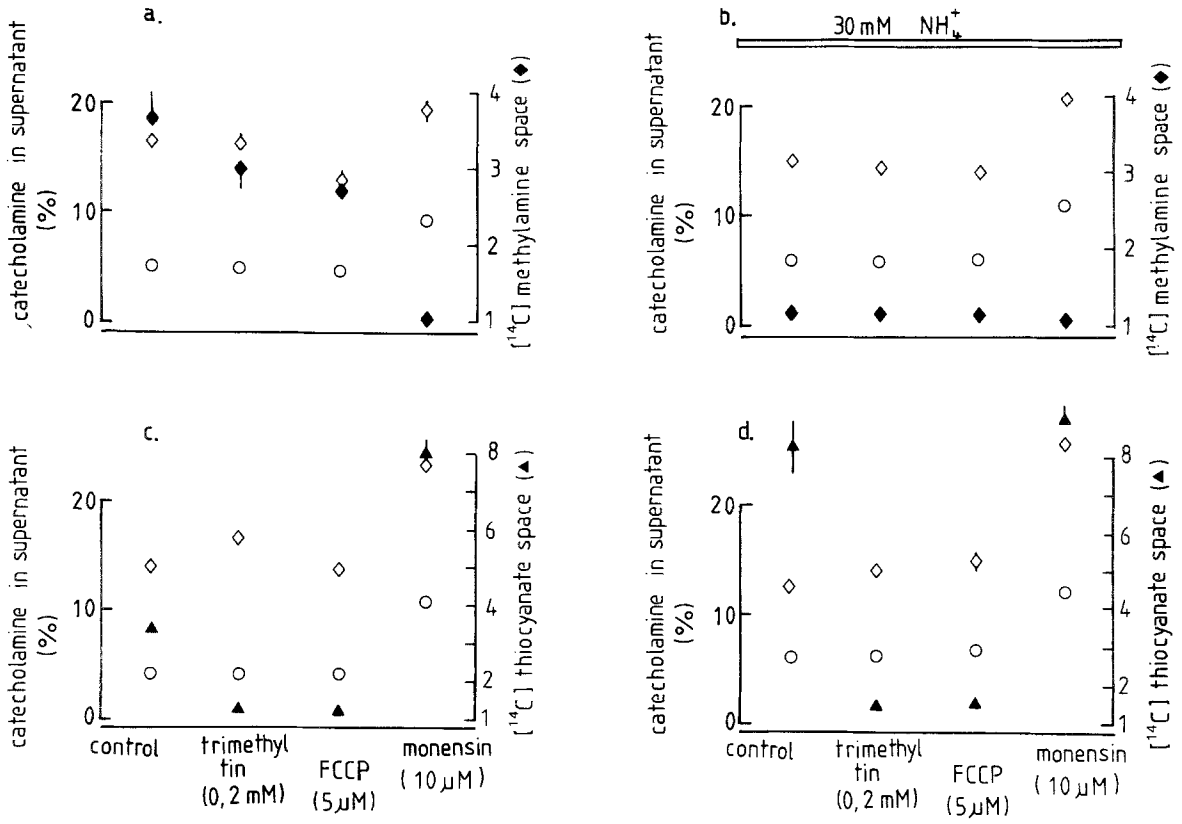

Fig. 12. Alteration in vesicular membrane potential and pH in the presence of FCCP, trimethyl tin, monensin, and ammonium and their effect on basal and evoked release of catecholamine. Cells in a medium of (mM): K glutamate, 75; Na glutamate, 75; HEPES, 20; EGTA, 0.4; glucose, 5; Mg acetate, 2; MgATP, 2; pH 7.4, were exposed to 10 electric fields 2 kV cm^{-1}, τ = 200 μsec and trace amounts of ^3H_2O (10 μCi ml^{-1}) together with [^{14}C]-SCN^{-1} (2 μCi ml^{-1}) or [^{14}C]-methylamine (2 μCi ml^{-1}) added. After a few minutes the cells were incubated either alone or with 2×10^{-4} M trimethyl tin, 5 μM FCCP, or 10 μM monensin, all in the presence (*b*, *d*) or in the absence (*a*, *c*) of 30 mequiv NH_4^+ (sulphate salt used). After 15 min incubation, aliquots were challenged with $CaSO_4$ (to give a final 0.2 mM Ca^{2+}) for a further 15 min before being centrifuged. The methylamine spaces (◆) and thiocyanate spaces (▲) correspond to the cells held in EGTA and are expressed as a ratio of the water space. The catecholamine in the supernatant of the cells in EGTA (○) and the cells challenged with Ca^{2+} (◇) are expressed as a percentage of the total in suspension. Temperature, 20°C. Data points reflect the mean and SEM of 4 determinations

Calcium-Dependent Exocytosis with Both ΔpH and ψ_m Close to Zero

By combining conditions that collapse the pH gradient (ammonium) with those that reduce or reverse the chromaffin granule potential (trimethyl tin and FCCP, respectively), it is possible to examine exocytosis in the virtual absence of both ΔpH and ψ_m. The results of such experiments are summarized in Figs. 10 and 12 and show that Ca-dependent exocytosis persists, both in terms of Ca sensitivity and extent, in the virtual absence of ΔpH and ψ_m, and also under conditions where ψ_m is increased (monensin and ammonium).

Discussion

This series of experiments appears to permit a rather simple conclusion: that Ca-dependent exocytosis in bovine adrenal medullary cells is little affected by the magnitudes of the secretory vesicle pH gradient and potential. This conclusion is, however, heavily dependent on the interpretation of [^{14}C]-SCN and [^{14}C]-methylamine spaces in terms of the potential and internal pH of the secretory vesicles. While contributions to these spaces from compartments other than secretory vesicles—for instance lysosomes—cannot be excluded, the close agreement between the properties of isolated chromaffin granules (Pollard et al., 1976; Johnson & Scarpa, 1976*a*; Casey et al., 1977; Phillips & Allison, 1978; Salama et al., 1980; Holz et al., 1983) with those of the thiocyanate and methylamine spaces in 'leaky' cells strongly supports the view that the secretory vesicles must make a major contribution to these spaces. Most notable are (i) the agreement between the internal pH and potential of the presumed vesicles in 'leaky' cells and those measured in isolated,

well-characterized vesicle preparations; (ii) the finding that both the acid pH of the isolated secretory vesicles and the methylamine spaces in 'leaky' cells are largely abolished by exposure to ammonium and monensin in the same concentration range, and (iii) the potential in isolated vesicles and the thiocyanate space in 'leaky' cells are collapsed by trimethyl tin or FCCP again in the same range of concentrations. In addition, maintenance of the thiocyanate space requires ATP, for which it displays a high affinity but quite broad nucleotide specificity. Both these features are also characteristic of the proton pump in isolated chromaffin granules (Pollard et al., 1976).

For these reasons, we feel fairly confident that the methylamine and thiocyanate spaces contain a significant contribution from the chromaffin granules and, as Ca-dependent exocytosis persists with both methylamine and thiocyanate spaces close to the $[^3H]$-H_2O space, it would appear that the chromaffin granule pH gradient and potential are not essential for Ca-dependent exocytosis. Holz et al. (1983) have recently reached a similar conclusion for the role of chromaffin granule pH in exocytosis from intact cells. Our results do, however, show a small, and in a few cases up to 50%, reduction in exocytosis in the presence of high concentrations of the various agents used to collapse or modify pH_i and ψ_m—suggesting that the secretory vesicle pH and potential may play a small modulating role in exocytosis.

Out data provide no evidence for a role, if any, of the plasma membrane potential in exocytosis. Because of the existence of electrically generated holes in the plasma membrane of 'leaky' cells, the potential across their limiting membrane is presumably close to zero, a condition that is confirmed by the use of voltage-sensitive dyes. Whether the kinetics of Ca-dependent exocytosis will prove to be different at plasma membrane potentials other than zero will have to await the measurement of exocytosis under conditions of voltage clamp.

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