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# CALCIUM REQUIREMENTS FOR SECRETION IN BOVINE CHROMAFFIN CELLS

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SUMMARY

1. Measurements of membrane capacitance and intracellular $Ca^{2+}$ concentration, $[Ca^{2+}]_i$, were used to examine the $Ca^{2+}$ dependence of secretion in single adrenal chromaffin cells.

2. Intracellular dialysis of $Ca^{2+}$, through a patch pipette, promoted secretion; the rate of secretion increased monotonically as $[Ca^{2+}]_i$ was elevated, while the total amount of secretion reached a maximum at 1·5 $\mu$M-$Ca^{2+}$ and declined at high $[Ca^{2+}]_i$.

3. Release of $Ca^{2+}$ from internal stores, using bradykinin or ionomycin, transiently elevated $[Ca^{2+}]_i$ and the rate of secretion.

4. Considering responses to both $Ca^{2+}$ dialysis and release from internal stores, it appears that the rate of secretion increases over a range of $[Ca^{2+}]_i$ levels above 0·2 $\mu$M and saturates at concentrations greater than 10 $\mu$M, if at all. Secretion appears to have a Hill coefficient for $Ca^{2+}$ of about 2. At $[Ca^{2+}]_i$ greater than 1–2 $\mu$M, prolonged elevation of $[Ca^{2+}]_i$, via dialysis, produced lower rates of secretion than transient elevation of $[Ca^{2+}]_i$ caused by release from internal stores. This may have been caused by a depletion of readily releasable chromaffin granules during prolonged elevation of $[Ca^{2+}]_i$.

5. Brief depolarizing pulses produced transient rises in both $[Ca^{2+}]_i$ and the rate of secretion. The ability of these pulses to evoke secretion 'washed out' during prolonged intracellular dialysis, due to both reduced $Ca^{2+}$ influx and a diminished ability of the cell to secrete in response to a given $Ca^{2+}$ load.

6. The kinetics of the secretory response depended upon the size of the depolarization-induced $Ca^{2+}$ load; small rises in $[Ca^{2+}]_i$ increased membrane capacitance only during the depolarization, while larger rises in $[Ca^{2+}]_i$ produced increases both during and following the depolarization. The secretory responses that outlasted the depolarization appeared to be due to persistent elevation of $[Ca^{2+}]_i$. Secretory responses were sometimes followed by a slower decline in membrane capacitance, probably due to endocytosis of membrane.

7. Comparison of the rates of secretion measured during depolarization to those produced by $Ca^{2+}$ dialysis or release from internal stores suggests that $[Ca^{2+}]_i$ at secretory sites can exceed 10 $\mu$M during depolarization. The spatially averaged

measurements of $[Ca^{2+}]_i$ indicate much smaller levels of $[Ca^{2+}]_i$; thus, there must be pronounced spatial gradients of $[Ca^{2+}]_i$ during depolarization.

## INTRODUCTION

Although numerous intracellular signals regulate secretion (Gomperts, 1986; Knight, 1988; Penner & Neher, 1988; Man-Song-Hing, Zoran, Lukowiac & Haydon, 1989), in many cases calcium ions are a key signal in the triggering of exocytosis (Douglas, 1968; Katz, 1969; Augustine, Charlton & Smith, 1987). Very little is known about the molecular mechanisms that allow $Ca^{2+}$ ions to promote exocytosis. Knowledge of the $Ca^{2+}$ requirements of secretion may provide an important clue toward identifying the molecules that mediate exocytosis (Creutz, Drust, Martin, Kambouris, Snyder & Hamman, 1988; Smith & Augustine, 1988; Burgoyne, 1990).

Two different technical approaches have been used to determine the $Ca^{2+}$ requirements of secretion. Studies of permeabilized secretory cells have shown that secretion is triggered by relatively low intracellular $Ca^{2+}$ concentrations ($[Ca^{2+}]_i$), typically in the order of 1 $\mu$M (Knight, 1988). Consistent with this view, direct measurements of $[Ca^{2+}]_i$ indicate levels in the order of 1 $\mu$M during secretion (e.g. Knight & Kesteven, 1983; O'Sullivan, Cheek, Moreton, Berridge & Burgoyne, 1989). Alternatively, theoretical predictions of the spatiotemporal properties of the $[Ca^{2+}]_i$ changes in the immediate vicinity of release sites predict that $[Ca^{2+}]_i$ at release sites can reach concentrations in the order of 10–100 $\mu$M, due to steep spatial gradients undetectable with available methods for measuring $[Ca^{2+}]_i$ (e.g. Simon & Llinas, 1985; Fogelson & Zucker, 1985; Smith & Augustine, 1988; Sala & Hernandez-Cruz, 1990; Roberts, Jacobs & Hudspeth, 1990). Although these two views are not necessarily contradictory, they none the less make very different predictions about the $Ca^{2+}$ requirements of exocytosis.

One means of reconciling these divergent views is to propose that while secretion can occur at the low levels of $[Ca^{2+}]_i$ typically used to evoke secretion from permeabilized cells (i.e. around 1 $\mu$M), the kinetics of secretion may be accelerated by higher $Ca^{2+}$ concentrations (Zimmerberg, Sardet & Epel, 1985). Such an increase in secretory rate might not be detected with the slow measurement techniques often applied to permeabilized cells. Therefore, it is possible that cells that produce rapid secretion in response to physiological stimuli, such as neurons (Llinas, Steinberg & Walton, 1981; Augustine, Charlton & Smith, 1985), chromaffin cells (Neher & Marty, 1982; Clapham & Neher, 1984; Schweizer, Schäfer, Tapparelli, Grob, Karli, Heumann, Theonen, Bookman & Burger, 1989) and anterior pituitary cells (Thomas, Suprenant & Almers, 1990), do so by elevating $[Ca^{2+}]_i$ to levels higher than those used in experiments on permeabilized cells.

We address this possibility by characterizing the $Ca^{2+}$ dependence of the rate of secretion from chromaffin cells of the bovine adrenal gland. In these cells, the whole-cell patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) can be used to measure membrane capacitance as an assay for secretion (Neher & Marty, 1982) and to deliver both $Ca^{2+}$ and $Ca^{2+}$-sensitive indicator dyes (Neher, 1989), permitting simultaneous measurement of the kinetics of secretion and $[Ca^{2+}]_i$ changes in single cells. A similar approach has recently been applied to anterior pituitary cells by Thomas *et al.* (1990). With such measurements we observe that the

rate of secretion does depend upon $[Ca^{2+}]_i$, but that elevating $[Ca^{2+}]_i$ by dialysis of $Ca^{2+}$ buffers or by release from internal stores renders an apparent $Ca^{2+}$ sensitivity very different from that observed during brief depolarizing pulses. Comparison of these measurements indicates that depolarization locally elevates $[Ca^{2+}]_i$ at the release sites to levels well above 10 $\mu$M. A preliminary report of some of this work has appeared (Augustine & Neher, 1990).

## METHODS

Experiments were performed on primary cultures of chromaffin cells from bovine adrenal gland, prepared as described previously (Marty & Neher, 1985). Cells were used starting 1 day after dissociation and a given culture was used until the cells no longer had the spherical morphology required for adequate spatial control of membrane potential (usually up to 5 days after dissociation). During experiments, cells were bathed in a solution containing (in mM): 120 NaCl, 5 (or occasionally 2) $CaCl_2$, 20 tetraethylammonium (TEA) chloride, 2 $MgCl_2$, 50 glucose and 10 Na–HEPES (pH 7·2). Tetrodotoxin (1 $\mu$M) was usually added to this solution to block current flowing through $Na^+$ channels. Drugs such as bradykinin and ionomycin (Calbiochem) were locally applied onto single cells by applying positive pressure pulses to a pipette containing the drugs. All experiments were performed at room temperature (22–26 °C).

### *Electrophysiological measurements*

Conventional whole-cell patch clamp methods were used to measure and control the membrane potential of single cells, as well as to measure transmembrane currents and to control the ionic composition of the cell interior (Hamill *et al.* 1981). Our standard solution was designed to optimize measurement of currents flowing through voltage-gated $Ca^{2+}$ channels. It contained (in mM): 145 caesium glutamate, 8 NaCl, 1 $MgCl_2$, 2 Mg-ATP, 0·3 GTP, 0·5 $K_2$–EGTA and 10 Na–HEPES (pH 7·2). Occasionally 20 mM-TEA-Cl was substituted for an equimolar amount of caesium glutamate, to maximize blockade of $K^+$ channels. Salts were obtained from Merck, CsOH (used for preparation of caesium glutamate) was from Aldrich Chemical Co. and other reagents were from Sigma Chemical Co., unless otherwise indicated. The annexin protein, p36, was obtained from Dr Volker Gerke (Max Planck Institute, Göttingen) and was prepared as described in Gerke & Weber (1984).

In order to manipulate $[Ca^{2+}]_i$, solutions buffered to various $Ca^{2+}$ concentrations were prepared using the $Ca^{2+}$ buffers, EGTA, *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA) or nitrilotriacetic acid (NTA) (Table 1, top). The procedure used to titrate these buffers with $Ca^{2+}$ is described in Neher (1988). Free $Ca^{2+}$ concentrations produced by these mixtures were calculated using the procedures described in Fabiato & Fabiato (1979), assuming $Ca^{2+}$ dissociation constants of 0·15 $\mu$M for EGTA, 3·16 $\mu$M for HEDTA and 118 $\mu$M for NTA, and $Mg^{2+}$ dissociation constants of 6 mM for EGTA, 2 mM for HEDTA and 1·18 mM for NTA. The actual $[Ca^{2+}]_i$ levels obtained by dialysis of these solutions was measured fluorimetrically, as described below.

The procedures described by Lindau & Neher (1988) were used to measure the capacitance of chromaffin cells. In brief, a sinusoidal voltage signal (800 Hz, 16 mV root mean square) was applied to the cell via the patch pipette and a two-phase lock-in amplifier was used to measure the alternating current (AC) admittance characteristics of the cell. The two orthagonal outputs of the lock-in amplifier, together with the direct current (DC) level, were sampled by a PDP 11/73 computer for on-line calculation of membrane capacitance and series resistance. These calculations were performed at frequencies of 2–10 Hz.

### *Optical measurements of $[Ca]_i$*

Fluorimetric methods were used to measure $[Ca^{2+}]_i$ of chromaffin cells. Fura-2 (Grynkiewicz, Poenie & Tsien, 1985) was used to measure $[Ca^{2+}]_i$ between 0·01 and about 2 $\mu$M, while Furaptra (Raju, Murphy, Levy, Hall & London, 1989; also known as Mag-fura-2) was used for higher $[Ca^{2+}]_i$ levels. Both indicators were purchased from Molecular Probes (Eugene, OR, USA) in their free acid forms and introduced into the cells via dialysis from a patch pipette. The fluorescence of these indicators was measured with the photomultiplier-based system described in Neher (1989), which produces a spatially averaged measure of $[Ca^{2+}]_i$. Fura-2 was excited with light alternated between

360 and 390 nm, while Furaptra was excited with 340 and 390–425 nm light. For experiments with Fura-2, a Leitz 50× water-immersion objective was used, while for Furaptra a Nikon 100× oil-immersion objective was used, along with a quartz collector lens and nosepiece, to optimize transmission of the 340 nm light. Emitted light was transmitted through a 425 nm dichroic mirror and 500–545 nm (or occasionally 500–530 nm) barrier filter before being detected by the photomultiplier.

TABLE 1. Composition of buffered $Ca^{2+}$ solutions used for intracellular dialysis

| $[Ca^{2+}]_{free}$ (μM) | $[Ca^{2+}]_{total}$ (mM) | [Buffer] (mM) | $[Mg^{2+}]_{total}$ (mM) | $[Mg^{2+}]_{free}$ (mM) |
|---|---|---|---|---|
| Ca dependence of exocytosis | | | | |
| 0 | 0 | 10 EGTA | 3 | 0·6 |
| 0·02 | 1 | 10 EGTA | 3 | 0·6 |
| 0·17 | 5 | 10 EGTA | 3 | 0·8 |
| 0·34 | 6·67 | 10 EGTA | 3 | 0·9 |
| 1·5 | 9 | 10 EGTA | 3 | 1·2 |
| 10 | 1·5 | 2 HEDTA | 1·5 | 0·2 |
| 101 | 4·6 | 10 NTA | 2 | 0·2 |
| 9000 | 10 | — | 2 | 1·6 |
| *In vivo* calibration of Furaptra | | | | |
| 0 | 0 | 10 EGTA, 3 NTA | 2 | 0·2 |
| 52 | 3 | 10 NTA | 2·3 | 0·2 |

$[Ca^{2+}]_i$ was calculated from the ratios ($R$) of the light emitted when these dyes were excited by the two excitation wavelengths (Grynkiewicz *et al.* 1985). For each dye, the relationship between $[Ca^{2+}]_i$ and $R$ is:

$$[Ca^{2+}]_i = K_{eff}(R - R_{min})/(R_{max} - R),$$

where $R_{min}$ and $R_{max}$ are the values of $R$ at very low and very high $Ca^{2+}$ concentrations, respectively, and $K_{eff}$ is the effective dissociation constant of the indicator under our particular experimental conditions. This $K_{eff}$ is quite different from the absolute $Ca^{2+}$ dissociation coefficient of the dye; the relationship between these two constants is discussed in Neher & Augustine (1992). To determine the calibration constants, $K_{eff}$, $R_{min}$ and $R_{max}$, we used the *in vivo* procedure described by Neher (1989). In this procedure, the patch pipette is used to dialyse buffered $Ca^{2+}$ solutions and indicators into the cell. In the case of Fura-2, $R_{min}$ and $R_{max}$ were determined from pooled measurements of $R$ in cells dialysed with internal solutions containing either 10 mM-EGTA or 10 mM-$CaCl_2$. These values, plus the value of $R$ in cells dialysed with a solution containing 0·34 μM-$Ca^{2+}$ (Table 1), were inserted into this equation to calculate $K_{eff}$. In the case of Furaptra, the calibration solutions used were somewhat different (see Table 1): the $[Ca^{2+}]_i$ level used to determine $K_{eff}$ was 52 μM (near the dissociation constant for this indicator under these conditions; Raju *et al.* 1989; Baylor, Hollingsworth & Konishi, 1989) and all solutions used with this buffer (except for the 10 mM-$Ca^{2+}$ solution used to saturate the dye) were adjusted to maintain a constant $Mg^{2+}$ concentration (0·2 mM) because this indicator is also sensitive to $Mg^{2+}$ (Raju *et al.* 1989).

At $Ca^{2+}$ levels around 2 μM, both Fura-2 and Furaptra are relatively insensitive to changes in $Ca^{2+}$ concentration. At 2 μM-$Ca^{2+}$, Fura-2 fluorescence is about 90% saturated and fluorescence at 390 nm, the $Ca^{2+}$-sensitive excitation wavelength, is small (Grynkiewicz *et al.* 1985). In chromaffin cells loaded with 100 μM-Fura-2, this limiting fluorescence is only about 4 times as large as the autofluorescence of the cells. Although cellular autofluorescence is taken into account during measurements and calibrations, in unloaded cells approximately half of this autofluorescence is dialysed out of the cells during a whole-cell recording. Thus, it is possible that the contribution of autofluorescence will change during both calibration and experimental measurements on the cells, resulting in some uncertainty in the $[Ca^{2+}]_i$ measuremets. As a worst-case estimate of this error, if it is assumed that the change in autofluorescence is comparable to the average amount of autofluorescence in a chromaffin cell, then this would yield a 25% error in the magnitude of the fluorescence signal at the long excitation wavelength. Given a typical set of calibration constants

($R_{min}$ = 0·53, $R_{max}$ = 96, $K_{eff}$ = 48·8 $\mu$M) this would yield an error in $[Ca^{2+}]_i$ of approximately 29% at 2 $\mu$M free $Ca^{2+}$ concentration. This error could be correspondingly higher in cells with larger amounts of autofluorescence.

The levels of $[Ca^{2+}]_i$ calculated with this method are approximately 2 times higher than those calculated based on *in vitro* calibrations (Fig. 1). This difference presumably occurs because of the effect of cytoplasmic conditions (such as viscosity) on the fluorescence properties of Fura-2

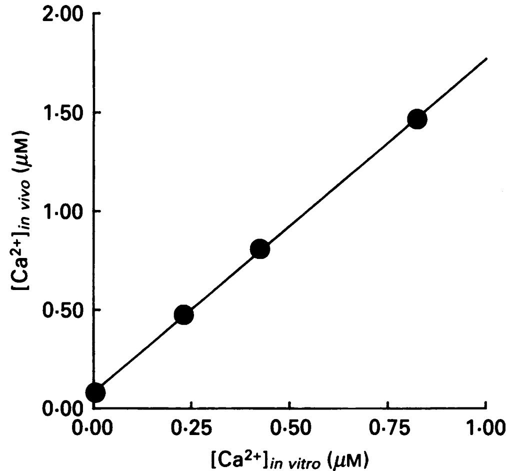


Fig. 1. Comparison of *in vivo* and *in vitro* calibrations of Fura-2 fluorescence ratios. Sets of calibration constants ($R_{min}$, $R_{max}$, $K_{eff}$) were obtained both under *in vivo* conditions, as described in the text, and by placing droplets of the same calibration solution into the recording chamber without cells (an *in vitro* calibration). Cellular Fura-2 ratio signals were then converted into $[Ca^{2+}]_i$ values for both sets of constants. Over the range of fluorescence ratios examined, $[Ca^{2+}]_i$ calculated from the *in vivo* constants, $[Ca^{2+}]_{in\ vivo}$, was approximately twice that determined from the *in vitro* constants, $[Ca^{2+}]_{in\ vitro}$.

(Grynkiewicz *et al.* 1985; Konishi, Olson, Hollingsworth & Baylor, 1988; Neher, 1989). We consider the *in vivo* calibration method to be more accurate because it is actually performed in the cell and, thus, takes cytoplasmic conditions into account. However, this difference must be kept in mind when comparing measurements made with the two calibration procedures.

## RESULTS

The goal of this study was to ask two related questions: (1) how much $Ca^{2+}$ is needed to trigger exocytosis from single chromaffin cells, and (2) how much $Ca^{2+}$ is available to trigger exocytosis when these cells are depolarized. To assay exocytosis, we have measured the changes in membrane capacitance ($C_m$) associated with the incorporation of vesicular membrane during exocytosis (Neher & Marty, 1982; Lindau & Neher, 1988). Intracellular $Ca^{2+}$ concentration, $[Ca^{2+}]_i$, was measured simultaneously by monitoring the fluorescence of Fura-2 or Furaptra in a spatially averaged fashion, with a photomultiplier. We began by quantifying the calcium requirements for exocytosis, using the assumption that $[Ca^{2+}]_i$ is elevated uniformly during dialysis (Oliva, Cohen & Mathias, 1988) so that the measured $[Ca^{2+}]_i$ reflects $Ca^{2+}$ levels at the release sites. We then used our measurements of the $Ca^{2+}$ dependence of the secretory response to estimate $[Ca^{2+}]_i$ at the secretory sites during depolarization.

*Secretion evoked by dialysis of $Ca^{2+}$ ions*

Dialysing micromolar concentrations of $Ca^{2+}$ into a chromaffin cell elevates the rate of exocytosis (Neher & Marty, 1982; Penner, Neher & Dreyer, 1986; Kim & Neher, 1988). An example of the effect of dialysing a single chromaffin cell with an

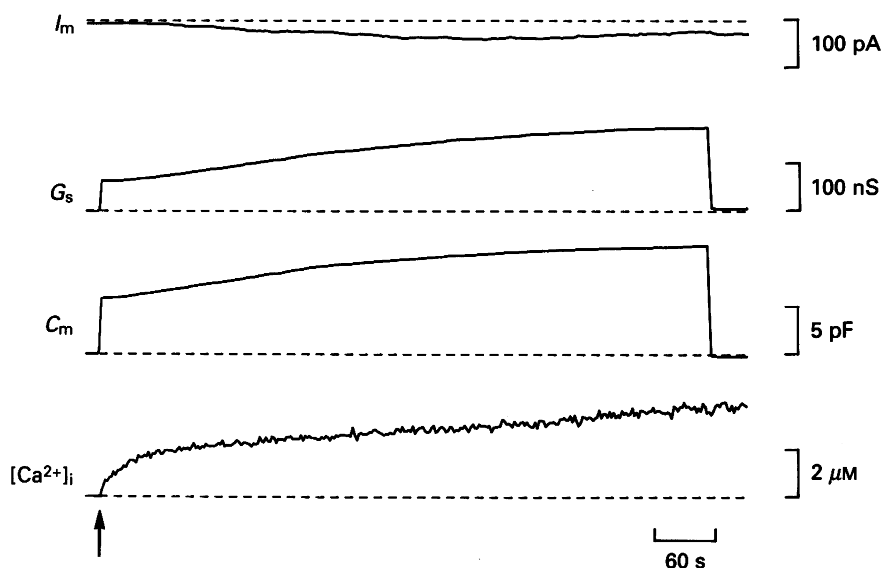


Fig. 2. Secretory activity monitored in a single chromaffin cell during $Ca^{2+}$ dialysis. Establishment of whole-cell recording conditions (at arrow) is indicated by an abrupt jump in measured series conductance ($G_s$) and capacitance ($C_m$). Dialysis caused the development of a small inward current ($I_m$), as well as an elevation of intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and a gradual rise in $C_m$, due to the 1·5 μM free calcium in the dialysis solution. At the end of the experiment the automatic $G_s$ and $C_m$ compensation feature of the EPC-9 patch clamp amplifier was activated to independently measure $C_m$ (10·5 pF) and $G_s$ (168 nS). Dashed lines indicate baselines for each trace.

internal solution buffered to 1·5 μM-$Ca^{2+}$ (containing 9 mM-$Ca^{2+}$ and 10 mM-EGTA) is shown in Fig. 2. In this cell, breakthrough of the patch of membrane separating the pipette lumen from the cell cytoplasm occurred at the arrow. $[Ca^{2+}]_i$ rose to approximately 2 μM within 60 s of breakthrough. The slow, secondary increase in $[Ca^{2+}]_i$ apparent through the rest of the experiment may have been an effect of dialysis-related changes in intracellular viscosity on Fura-2 fluorescence, rather than a genuine increase in $[Ca^{2+}]_i$ (Neher, 1989). Breakthrough was associated with an abrupt rise in the capacitance ($C_m$) signal, reflecting the resting capacitance of the cell, and was followed by a more gradual, sigmoidal increase in $C_m$ up to a sustained level. The increase in $C_m$ above the resting level is due to exocytosis and is triggered by the $Ca^{2+}$ dialysed into the cell. In all subsequent examples of $C_m$ recordings, the resting capacitance of the cell has been subtracted to emphasize the changes in $C_m$ associated with exocytosis.

In the experiment shown in Fig. 2, dialysis also caused a gradual increase in access conductance between the pipette and cytoplasm. In this experiment a small inward

current was also produced, due to a chloride current that is regulated by second messengers and cell swelling (Doroshenko, Penner & Neher, 1991). Whilst either of these changes could be seen in a given experiment, they were not a consistent feature of the recordings.

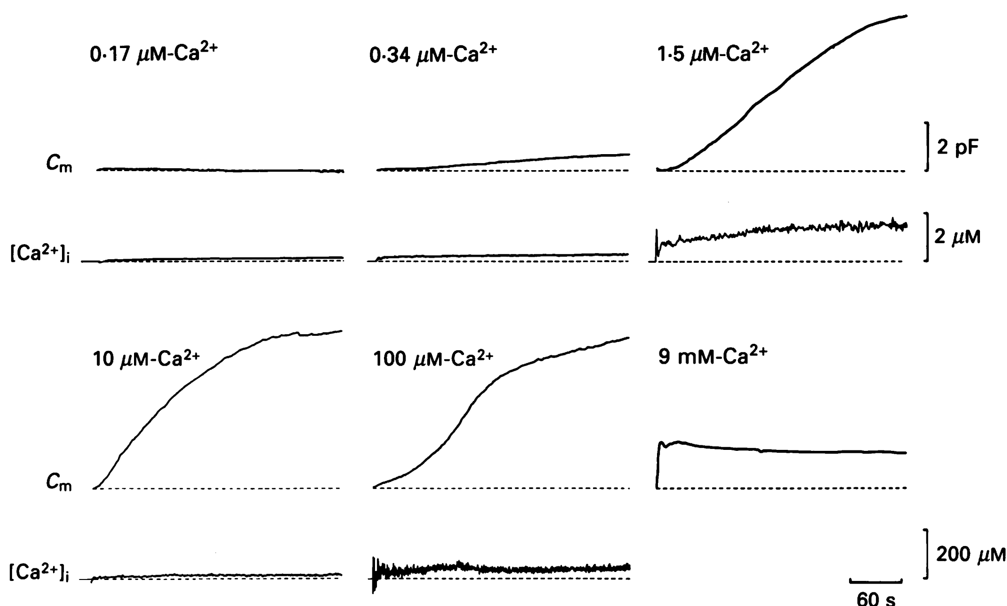


Fig. 3. Changes in $C_m$ induced by dialysis of buffered $Ca^{2+}$ solutions. Elevating $[Ca^{2+}]_i$ produced concentration-dependent changes in the maximum level and rate of rise of $C_m$. $[Ca^{2+}]_i$ was measured with Fura-2 (upper set of records) or Furapatra (lower set of records). Each pair of $C_m$ and $[Ca^{2+}]_i$ records was taken from a different cell dialysed with internal solutions buffered to the indicated $Ca^{2+}$ concentrations. Initial values for $C_m$, measured at breakthrough, are indicated by dashed lines.

Manipulating the concentration of $Ca^{2+}$ within the pipette altered $[Ca^{2+}]_i$ and the resultant changes in $C_m$. In all cases, within 60 s $[Ca^{2+}]_i$ reached a plateau that was close to the $Ca^{2+}$ concentration predicted from the amounts of $Ca^{2+}$ and $Ca^{2+}$ buffer added to the dialysis solution. Examples of responses produced by dialysis of various buffered $Ca^{2+}$ solutions are shown in Fig. 3. $[Ca^{2+}]_i$ levels higher than 0·2 μM produced increases in $C_m$, while lower $[Ca^{2+}]_i$ levels produced no significant increases in $C_m$. In fact, at 0·02 μM-$[Ca^{2+}]_i$, slight decreases in $C_m$ were sometimes observed, presumably due to endocytosis (see also Almers & Neher, 1987; Thomas *et al.* 1990). For levels of $[Ca^{2+}]_i$ that triggered exocytosis, both the total amount of exocytosis, measured as the peak increase in $C_m$, and the rate of exocytosis, measured as the rate of rise in $C_m$, depended upon $[Ca^{2+}]_i$. The peak increase in $C_m$ occurred late in the experiment, usually 10–20 min after beginning dialysis. However, the fastest rate of increase in $C_m$ occurred at about the time that $[Ca^{2+}]_i$ reached a maximum. The experiments using 9 mM-$Ca^{2+}$ in the pipette were exceptions to both of these generalizations; under these conditions the $C_m$ trace usually reached a peak within

the first 10 or 20 s and the most rapid rate of change in $C_m$ occurred even earlier, while $[Ca^{2+}]_i$ presumably was still increasing (in these conditions we could not measure $[Ca^{2+}]_i$ accurately with Fura-2 or Furaptra because of indicator saturation).

The rate of exocytosis increased monotonically as $[Ca^{2+}]_i$ was raised, while the total amount of exocytosis was largest at 1·5 $\mu$M-$[Ca^{2+}]_i$ and declined at very high

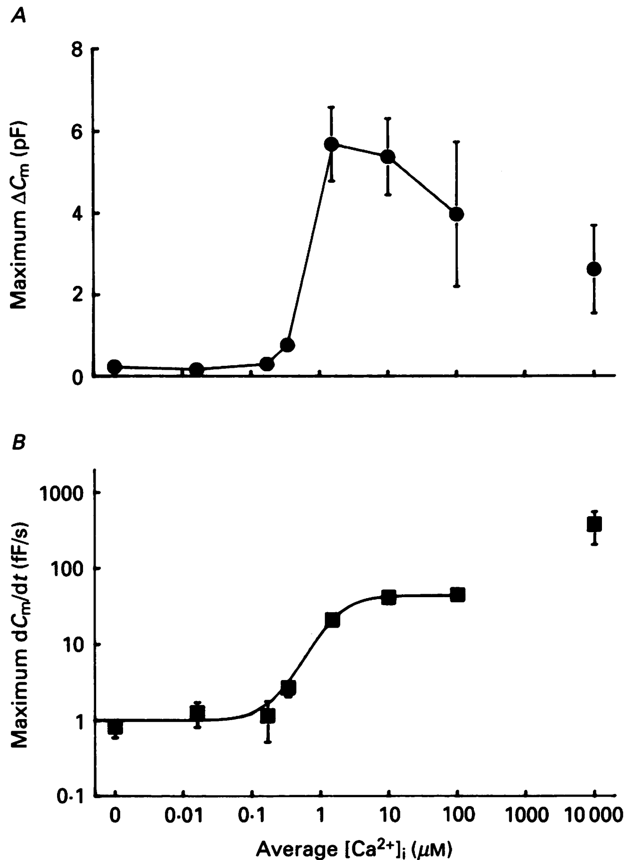


Fig. 4. $[Ca^{2+}]_i$ dependence of capacitance changes induced by $Ca^{2+}$ dialysis. *A*, the peak change in $C_m$ (measured at its maximum, or after 10–20 min of dialysis, whichever occurred first) increased sharply at $[Ca^{2+}]_i$ in the order of 1 $\mu$M and declined at very high $[Ca^{2+}]_i$. *B*, the maximum rate of increase in $C_m$ also increased with elevated $[Ca^{2+}]_i$, reaching a plateau at approximately 10 $\mu$M but increasing still more at very high $Ca^{2+}$. The smooth curve is fitted to the data points measured at 100 $\mu$M or lower $[Ca^{2+}]_i$; it has a Hill coefficient of 1·85 and a $K_D$ for calcium of 1·6 $\mu$M. In both graphs, the points represent mean values determined from 4–17 experiments and the error bars indicate ± S.E.M.

(nominally 9 mM) $[Ca^{2+}]_i$. The quantitative relationships between $[Ca^{2+}]_i$ and these two parameters are shown in Fig. 4. Excluding the point at 9 mM-$Ca^{2+}$, the $[Ca^{2+}]_i$ dependence on the rate of change in $C_m$ could be fitted with a co-operative function, with a Hill coefficient of about 2 and a $K_{Ca}$ of about 2 $\mu$M (smooth curve in Fig. 4*B*). However, the marked elevation in the rate of increase in $C_m$ that occurred with 9 mM-$Ca^{2+}$ indicates that the secretion may saturate at $[Ca^{2+}]_i$ levels much higher than

2 $\mu$M, if at all. The $[Ca^{2+}]_i$ dependence on the maximum change in $C_m$ was more complex, but was a steeper function of $[Ca^{2+}]_i$ and was half-maximal at about 1 $\mu$M-$[Ca^{2+}]_i$ (Fig. 4*A*).

*Secretion evoked by release of $Ca^{2+}$ from internal stores*

While the delivery of $Ca^{2+}$ to cells via the patch pipette is an effective means of elevating $[Ca^{2+}]_i$, it is possible that the resultant continuous elevation of $[Ca^{2+}]_i$ could

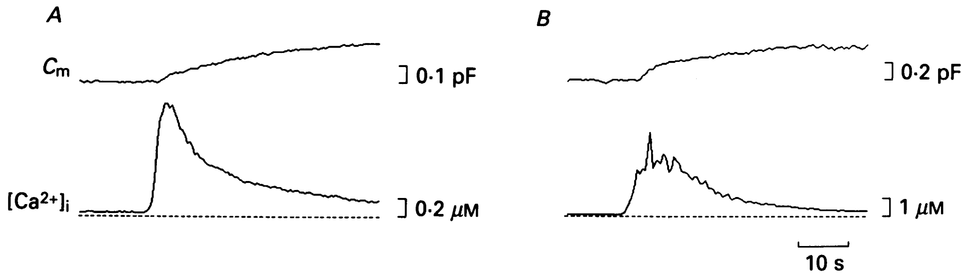


Fig. 5. Secretory activity induced by $Ca^{2+}$ release from internal stores. *A*, changes in $C_m$ and $[Ca^{2+}]_i$ produced by application of 1 $\mu$M-bradykinin onto a chromaffin cell. *B*, changes in $C_m$ and $[Ca^{2+}]_i$ produced by application of ionomycin (1·4 $\mu$M) onto another chromaffin cell bathed in $Ca^{2+}$-free external solution.

have secondary effects upon the secretory response (see Discussion). We therefore tried to produce transient changes in $[Ca^{2+}]_i$ through application of agents that release $Ca^{2+}$ from intracellular stores. In these experiments, we decreased the calcium buffering capacity of the internal solution (0·1 or 0·2 mM-Fura-2, no EGTA) to minimize damping of the $[Ca^{2+}]_i$ signal by exogenous buffers.

Bradykinin is known to release $Ca^{2+}$ from internal stores in these cells, via the generation of the intracellular messenger, $IP_3$ (O'Sullivan & Burgoyne, 1989). Extracellular application of bradykinin (1 $\mu$M) produced a transient rise in $[Ca^{2+}]_i$, up to 2 $\mu$M, and a resultant increase in $C_m$ (Fig. 5*A*). The calcium ionophore, ionomycin (1·4–2·8 $\mu$M), also produced transient rises in $[Ca^{2+}]_i$ and an increase in $C_m$ (Fig. 5*B*). While ionomycin is known to promote $Ca^{2+}$ influx across the plasma membrane, the transient rises in $[Ca^{2+}]_i$ produced by ionomycin application under the conditions used here were not eliminated by removing external $Ca^{2+}$. Thus, it appears that ionomycin can release $Ca^{2+}$ from internal stores in chromaffin cells (Albert & Tashjian, 1984; Stauderman & Pruss, 1989). Indeed, most of these experiments were performed in the absence of external $Ca^{2+}$ to avoid $[Ca^{2+}]_i$ gradients at the membrane.

In contrast to the responses to dialysis of $Ca^{2+}$, release of $Ca^{2+}$ from internal stores never produced an increase in total capacitance larger than 1·5 pF. In ten experiments where the rise in $[Ca^{2+}]_i$ was greater than 1 $\mu$M, the mean total change in $C_m$ was 0·56 ± 0·19 pF (S.E.M.). In many cases, such as in Fig. 5*B*, the rate of rise in capacitance was very high for the first 2 or 3 s of $[Ca^{2+}]_i$ elevation but then dropped despite continued elevation of $[Ca^{2+}]_i$. The rapid initial bout of secretion may be due

to the presence of a limited population of chromaffin granules that are present at the membrane and are rapidly releasable, while the slower late phase of secretion may be limited by a slower mobilization of vesicles toward the release sites at the membrane (Perrin, Langley & Aunis, 1987; Bookman & Schweizer, 1988; Schweizer *et al.* 1989; Eberhard, Cooper, Low & Holz, 1990; Thomas *et al.* 1990).

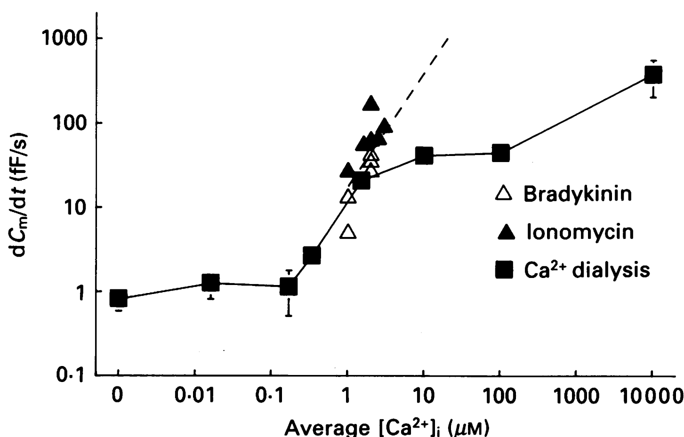


Fig. 6. $[Ca^{2+}]_i$ dependence of capacitance changes induced by release of $Ca^{2+}$ from internal stores. Maximum rates of change in $C_m$ induced by 1 µM-bradykinin (△) and 1·4–2·8 µM-ionomycin (▲). Dashed line has a slope of 2 and is an extrapolation of the data points for responses induced by $Ca^{2+}$ release. For comparison, rates obtained by dialysis of $Ca^{2+}$, taken from Fig. 4*B*, are indicated by ■——■.

The relationship between the changes in $[Ca^{2+}]_i$ and the maximum slope of the increase in $C_m$ caused by these agents is shown in Fig. 6. For $[Ca^{2+}]_i$ levels of 1–2 µM or lower, this relationship was very similar to that found for $Ca^{2+}$ dialysis (Fig. 6, ■). However, for $[Ca^{2+}]_i$ between 2 and 4 µM the response rates exceeded the plateau found for dialysis of $Ca^{2+}$ to similar levels. On logarithmic coordinates, the relationship between $[Ca^{2+}]_i$ and rate of increase in $C_m$ produced by $Ca^{2+}$ release from internal stores was fitted by a function with an exponent of about 2, which is consistent with the Hill coefficient of 2 found with responses to $Ca^{2+}$ dialysis (Fig. 4*B*).

In summary, the $Ca^{2+}$ dependence of membrane capacitance changes apparently depends on the experimental procedures used to elevate $[Ca^{2+}]_i$ and to measure $C_m$. *$Ca^{2+}$ dialysis* produces large and long-lasting rises in $[Ca^{2+}]_i$ and $C_m$, while *release from internal stores* produces transient rises in these parameters. When the *total amount* of secretion is measured (during dialysis), it saturates at $[Ca^{2+}]_i$ levels of a few micromolar and decreases at very high $[Ca^{2+}]_i$. However, when the *rate* of secretion is measured (with either delivery method), it increases as a more-or-less continuous function of $[Ca^{2+}]_i$. With dialysis it reaches a plateau at $[Ca^{2+}]_i$ levels greater than a few micromolar. In all cases, secretion seems to have a Hill coefficient for $Ca^{2+}$ of about 2, suggesting the involvement of multiple $Ca^{2+}$ binding sites in triggering exocytosis.

*Secretion evoked by membrane potential depolarizations*

The above information on the $[Ca^{2+}]_i$ sensitivity of exocytosis permits the use of the secretory response as an indicator of $[Ca^{2+}]_i$ levels produced at these sites during depolarization. We first describe the nature of the secretory response produced by

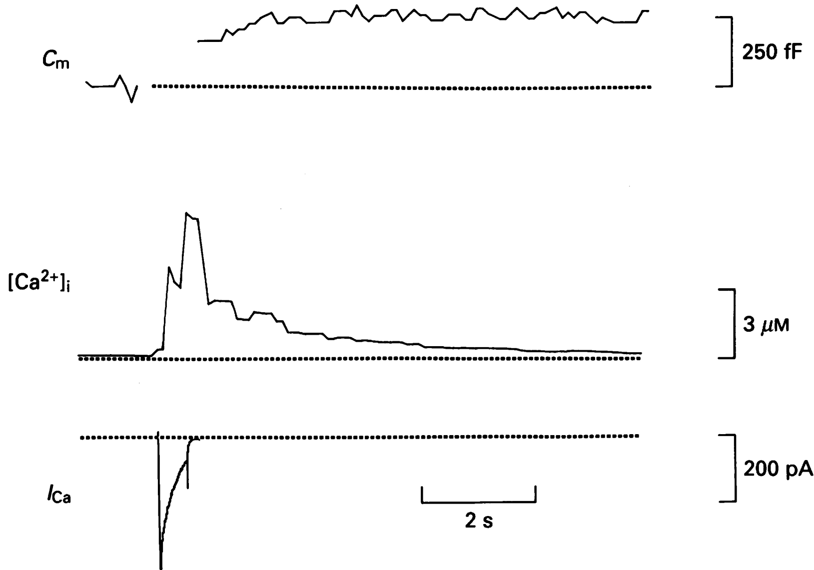


Fig. 7. Secretory activity evoked by a depolarizing pulse. A 500-ms-long depolarization to +21 mV elicited an inward $Ca^{2+}$ current ($I_{Ca}$), as well as a rise in $[Ca^{2+}]_i$ and a step-like increase in $C_m$. Dashed lines indicate pre-depolarization baselines for $C_m$ and $I_{Ca}$ traces and zero level for $[Ca^{2+}]_i$. The gap in the $C_m$ trace is due to the transient rise in membrane conductance during the depolarization.

depolarizations and then use secretion rates to derive an experimental estimate of $[Ca^{2+}]_i$ at the release sites.

*Stationarity of responses to depolarization.* Early attempts to measure secretory responses during depolarization of single chromaffin cells were limited by the stability of the baseline $C_m$ (e.g. Clapham & Neher, 1984). In the present study, we were able to obtain relatively stable values for baseline $C_m$, presumably because the 0·5 mM-EGTA in our internal solution maintained resting $[Ca^{2+}]_i$ at a low and constant level (see also Schweizer & Bookman, 1988). Despite the presence of EGTA, brief depolarizations of the membrane potential elicited an inward $Ca^{2+}$ current and a transient rise in $[Ca^{2+}]_i$ (Fig. 7). The relationship between this $Ca^{2+}$ current and resultant $[Ca^{2+}]_i$ changes are explored in the following paper (Neher & Augustine, 1992). The depolarization also produced an abrupt increase in $C_m$ (Fig. 7). Although the magnitude of these responses varied from cell to cell, and from culture to culture, more than 90% of the cells in which acceptable sealing and breakthrough were achieved produced responses similar to those shown in Fig. 7.

One limitation of the whole-cell recording method is that physiological responses may decline with time after establishing continuity between the recording pipette

and cytoplasm (e.g. Fenwick, Marty & Neher, 1982; Penner, Pusch & Neher, 1987). Such a 'wash-out' of $Ca^{2+}$ current was present in our recording conditions (Fig. 8*A*). Further, changes in $C_m$ elicited by depolarization also declined with time (Fig. 8*B*). Because the wash-out of depolarization-induced $C_m$ changes is influenced by the loss

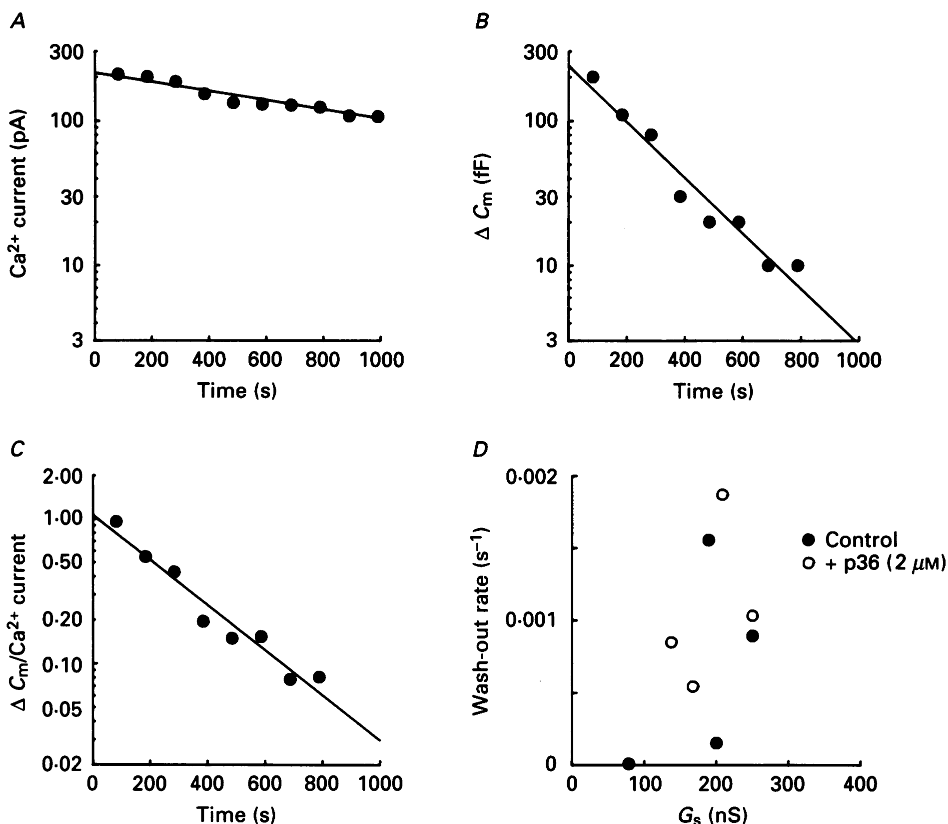


Fig. 8. Wash-out of $Ca^{2+}$ currents and exocytotic activity during cell dialysis. Repetitive depolarizing pulses (to +31 mV for 500 ms) were used to evoke $Ca^{2+}$ currents and changes in $C_m$ in a single cell ($G_s$ = 189 nS). *A*, progressive decline in amplitude of $Ca^{2+}$ currents during dialysis. Straight line is a linear regression fit to the data and represents an exponential with a time constant of 1374 s. *B*, progressive decline in amplitude of changes in $C_m$ during dialysis. Straight line is a linear regression fit with a time constant of 226 s. *C*, after correction for decline in $Ca^{2+}$ influx, by dividing the $Ca^{2+}$ current amplitude, wash-out of $C_m$ changes is still evident. Straight line is a linear regression fit with a time constant of 279 s. *D*, rate of wash-out, determined for several cells as the negative of slopes of plots like the one shown in *C*, increases with high series conductance ($G_s$). Inclusion of 2 μM-p36 in the internal dialysis solution had little detectable effect on wash-out rate.

of the $Ca^{2+}$ current which triggers the $C_m$ changes, we took into account the loss of $Ca^{2+}$ entry by dividing the magnitude of $C_m$ changes by the size of the $Ca^{2+}$ current that elicited them (Fig. 8*C*). Even after correcting for partial loss of $Ca^{2+}$ current, secretory responses washed out, with an exponential time course that was substantially more rapid than that of wash-out of $Ca^{2+}$ current (compare Fig. 8*C* to 8*A*).

Because the series conductance limits the rate of exchange between the pipette and cytoplasm (Pusch & Neher, 1988; Oliva *et al.* 1988), the time course of the wash-out of the secretory response should depend upon the size of the series conductance (Marty & Zimmerberg, 1989). This was the case for chromaffin cells, with wash-out occurring more quickly when the series conductance was higher (Fig. 8*D*). Although it has been reported that the $Ca^{2+}$-binding protein, p36, reverses wash-out of secretion from permeabilized chromaffin cells (Ali, Geisow & Burgoyne, 1989), addition of p36 (2 $\mu$M) to the patch pipette solution did not noticably slow wash-out in our experiments (open symbols in Fig. 8*D*). To avoid complications caused by wash-out, all of the measurements described below were limited to the first 100–200 s following the start of whole-cell recording.

*Kinetics of responses to depolarizations.* Depolarizing pulses produced transient rises in $[Ca^{2+}]_i$ that led to transient increases in the rate of secretion, evident as an elevation of $C_m$. The time course of these changes can be seen in the examples of responses to depolarizations to a range of potentials, shown in Fig. 9*A*. Depolarizations that elicited significant elevation of $[Ca^{2+}]_i$ led to a rise in $C_m$. The kinetics of the $C_m$ changes occurring during the depolarizations could not be resolved, because of the large membrane conductance during the depolarization (Lindau & Neher, 1988). However, the rise in $C_m$ could be detected after the depolarization as an increment with respect to the baseline level measured before the depolarization.

In addition to such abrupt increases in $C_m$, depolarizations that produced large rises in $[Ca^{2+}]_i$, such as the +11 and +51 mV depolarizations in Fig. 9*A*, often caused continuing rises in $C_m$ that outlasted the depolarization by several seconds. Because such stimuli cause slowly decaying $[Ca^{2+}]_i$ changes that remain within the range of $[Ca^{2+}]_i$ that triggers secretion (see Fig. 6), it is possible that they are due to the residual $[Ca^{2+}]_i$ signal (see also Bookman & Schweizer, 1988; Thomas *et al.* 1990). We tested this possibility by measuring the rate of secretion during such episodes and comparing it to $[Ca^{2+}]_i$ signals measured simultaneously. The resultant relationship was very similar to that found for responses to $Ca^{2+}$ dialysis or $Ca^{2+}$ release from internal stores (Fig. 9*B*), suggesting that residual $Ca^{2+}$ is indeed the cause of the persistent secretory activity.

Depolarization-evoked increases in $C_m$ often were followed by slow decreases in $C_m$; an unusually obvious example of such a decrease is shown in the response to the depolarization to +31 mV in Fig. 9*A*. Although these decreases typically occurred over time spans of tens of seconds, occasionally $C_m$ partially relaxed towards the baseline within 4–10 s. Such decreases in $C_m$ presumably are caused by activation of endocytosis (Neher & Marty, 1982; Patrick, Böck, Fischer-Colbrie, Schauenstein, Schmidt, Lingg & Winkler, 1984; von Grafenstein, Roberts & Baker, 1986). Because these declines in $C_m$ were much slower than the step increase in $C_m$, they should not interfere with measurements of exocytosis during depolarizations. However, they could influence measurements of exocytosis produced by $Ca^{2+}$ dialysis, if endocytosis occurs under such conditions. The slow declines in $C_m$ were typically associated with stimuli that produced larger initial jumps in $C_m$ (e.g. Fig. 9*A*) and were more obvious in cells that had recently undergone extensive exocytosis. Thus, it appears that the rate of endocytosis is somehow coupled to exocytotic activity in these cells.

During trains of repetitive depolarizations, even more complex changes in $C_m$ could be observed (see also Clapham & Neher, 1984; Bookman & Schweizer, 1988). Even though $Ca^{2+}$ currents elicited by the depolarizations usually declined throughout the train due to inactivation (Fenwick *et al.* 1982), $C_m$ responses were

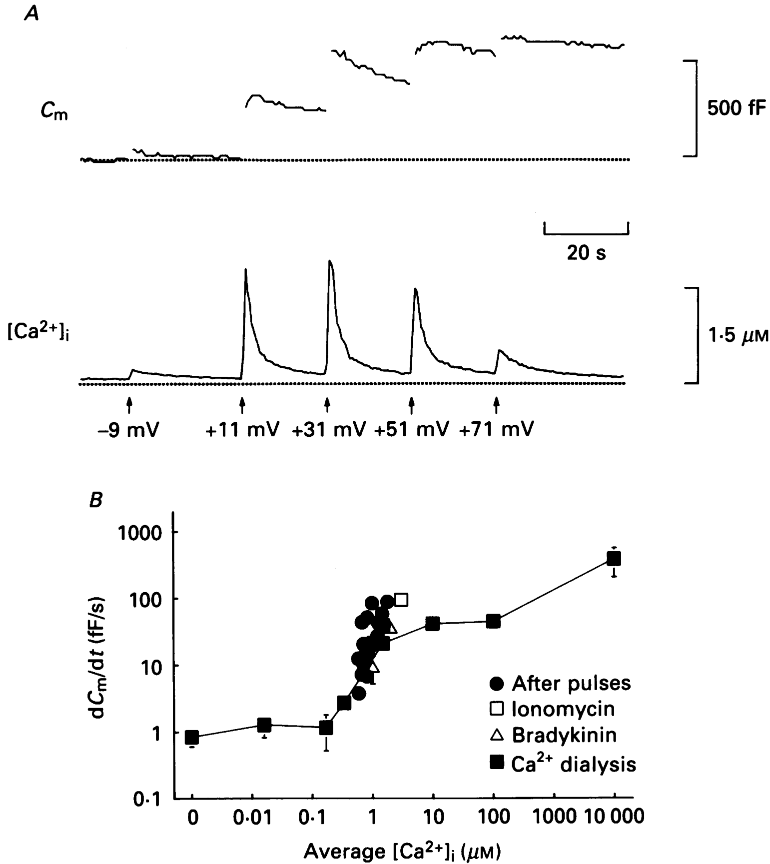


Fig. 9. $C_m$ changes evoked by depolarizations to different membrane potentials. *A*, changes in $C_m$ and $[Ca^{2+}]_i$ produced by 500-ms-long depolarizations to different membrane potentials (at arrows). The trace shown here begins 70 s after breakthrough. Dashed lines indicate baseline $C_m$ before depolarization and zero level of $[Ca^{2+}]_i$. *B*, $Ca^{2+}$ dependence of secondary rises in $C_m$ occurring after 500-ms-long depolarizations in three cells where endocytosis was not evident. ● indicate relationship between rate of increase in $C_m$ and residual $[Ca^{2+}]_i$ changes, measured at several time points after the depolarizing pulses. For comparison, also plotted are $C_m$ responses to dialysis of $Ca^{2+}$ (■, from Fig. 4*B*) and release of $Ca^{2+}$ from internal stores (open symbols, data taken from Fig. 6 and binned).

usually facilitated at the beginning of a train so that depolarizations produced increasing changes in $C_m$ (Fig. 10). Because the slowly decaying $[Ca^{2+}]_i$ transients summed during the train, to produce an elevated envelope of $[Ca^{2+}]_i$, it is possible that this caused the facilitation (Katz & Miledi, 1968; Charlton, Smith & Zucker, 1982; Bookman & Schweizer, 1988; Thomas *et al.* 1990). The facilitation often was followed by a slower decline or depression of $C_m$ changes (see also Fidler, Nowycky

& Bookman, 1990), so that the $C_m$ signal produced by a pulse train usually had a sigmoidal appearance. The depression may have been caused by inactivation of $Ca^{2+}$ current and/or depletion of vesicles readily available for exocytosis; our observation that depression occurred at persistently elevated $[Ca^{2+}]_i$ levels argues in favour of the latter explanation.

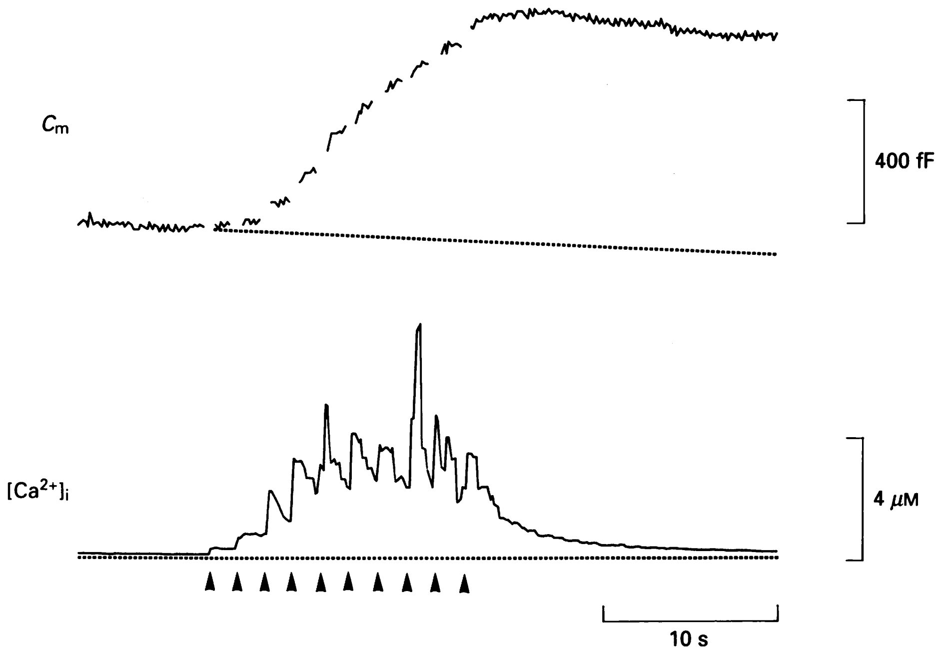


Fig. 10. $C_m$ changes evoked by repetitive depolarizations. A train of ten depolarizations (to +31 mV for 200 ms, at arrowheads) produced complex changes in $C_m$ and $[Ca^{2+}]_i$. Dashed lines indicate extrapolated baseline for $C_m$ trace and zero level for $[Ca^{2+}]_i$.

*Voltage dependence of responses*. The magnitude of the $C_m$ changes depended upon the potential level reached during the depolarization (Fig. 9*A*). Most of this voltage dependence apparently is due to the bell-shaped relationship between membrane potential and $Ca^{2+}$ accumulation in the cell (Fig. 11*A*), which is caused by the voltage dependence of the $Ca^{2+}$ current (Fenwick *et al*. 1982; Hoshi & Smith, 1987; Kim & Neher, 1988).

In addition to the large effect of membrane potential on average $[Ca^{2+}]_i$ levels, there appears to be a second, smaller effect of membrane potential on secretory activity. This is evidence in Fig. 11*A* when comparing the $[Ca^{2+}]_i$ and $C_m$ responses produced by depolarizations to +6 and +66 mV. Even though these potentials produced very similar changes in average $[Ca^{2+}]_i$, the changes in $C_m$ were much larger at +6 mV. This can also be seen by plotting the changes in $C_m$ as a function of the average $[Ca^{2+}]_i$ changes produced by varied depolarizations (Fig. 11*B*). Such a voltage dependence suggests that depolarization-induced secretion depends upon considerations in addition to the net $Ca^{2+}$ current and the average change in $[Ca^{2+}]_i$. It can be explained if secretion depended on the magnitude of the $[Ca^{2+}]_i$ changes

occurring within unitary domains around single open $Ca^{2+}$ channels (Chad & Eckert, 1984; Simon & Llinas, 1985; Fogelson & Zucker, 1985; Smith & Augustine, 1988), because the size of these $[Ca^{2+}]_i$ changes should decrease with depolarization as the single-channel $Ca^{2+}$ current declines (e.g. Fenwick *et al.* 1982).

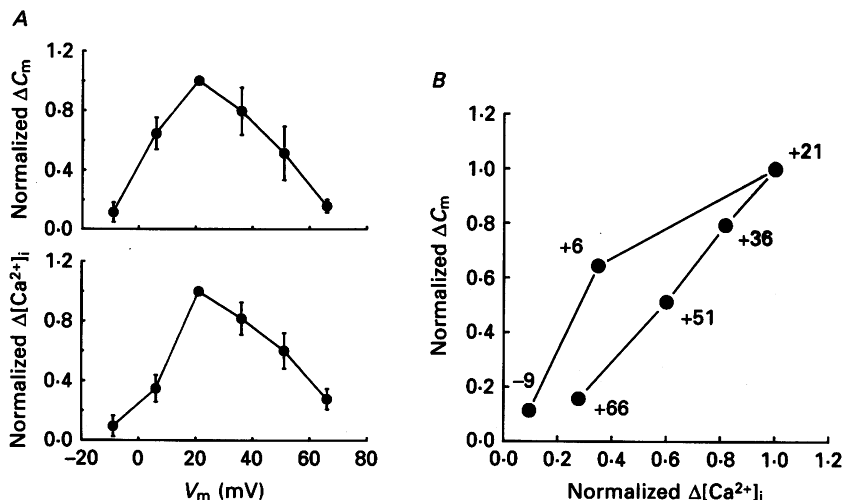


Fig. 11. Voltage dependence of secretion. *A*, influence of membrane potential ($V_m$) on $C_m$ (top) and $[Ca^{2+}]_i$ changes (bottom). Points represent mean values obtained from four experiments using 100-ms-long depolarizations. Bars indicate ± S.E.M. *B*, correlation between $C_m$ and $[Ca^{2+}]_i$ changes evoked by depolarizations to the indicated potentials. Data are from the same experiments shown in *A* and have been normalized by dividing by the maximum value measured in each cell.

*Calcium dependence of responses.* During 100-ms-long depolarizations, average $[Ca^{2+}]_i$ levels approached 1 $\mu$M and produced rapid initial jumps of $C_m$. The rate of increase in $C_m$, measured as the magnitude of the jump in $C_m$ divided by the duration of the depolarization, increased as much as several hundred femtofarads per second during 100-ms-long depolarizations (◆ in Fig. 12*A*). These rates are much higher than expected based on elevation of $[Ca^{2+}]_i$ to similar levels with dialysis or release from internal stores. A likely explanation for these discrepancies is the presence of gradients of $[Ca^{2+}]_i$ during these brief depolarizations. These gradients would cause the measurements of average $[Ca^{2+}]_i$ to underestimate the actual level of $[Ca^{2+}]_i$ at the sites of secretion (O'Sullivan *et al.* 1989; Neher & Augustine, 1992). A further indication of this comes from experiments using longer (500-ms-long) depolarizations, which evoked larger responses with $dC_m/dt$ that could be in excess of 1000 fF/s. For these responses, the apparent relationship between rates of $C_m$ increase and average $[Ca^{2+}]_i$ was shifted (◇ in Fig. 12*A*), presumably because the average $[Ca^{2+}]_i$ values measured during the longer pulses more closely approximate the changes in $[Ca^{2+}]_i$ at the secretory sites.

As long as $Ca^{2+}$ is entering the cell through the $Ca^{2+}$ channels, gradients of $[Ca^{2+}]$ will occur (Neher & Augustine, 1992). Thus, average $[Ca^{2+}]_i$ signals will underestimate the concentration of $Ca^{2+}$ at the secretory sites even during the 500-ms-long depolarizations. However, following the depolarizing pulse, $[Ca^{2+}]_i$ will equilibrate

spatially within a fraction of a second (Neher & Augustine, 1992). Consistent with this expectation, the $C_m$ increases measured following depolarizing pulses, while $[Ca^{2+}]_i$ decayed from micromolar levels back to the baseline levels of resting $[Ca^{2+}]_i$, were shifted along the $[Ca^{2+}]_i$ axis relative to $C_m$ changes measured during 500-ms-

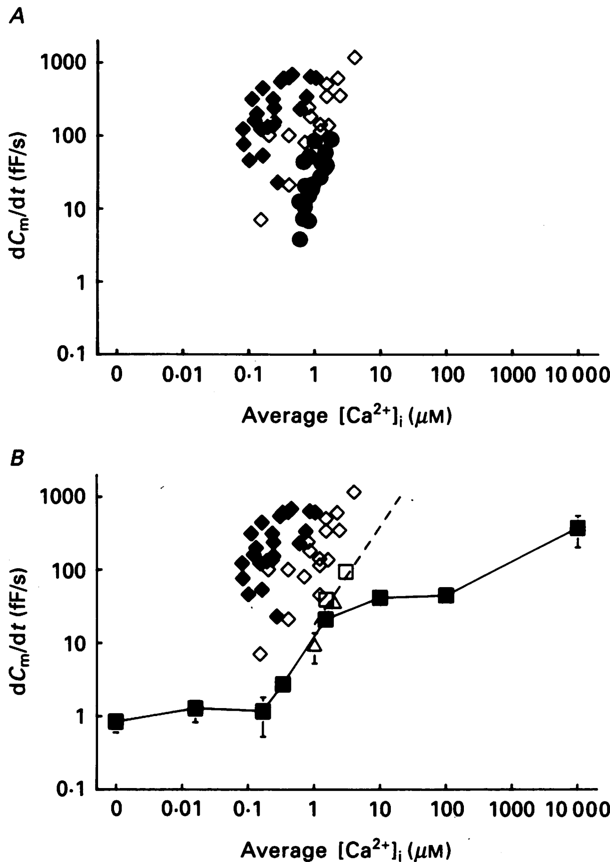


Fig. 12. $[Ca^{2+}]_i$ dependence of $C_m$ changes induced by depolarizations. *A*, jumps in $C_m$ produced by 100-ms-long (◆) and 500-ms-long (◇) depolarizations to various membrane potentials are plotted as a function of the peak change in average $[Ca^{2+}]_i$ level, measured with Fura-2. Also shown is the $Ca^{2+}$ dependence of the slow increases in $C_m$ following 500-ms-long pulses (●, from Fig. 9*B*). *B*, comparison between $Ca^{2+}$ dependence of rate of change in $C_m$ evoked by depolarizations (diamonds; from Fig. 12*A*) and release of $Ca^{2+}$ from internal stores (△ and □, from Fig. 9*B*) or $Ca^{2+}$ dialysis (■, from Fig. 4*B*).

long depolarizations (Fig. 12*A*). The similar $[Ca^{2+}]_i$ dependence of these persistent depolarization-induced responses and responses to dialyis or release from internal stores (Fig. 9*B*) is another indication that $[Ca^{2+}]_i$ is spatially uniform soon after a depolarization ends.

A quantitative comparison of secretory rates derived from $Ca^{2+}$ dialysis with those from depolarizations can be used to estimate the levels of $[Ca^{2+}]_i$ present at the secretory sites during depolarization, using the depolarization-induced secretory response as a $Ca^{2+}$ detector (Fig. 12*B*). Small depolarizations that raised average

$[Ca^{2+}]_i$ by a few hundred nanomolar produced changes in $C_m$ in the order of a few hundred femtofarads per second; in order to achieve this rate of secretion by release from internal stores, $[Ca^{2+}]_i$ must be elevated to a few micromolar. When raising $[Ca^{2+}]_i$ by dialysis even 100 $\mu$M $[Ca^{2+}]_i$ did not reach these rates. The largest depolarization-induced rises in $C_m$ exceeded 1000 fF/s, which would require a release of $Ca^{2+}$ from internal stores in excess of 10 $\mu$M (estimated by extrapolation). Thus, depolarization apparently raises $[Ca^{2+}]_i$ at the release sites to levels at least an order of magnitude higher than evident from average $[Ca^{2+}]_i$ measurements. At their highest, these levels exceed 10 $\mu$M and could be in the range of 100 $\mu$M.

## DISCUSSION

In this paper we have examined the $[Ca^{2+}]_i$ dependence of secretion in single chromaffin cells. Using dialysis or release of $Ca^{2+}$ from internal stores, we have found that the rate of secretion is sensitive to $[Ca^{2+}]_i$ and accelerates by several orders of magnitude as $[Ca^{2+}]_i$ is raised above 0·2 $\mu$M. Depolarization-induced rises in $[Ca^{2+}]_i$ also accelerate the rate of secretion; comparison of the rates achieved during depolarization to those measured during $Ca^{2+}$ dialysis or release from internal stores suggests that depolarization can raise $[Ca^{2+}]_i$ at the secretory sites to 10 $\mu$M or higher. Because the spatially averaged $[Ca^{2+}]_i$ levels measured during such depolarizations are much smaller than this estimate of $[Ca^{2+}]_i$ at the secretory site, spatial $[Ca^{2+}]_i$ gradients must occur during depolarization.

### *Wash-out of the secretory response*

As has been found for permeabilized chromaffin cells (Sarafian, Aunis & Bader, 1987; Ali *et al.* 1989), the ability to support $Ca^{2+}$-dependent exocytosis washes out during dialysis of chromaffin cells with a patch pipette. However, unlike permeabilized chromaffin cells (Ali *et al.* 1989), the wash-out of exocytosis could not be prevented by adding p36 to the internal solution. The reason for this discrepancy is not clear, but could be due to differences in experimental technique.

Knowledge of the series resistance of the patch pipette allows us to estimate the effective molecular mass of the molecule that is lost during wash-out. In chromaffin cells, the relationship between molecular mass, $M$ and series resistance, $R_A$ is:

$$M = (\tau/(0{\cdot}6\,R_A))^3$$

where $\tau$ is the time constant for wash-out (Pusch & Neher, 1988). Based on the data shown in Fig. 8*C*, the time constant for wash-out is 280 s for a series resistance of 5·3 $\Omega$. This corresponds to an effective $M$ of $6{\cdot}8 \times 10^5$ g/mol, suggesting the involvement of a very large molecule in maintaining exocytosis. If the washed out molecule binds to intracellular structures, then its diffusion will be retarded and its molecular mass would be lower than this estimate.

One of the most interesting applications of the patch clamp in the study of exocytosis is to deliver molecular probes to the interior of secretory cells (Fernandez, Neher & Gomperts, 1984; Penner *et al.* 1986; Schweizer *et al.* 1989). However, the presence of wash-out requires careful experimental design, to avoid confusing wash-out during probe delivery with an actual inhibitory action of a probe. In such

experiments the relevant consideration is the rate of delivery of the probe relative to the rate of wash-out of exocytosis. One way to promote the rapid delivery of the probe is to make its concentration within the pipette very high, so that the intracellular concentration is as high as possible before wash-out is complete. Regardless of the speed of probe delivery, it is necessary to measure the series conductance for each experiment to anticipate the rate of loss of secretory activity expected due to wash-out (as in Fig. 8*D*).

*$Ca^{2+}$ requirements for secretion*

One of the goals of our study was to determine how much calcium is needed to trigger exocytosis from single chromaffin cells. This goal was complicated by several problems. First, our measurements of $C_m$ report membrane area, which is influenced not only by exocytosis but also by endocytosis. In dialysis experiments it is likely that both processes are occurring simultaneously, so that measurements of net changes in area will underestimate the amount of exocytosis. Responses to the other, more rapid procedures that we used to elevate $[Ca^{2+}]_i$ should be less affected, because of the temporal separation between endocytosis and exocytosis. Second, dialysis of $Ca^{2+}$ and entry through voltage-gated $Ca^{2+}$ channels elevates $[Ca^{2+}]_i$ in different time domains: $[Ca^{2+}]_i$ reaches a maximum over tens of seconds to minutes during dialysis, while depolarization should maximally elevate $[Ca^{2+}]_i$ during the 50–500-ms-long stimulus. The responses to $Ca^{2+}$ release from internal stores, which occurred over a few seconds (Fig. 5), thus are more comparable to responses to depolarizations.

A possible additional complication is that studies on chromaffin cells (Perrin *et al.* 1987; Schweizer *et al.* 1989; Eberhard *et al.* 1990; Burgoyne, 1990), other endocrine cells (Thomas *et al.* 1990) and neurons (reviewed in Kelly, 1988) suggest that there might be a relatively small pool of membrane-docked, release-ready vesicles that can be exocytosed at high rates. This idea is consistent with several observations we have made (see also Bookman & Schweizer, 1988) and can be used to interpret some of our findings. The fast, step-like change of capacitance produced by depolarization may arise from this readily releasible pool and the depression of secretion during repetitive depolarizations could result from depletion of this pool. Slow elevation of $[Ca^{2+}]_i$ via dialysis should cause depletion to occur, so that measured rates of secretion will be limited by the rate of mobilization of vesicles rather than the rate of exocytosis once the vesicles are docked. This could account for the progressive decline in the rate of secretion during dialysis, which occurred even though $[Ca^{2+}]_i$ remained high. It could also account for our observation that dialysis of 10–100 $\mu$M-$Ca^{2+}$ produced an apparent saturation of secretory rates, yet higher rates were produced transiently by dialysis of 9 mM-$Ca^{2+}$ or by depolarization. According to this view, exocytosis of docked vesicles can briefly occur at rates in excess of 1000 fF/s, while mobilization-limited exocytosis is slowed to approximately 50 fF/s. Such considerations might also explain the small total amount of secretion observed with dialysis with 9 mM-$Ca^{2+}$, if it is assumed that this high $[Ca^{2+}]_i$ level triggered exocytosis but inhibited the mobilization process. The small total amount of capacitance increase during release of $Ca^{2+}$ from internal stores might result from depletion of the releasible pool and, as mentioned in the Results, the decline in the rate of secretion during sustained release of $Ca^{2+}$ from internal stores is also

consistent with this explanation. Alternatively, some of these phenomena might have other causes, such as a direct inhibitory effect of high $[Ca^{2+}]_i$ on the exocytotic apparatus (Knight & Baker, 1982). Further work will be necessary to evaluate these alternative explanations and to determine the physiological relevance of these findings.

Despite these complexities, the relationship between $[Ca^{2+}]_i$ and $dC_m/dt$ was rather similar regardless of whether it was measured during dialysis of $Ca^{2+}$, release of $Ca^{2+}$ from internal stores or during the slow changes in $C_m$ that follow depolarizations (Fig. 9*B*). Thus, we conclude that all methods that increase $[Ca^{2+}]_i$ in a slow and spatially uniform manner are characterized by a single 'dose–response' relationship. Changes in $C_m$ measured during depolarization clearly are much faster than expected based on the resultant changes in average $[Ca^{2+}]_i$ and lead to $C_m$ signals that are incompatible with this relationship, presumably due to spatial gradients of $[Ca^{2+}]_i$ (see below).

Our results contrast with those of some previous studies (e.g. Kim & Westhead, 1989; Cheek, Jackson, O'Sullivan, Moreton, Berridge & Burgoyne, 1989; but see Warashina, Fujiwara & Shimoji, 1990) reporting that $Ca^{2+}$ released from internal stores produced no measurable exocytosis. In our experiments, release from internal stores triggered exocytosis; however, because $[Ca^{2+}]_i$ was only transiently elevated, the rate of secretion produced by $Ca^{2+}$ release from internal stores was transient (Fig. 5) and the total amount of secretion was only about 10% of that obtained by prolonged dialysis of $Ca^{2+}$ (compare Figs 3 and 5). These features of the response could make it difficult to detect with chemical assays of secretion, as were used in the previous studies. Therefore, we conclude that $Ca^{2+}$ released from internal stores is quite capable of inducing secretion, but that it lacks the advantageous localization of the voltage-gated $Ca^{2+}$ channels (O'Sullivan *et al.* 1989).

While our results do not allow us to completely define the relationship between $[Ca^{2+}]_i$ and rate of secretion, we can make some inferences about this relationship. First, we can conclude that the rate of secretion is sensitive to $[Ca^{2+}]_i$, accelerating more than three orders of magnitude as $[Ca^{2+}]_i$ at the release site is elevated. Limitations in our ability to rapidly raise $[Ca^{2+}]_i$ to defined values above a few micromolar prevent better resolution of the maximum rate of secretion, but rates as rapid as 1000 fF/s (approximately 1000 exocytotic events per second, assuming a single chromaffin granule to have a capacitance of 1 fF) were observed during depolarization and during dialysis of 9 mM-$Ca^{2+}$. Second, it appears that $Ca^{2+}$-dependent secretion requires $[Ca^{2+}]_i$ levels above 0·2 $\mu$M. Third, because we estimate $[Ca^{2+}]_i$ levels greater than 10 $\mu$M during depolarization (Fig. 12*B*) and because of the increased rate of secretion seen when raising the $Ca^{2+}$ concentration from 100 $\mu$M to 9 mM in the dialysis experiments (Fig. 4*B*), we can conclude that secretion does not saturate at $[Ca^{2+}]_i$ below 10 $\mu$M. The observed saturation of this relationship seen with dialysis of 10–100 $\mu$M-$[Ca^{2+}]_i$ presumably is due to depletion of releasible vesicles and/or overlapping endocytotic activity, as discussed above.

Most measurements of secretion from populations of permeabilized chromaffin cells have indicated that secretion saturated at $Ca^{2+}$ concentrations well below 10 $\mu$M (Knight & Baker, 1982; Dunn & Holz, 1983; cf. Wilson & Kirshner, 1983). Because of the limited time resolution of these measurements, they measure the total amount

of secretion, rather than the rate of secretion. These measurements, therefore, should be more comparable to those shown in Fig. 4*A* and there is good correspondence between the form of the two sets of measurements. However, the rate of secretion should provide the better indicator of the actual affinity of the exocytotic apparatus for $Ca^{2+}$, because the total amount of secretion may just reflect the total number of chromaffin granules available for exocytosis. Thus, it does not appear that the exocytotic apparatus saturates at $[Ca^{2+}]_i$ below 10 $\mu$M.

Biochemical studies have identified a large number of calcium-binding proteins which are found in secretory cells, often in association with vesicles, and which may play a role in mediating secretion (Reichardt & Kelly, 1983; Augustine *et al.* 1987; Burgoyne, 1990). However, it has proved difficult to determine which of these proteins are actually involved in secretion. One means of distinguishing between these various proteins is in their calcium-dependence, because their affinity for calcium varies from less than 1 $\mu$M to greater than several hundred micromolar (e.g. Creutz *et al.* 1988). Previous studies have concentrated on molecules with high $Ca^{2+}$ affinities (in the order of 1 $\mu$M), because of the apparent saturation of the secretory apparatus at higher $[Ca^{2+}]_i$ in permeabilized cells. Our conclusion that the exocytotic apparatus responds to elevation of $[Ca^{2+}]_i$ above 10 $\mu$M suggests that molecules with a fairly low $Ca^{2+}$ affinity could be involved in triggering exocytosis in chromaffin cells. The apparent Hill coefficient of about 2, evident when elevating $[Ca^{2+}]_i$ with any of the methods we have employed, suggests a requirement for multiple $Ca^{2+}$-binding sites in this process, as has already been proposed for chromaffin cells (e.g. Knight & Baker, 1982). It is interesting that higher coefficients have been reported for neurons (Dodge & Rahamimoff, 1967; Augustine & Charlton, 1986) and anterior pituitary cells (Thomas *et al.* 1990).

### *Intracellular $Ca^{2+}$ gradients during depolarization*

The second goal of our study was to define the levels of $[Ca^{2+}]_i$ present at secretory sites during depolarization, a goal complicated by the fact that our measurements of average $[Ca^{2+}]_i$ apparently underestimate the $[Ca^{2+}]_i$ levels actually occurring at these sites. The most obvious indication of this complication was the discrepancy between plots of the rate of secretion as a function of average $[Ca^{2+}]_i$: responses to depolarizations appeared to require lower $[Ca^{2+}]_i$ than those produced by treatments that should make $[Ca^{2+}]_i$ rise in a more spatially uniform manner, such as dialysis or release of $Ca^{2+}$ from intracellular stores (Fig. 12*B*). Another indication was that these plots become more similar when the duration of depolarization was increased (Fig. 12*A*) and became identical when measured after the depolarization (Fig. 9*B*). We propose that these discrepancies are a consequence of spatial gradients of cytoplasmic $Ca^{2+}$ during depolarization, due to the localization of voltage-gated $Ca^{2+}$ channels at the plasma membrane of these cells. Video imaging of $[Ca^{2+}]_i$ signals in these cells provides direct support for this idea (O'Sullivan *et al.* 1989; Neher & Augustine, 1992), although even such measurements probably underestimate the steepness of these gradients (Neher & Augustine, 1992).

Quantitative analysis of the rates of secretion during depolarization suggests that $[Ca^{2+}]_i$ at the secretory sites reaches levels in excess of 10 $\mu$M. This is higher than earlier estimates based on measurements of depolarization-induced $[Ca^{2+}]_i$ signals

(Knight & Kestevian, 1983; O'Sullivan *et al.* 1989; Cheek *et al.* 1989) or the $[Ca^{2+}]_i$ requirements for secretion in permeabilized chromaffin cells (reviewed in Knight, 1988). However, it is in line with experimental estimates of $[Ca^{2+}]_i$ at exocytotic sites in nerve terminals (Roberts *et al.* 1990; Adler, Augustine, Duffy & Charlton, 1991) and theoretical studies of $Ca^{2+}$ diffusion in the vicinity of single open $Ca^{2+}$ channels (Chad & Eckert, 1984; Simon & Llinas, 1985; Fogelson & Zucker, 1985; Smith & Augustine, 1988). These theoretical studies also indicate that the secretory sites of chromaffin cells must be located in close proximity to the voltage-gated $Ca^{2+}$ channels in order to encounter such high levels of $[Ca^{2+}]_i$. Consistent with such an idea, our measurements of the relationship between average $[Ca^{2+}]_i$ and rate of exocytosis are sensitive to the magnitude of $Ca^{2+}$ entry through single $Ca^{2+}$ channels (Fig. 11*B*), as would be expected if the release sites are located close to the channels (Simon & Llinas, 1985). A similar structural arrangement is found in nerve terminals (Heuser, Reese & Landis, 1974; Pumplin, Reese & Llinas, 1981; Augustine, Buchanan, Charlton, Osses & Smith, 1989; Robitaille, Adler & Charlton, 1990) and it may be that close apposition between $Ca^{2+}$ channels and secretory sites is characteristic of all cells that use voltage-gated $Ca^{2+}$ channels to trigger exocytosis in the millisecond (or faster) time domain.

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