Increased Cytosolic Calcium Stimulates Exocytosis in Bovine Lactotrophs

Direct Evidence from Changes in Membrane Capacitance

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ABSTRACT The patch-clamp technique has been used to measure changes in membrane capacitance ($C_m$) of bovine lactotrophs in order to monitor fluctuations in cell surface area associated with exo- and endocytosis. Cells were prepared by an enrichment procedure and cultured for up to 14 d before use. Under whole-cell recording, cell cytoplasm was dialyzed with various Ca$^{2+}$-containing solutions. The resting $C_m$ of 6.05 ± 1.68 pF was found to correlate well with squared cell radius, suggesting a specific $C_m$ of 0.8 μF/cm$^2$. Discrete $C_m$ steps of 2-10 fF were recorded, which most likely reflect single fusion and retrieval events of prolactin-containing granules (0.2-0.6 μm in diameter). High Ca$^{2+}$ resulted in a $C_m$ increase of 20-50% from the resting value, demonstrating a role for [Ca$^{2+}$] in stimulus-secretion coupling. Spontaneous $C_m$ changes have also been recorded, which presumably reflect prolactin secretion supported by a tonic influx of Ca$^{2+}$ through the membrane. This is supported by the following findings: addition of Co$^{2+}$ diminished or reversed the spontaneous $C_m$ changes and decreased resting [Ca$^{2+}$]; and membrane depolarization increased $C_m$, indicating the role of voltage-activated channels in stimulus–secretion coupling. As bovine lactotrophs have been found to be largely devoid of spontaneous electrical activity, a mechanism involving modulation of a tonic Ca$^{2+}$ influx is proposed; this is shown to provide adequate control of basal and triggered secretion monitored by $C_m$.

INTRODUCTION

Since Douglas (1968) proposed that intracellular calcium plays an important role in stimulus–secretion coupling, compelling evidence has accumulated in support of this hypothesis. Adenohypophysal cells exposed to excess K$^+$ secrete hormone (Vale et al., 1977), and this requires extracellular calcium. Furthermore, electrophysiological studies have revealed that tumor (Kidokoro, 1975) and normal cells (Taraskevich and...
Douglas, 1977; Ozawa and Sand, 1978) of adenohypophysis possess voltage-activated calcium channels and are excitable. Because of this it has been suggested that control of the firing frequency of Ca$^{2+}$-dependent action potentials might play a key role in basal and stimulated secretion (Taraskевич and Douglas, 1984; Ozawa and Sand, 1986). However, an influx of Ca$^{2+}$ ions in the absence of action potentials was also proposed to play a role (Kidokoro et al., 1982; Tomiko et al., 1984; Mason and Waring, 1985, 1986). Recently it was demonstrated that generation of an action potential in clonal adenohypophyseal cells leads to a transient increase in intracellular calcium activity (Schlegel et al., 1987; Benham, 1989).

We have investigated the influence of intracellular calcium activity ([Ca$^{2+}$]) on exocytosis of bovine lactotrophs. The patch-clamp technique (Hamill et al., 1981) was used to study secretion at the cellular level by monitoring cell membrane capacitance ($C_m$). $C_m$ is proportional to the membrane surface area; therefore fusion and retrieval of vesicles will be reflected in fluctuations of $C_m$, and an increase in $C_m$ will be indicative of increased cellular secretory activity (Neher and Marty, 1982). We have altered intracellular Ca$^{2+}$ activities by dialyzing the cytosol with different Ca$^{2+}$-containing pipette solutions. We have also investigated the effect of membrane depolarization on $C_m$ to study the role of voltage-activated channels in stimulus-secretion coupling.

Our results demonstrate the Ca$^{2+}$ dependence of exocytosis in single bovine adenohypophyseal cells. In addition, we have found that a sustained train of depolarizing pulses can induce a small increase in $C_m$, which demonstrates a possible role of voltage-activated channels in stimulus-secretion coupling. However, the physiological relevance of this response is doubtful as these cells are largely devoid of such electrical activity (Ingram et al., 1986; Cobbett et al., 1987a). Therefore, we propose an alternative mechanism for the regulation of hormone secretion, which involves the modulation of a tonic influx of Ca$^{2+}$ into the cytosol. We have found that at resting membrane potential, influx of Ca$^{2+}$ through channels (possibly voltage-activated channels) is sufficient to support exocytosis. Some of these results have been presented as abstracts (Mason et al., 1988b; Zorec et al., 1988).

**MATERIALS AND METHODS**

**Cell Culture**

The techniques for cell dispersion, cell separation, and cell culture have been described elsewhere (Mason and Ingram, 1986; Ingram et al., 1988). Briefly, lactotrophs were prepared from the pars distalis of the bovine pituitary gland obtained from a local abattoir. Glands were enzymatically dispersed, and a lactotroph-rich population was produced by separation of cells on a discontinuous Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient. Cells from the interface of the 1.062 and 1.074 mg/ml density layers were removed, washed, and plated on poly-L-lysine-coated glass coverslips in 35-mm plastic Petri dishes (Nunc, Roskilde, Denmark). The cells were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) with 10% newborn calf serum (Uniscience, Cambridge, UK) and antibiotics at 36°C in a humidified atmosphere containing 5% CO$_2$. This cell population contains ~70% lactotrophs as determined by immunocytochemistry (Ingram et al., 1988). However, the probability of selecting a lactotroph for an experiment was higher as we were
recording only from the large (12–18 μm in diameter) spherical cells in the field viewed under the microscope (×40 or ×32 objectives) (Ingrain et al., 1986, 1988), giving a probability of >99% that the recorded cell was a lactotroph. Cell-coated coverslips were selected for experimentation 6–14 d after dispersion. No difference was detected in the properties of cells in relation to time in culture (Fig. 4 B), and the data are therefore treated as such. All experiments were performed at room temperature (19–22°C).

**Solutions**

Before the experiment, cell-coated coverslips were transferred into the recording chamber (see Fig. 1 of McBurney and Neering, 1985) mounted on an inverted microscope (E. Leitz, Inc., Rockleigh, NJ). Unless indicated otherwise, the cells were bathed in recording medium (1 ml) of the following composition (in mM): 127 NaCl, 5 KCl, 2 MgCl₂, 0.5 NaH₂PO₄, 5 NaHCO₃, 10 HEPES, 10 d-glucose, and 5 CaCl₂, pH = 7.2 (NaOH). In a few experiments CaCl₂ was reduced to 1.8 mM, and therefore the NaCl concentration was raised to 131.8 mM. In some experiments cobalt ions (Co²⁺; 2–8 mM), tetrodotoxin (TTX; 1–5 μM), and tetraethylammonium ions (TEA; 5 mM) were added to the bathing medium directly by a bolus application from stock solutions of 100, 1, and 100 mM for Co²⁺, TTX, and TEA, respectively.

Pipette-filling solution contained (in mM): 140 K-glutamate, 10 NaCl, 2 MgCl₂, EGTA (Ca²⁺-free), and Ca-EGTA (Ca²⁺-saturated EGTA), as indicated, and 10 HEPES, pH = 7.2 (Trizma base; Sigma Chemical Co., St. Louis, MO). In experiments where the role of voltage-activated conductances on Cₘ was studied, Na₄ATP (1–3 mM; Sigma Chemical Co.) was included to prevent the “rundown” of voltage-activated Ca²⁺ currents (Cobbett et al., 1987b), and K-glutamate was replaced by Na-glutamate to isolate voltage-activated Ca²⁺ currents. ATP stock solutions (100 mM) were aliquotted and stored at −20°C. Ca-EGTA buffers were prepared by a procedure similar to that described by Neher (1988) to allow for possible impurities in EGTA (Miller and Smith, 1984), and stored in aliquots at −20°C. Intracellular Ca²⁺ activity ([Ca²⁺]ᵢ) was calculated assuming an apparent dissociation constant (Kᵢ) for the Ca-EGTA complex of 0.15 μM (Grynkiewicz et al., 1985). Pipette-filling solutions were prepared from high grade water (Milli Q; Millipore/Continental Water Systems, Bedford, MA) and salts of the highest purity obtainable (Sigma Chemical Co., Aldrich Chemical Co., Milwaukee, WI). Osmolarity of bathing and pipette-filling solutions was measured by freezing point depression to be 294 ± 4 (n = 17) and 287 ± 9 (n = 14) mosM, respectively (Camlab, Cambridge, UK).

With these pipette-filling solutions a liquid junction potential of around −5 to −8 mV (negative inside pipette, with K-glutamate in the pipette) developed while the pipette was immersed in bathing medium. No corrections were made for these junction potentials.

Bolus applications introduced in some recordings a d.c. shift (typically ~100 fF) in the out-of-phase signal, but not in the in-phase signal, presumably because of altering the stray capacitance of the bath–pipette–reference electrode assembly. This offset was therefore subtracted from the signal. If there was a simultaneous change in the conductance signal, the experiment was rejected.

**Fura-2 Fluorescence Measurements**

Levels of Ca²⁺ contamination of the Ca²⁺-free solutions were tested using fura-2 measurements as follows: solutions containing 0.1 mM EGTA and 1 μM fura-2 (free acid; Cambridge BioScience, Cambridge, UK) were put in a chamber on a fluorescence microscope (Nikon Inc., Garden City, NY) equipped for dual excitation (340/380 nm) and fluorescence intensity was measured at 510 nm with an intensified CCD camera (Photonic Science, Robertsbridge, UK). Readings on a digital voltmeter from the output of the camera were compared with the
calibration table obtained according to the method of Grynkiewicz et al. (1985), using an apparent $K_d$ for the fura-2/Ca$^{2+}$ complex of 135 nM. Intracellular solutions contained $<30$ nM Ca$^{2+}$.

Measurements of resting [Ca$^{2+}$]$_i$ were performed on fura-2 acetoxymethylester (fura-2/AM; Cambridge BioScience) loaded cells (30 min at room temperature in recording medium with added 1 $\mu$M fura-2/AM). Details of data capture have been previously described (O'Sullivan et al., 1989). Briefly, fura-2 was excited as above, and ratio images, recursively filtered with a 0.2-s time constant filter, stored on a Sony U-matic videotape. Formation of a ratio image was implemented in a look-up table using the formula of Grynkiewicz et al. (1985). Constants were determined empirically using bulk solutions of CaCl$_2$/10 mM EGTA with 5 $\mu$M fura-2 (free acid). Recorded data were played back either through the Imagine system (Synoptics, Cambridge, UK) or on the Magical image analysis system (Joyce Loehl, Gateshead, UK), allowing capture of individual ratio frames and calculation of [Ca$^{2+}$], from the ratio values. Averaging was used (four ratio frames, giving a frequency response of $\sim$0.5 Hz) to diminish the noise level. Continuous traces of [Ca$^{2+}$]$_i$ were obtained by defining a region around a cell and thresholding at an intensity value of $\sim$ 10 nM [Ca$^{2+}$]$_i$ to ignore the background. This value was chosen after inspection of the pixel amplitude histogram. The mean [Ca$^{2+}$]$_i$ was calculated over the whole of the defined cell using calibration data of [Ca$^{2+}$] versus ratio pixel intensity. Resultant data files were plotted with the aid of a spreadsheet (Lotus 123). This allowed us to standardize measurements of different cells. It should be noted that [Ca$^{2+}$]$_i$ in certain parts of the cell may be much higher than the mean values obtained. The cells were superfused with recording medium at a rate of $\sim$ 1 ml/min, and Co$^{2+}$ (2–5 mM) was applied to cells by superfusion from a main reservoir.

Electrophysiological Recordings

Recording pipettes were prepared with an outer diameter of $\sim$ 1–2 $\mu$m (resistances in the range 5–15 M$\Omega$, typically 7 M$\Omega$), coated with Sylgard (Dow Corning Corp., Midland, MI), and heat polished according to the methods of Corey and Stevens (1983).

The tight-seal whole-cell recording technique was used in our experiments (Marty and Neher, 1983). Measurements were made using a patch-clamp amplifier (Henigman et al., 1987; Zorec et al., 1991), which contained fast ($C_i$; 0–10 pF) and slow ($C_c$; 0–1, 0–10, and 0–100 pF ranges) capacitance compensation circuits, as well as a circuit to cancel the series resistance ($R_s$; 1–101 M$\Omega$, or 0–1 $\mu$S). Each of these could be set by a calibrated potentiometer (0.25% accuracy). In addition, a two-phase lock-in amplifier was incorporated into the patch-clamp amplifier, including a phase delay facility to adjust for the phase shifts introduced by the $R_s$, $C_{ac}$. Calibrations were performed with capacitors, resistors, and model cells for which values were determined by an RLC meter (0.25% accuracy). Cell $C_{ac}$ was determined using approaches similar to those already described (Neher and Marty, 1982; Joshi and Fernandez, 1988; Lindau and Neher, 1988).

Briefly, under voltage clamp a sinusoidal voltage (10 or 1 mV p-p [peak-to-peak], 1,600 Hz) was summed with the membrane potential (usually held at around $\sim$ 70 mV) and the resulting current was measured by the lock-in amplifier at two mutually orthogonal phase angles. Summation of the sinusoidal signal to the holding potential did not activate any voltage-activated channels, and therefore cell conductance was treated as a linear element (Lindau and Neher, 1988). Measurements were performed with the 500 M$\Omega$ resistor switched into the feed-back loop of the patch-clamp amplifier headstage, as the noise generated by the instrumentation (0.020 fF$^2$/Hz) was lower than the noise generated by the cell in the whole-cell recording mode (noise generated by the model cell was 0.055 fF$^2$/Hz; $C_{ac} = 5.2$ pF, $R_s = 4.7$ M$\Omega$, $R_m = 10$ G$\Omega$ [membrane resistance]). Spectra were measured in the range 0–25 Hz (3 dB). These values compare well with those reported (Neher and Marty, 1982; Lindau and
Neher, 1988). Membrane d.c. current (0–10 Hz, 3 dB) and both outputs from the lock-in amplifier were monitored on a four-channel chart recorder (Gould Inc., Cleveland, OH), at a bandwidth of 0–25 Hz (~3 dB) and stored on a magnetic tape (Racal 4DS) for subsequent analysis.

Two different Cm measurements were used in these experiments: (a) microscopic (fF range) and (b) macroscopic measurements (pF range).

(a) Microscopic fluctuations in Cm were monitored in the “compensated” mode of recording (Lindau and Neher, 1988). Briefly, the contributions of the pipette to the lock-in signals were cancelled during the cell-attached configuration before patch rupture. Capacitive currents evoked either by sinusoidal or square pulse stimulation were then electronically subtracted by the Cc and Rs potentiometers. The phase angles were set such that one of the lock-in signals represented changes in Cm (capacitance signal), whereas the other one was influenced by changes in both series resistance and membrane conductance (conductance signal) (Neher and Marty, 1982; Lindau and Neher, 1988). Phase angle setting was regularly checked by simulating small changes of cell Cm by altering the level of the capacitance cancellation (artifacts on Figs. 2A, 5, and 8). Signals were low pass filtered (5–25 Hz, −3 dB, Bessel) to resolve discrete Cm steps.

(b) Macroscopic changes of Cm were monitored by “compensated” and by “uncompensated” modes of recording (Lindau and Neher, 1988). With the former method, the response of the cell was constructed from sections of continuous records obtained as for microscopic measurements, but the attenuation of the signal by the series resistance was also taken into account using the following equation:

\[ k = \frac{1 + G_m G_s}{1 + (2\pi f C_m/G_s)^2} \]  

where \( k \) represents the attenuation factor, \( G_m = 1/R_m \), \( G_s = 1/R_s \), and \( f \) is frequency. Note that \( k \) equals \( 1/T \), where \( T \) is a complex parameter described by Lindau and Neher (1988). Readings from the Cc and Rs potentiometers were used to calculate \( k \). The readings of Cc were made with an error of 2–5% if the 0–100-pF range was used, and were 10 times lower for the 0–10-pF range. However, these errors are significantly smaller when compared with the variability of resting Cm values (and Rs) of ~30%. The estimation of \( k \) is only sensitive to errors introduced by the readings of Rs and Cc if Rs values are relatively high (~20–25 MΩ), where the error introduced by the reading of Rs is <2%. Therefore \( k > 2 \) would be more influenced by errors introduced by Rs and Cc, because the second term of Eq. 1 increases with the square, whereas \( k < 2 \) would be less sensitive because the second term of Eq. 1 (<1) is diminished by squaring. The average value of \( k \) in our experiments was 1.86 ± 0.55 (\( n = 39 \)), and the reconstruction of macroscopic Cm responses were therefore not subject to error larger than the biological variability. This is shown in Fig. 2A, where measurements of Cm by the Cc potentiometer (filled circles) fits well with the reconstructed signal recorded by the microscopic (compensated) measurements.

Gm was usually constant during the experiment (see Figs. 2, B and C, 8, 9, and 10), whereas Rs and Cm were changing, and therefore \( k \) was also changing. Capacitance signals were corrected by attenuation factors that were obtained by linear interpolation every 80 s in a given recording section. Such sections are separated by gaps as seen in Fig. 2A, during which Cc and Rs potentiometers were reset. The macroscopic Cm response was obtained by playing the capacitance signal into the PDP 11/73+ computer and multiplying each 80-s segment with appropriate attenuation factors using the computer program Anadisk (T. Lamb, Cambridge University, Cambridge, UK).

The macroscopic response of the cell was also measured in the “uncompensated” mode of recording with the aid of a PC (Lindau and Neher, 1988). Signals from the lock-in amplifier (low pass filtered at 1 Hz, −3 dB), together with the d.c. current (low pass filtered at 10 Hz, −3 dB), were recorded on a four-channel chart recorder (Gould Inc., Cleveland, OH), at a bandwidth of 0–10 Hz (~3 dB) and stored on a magnetic tape (Racal 4DS) for subsequent analysis.

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dB) were digitized at 10–100 Hz (CED 1401; Cambridge Electronic Design, Cambridge, UK). The program (provided by J. Dempster, University of Strathclyde; see Lindau and Neher, 1988) used these three quantities and the value of the driving potential (holding potential – reversal potential for the d.c. current, measured as −50 mV), to calculate the parameters of the equivalent circuit: series resistance, membrane capacitance, and parallel combination of leak and membrane conductance. In Fig. 4 A the holding potential was also played into the computer. In these experiments the amplitude of the excitation signal was 1 mV p-p. All approaches to determine macroscopic Cm changes yielded similar results.

All statistics are in the form of mean ± SD unless otherwise stated; n is the number of measurements.

RESULTS

General Cell Membrane Capacitance Properties

Immediately after establishing whole-cell recording, resting Cm was determined by nullifying the capacitive currents evoked by sinusoidal or square pulse stimulation. Results obtained with both of these pulse protocols were not significantly different and were pooled. We assumed that these cells were spherical and therefore we expected resting Cm to equal \( 4\pi r^2 \epsilon \), where \( \epsilon \) equals specific membrane capacitance and \( r \) is cell radius. Cell diameter was measured and linear regression of such data gave the following relationship:

\[
C_m = (0.102 \pm 0.011) r^2 + (0.617 \pm 0.608) \quad (2)
\]

(\( C_m \) is in pF, \( r \) is \( \mu m \)) with a significant correlation coefficient of 0.788 (\( n = 53, P < 0.001 \)). Thus the mean capacitance of 6.05 ± 1.68 pF corresponds to a mean cell radius of 7.65 \( \mu m \). From the slope of the regression line, a specific membrane capacitance of 0.81 ± 0.09 \( \mu F/cm^2 \) was estimated. This value compares well with results reported for other biological membranes (Pusch and Neher, 1988; see also Almers, 1978). These results show that Cm is a good measure of membrane surface area. Scattering of the data used in regression analysis is probably due to the statistical error introduced by electrical and morphological measurements, and accounts for the small constant term in Eq. 2.

Importantly, methods for recording Cm are usually based on the assumption that the whole-cell configuration can be adequately represented by an electrical circuit consisting of a capacitor (\( C_m \)) and resistor (\( R_m \)) in parallel, and with a series resistor (\( R_s \)). The current response (Fig. 1 A, middle trace) to a 30-mV voltage step in a voltage-clamped cell can be subtracted by use of the \( C_s \) and \( R_s \) capacitance compensation circuit (Fig. 1 A, lower trace). This indicates that our recording arrangement is consistent with a single time constant circuit. In agreement with this, the decay of the uncompensated current transient is also well fitted with one exponential function (Fig. 1 B).

Ca-induced Membrane Capacitance Increase (Macroscopic Measurements)

To investigate the role of [Ca\(^{2+}\)]\(_i\) on Cm, the cell cytosol was dialyzed with pipette-filling solutions containing different Ca\(^{2+}\) activities. In Fig. 2 A (top trace) a cell was dialyzed with a high Ca\(^{2+}\)-containing solution, in which estimated [Ca\(^{2+}\)] was 750 nM. An increase in Cm by 33% more than the resting Cm was observed, taking 310 s to
reach the half-maximal value. This maximal value was usually stable, although 1 of 23 cells studied showed a slow decline following this maximum value. In four cells using 750 nM [Ca$^{2+}$], the maximal change in $C_m$ was 21–52% (33 ± 14%), with half-maximal delays ranging from 160 to 545 s (300 ± 178 s). A similar response was observed in cells dialyzed with 1,050 and 1,500 nM [Ca$^{2+}$] in Figs. 2 B and 10, respectively (see also Fig. 3).

When cells were dialyzed with a low Ca$^{2+}$-containing solution (2.85 or 10 mM EGTA in pipette solution), large increases in $C_m$ were never seen (Fig. 2 A, lower

**Figure 1.** (A) Current measured in response to a −30-mV step from a holding potential of −60 mV recorded in the tight-seal, whole-cell configuration on a bovine lactotroph cell. Stray capacitance due to the head-stage–pipette–pipette holder assembly was compensated in the cell-attached configuration before patch disruption. Middle trace, uncompensated capacitive currents; lower trace, transients canceled by slow capacitance cancellation ($C_c = 5.4$ pF; 0–100-pF range) and series resistance compensation ($R_s = 6.4$ MΩ; 1–101-MΩ range). The signal was recorded on FM tape (Racal 4DS) at 20 kHz bandwidth, played into the PDP 11/73+ computer at 16 times lower speed, low pass filtered (4 kHz, −3 dB; Kemo Filters, Beckenham, Kent, UK) and sampled at a rate of 8 kHz. The cell was cultured for 6 d before the experiment. The pipette-filling solution contained 0.1 mM EGTA ([Ca$^{2+}$]~30 nM). (B) 25 capacitive transients from the same cell were averaged and an exponential function of the form $I(t) = I_0 e^{-t/\tau}$ was fitted to the decay phase of the transient, where $t$ is time after the voltage step, $\tau$ is the time constant, $I(t)$ is the current at time $t$, and $I_0$ is peak current (strictly, $I$ at $t = 0$). It was assumed that the steady-state current during the voltage pulse was equal to the baseline current, as the parallel combination of leak and membrane resistance was typically ~ 10 GΩ. Estimated time constant using $C_m$, $R_s$ (as above), and $R_m = 10$ GΩ is 35 μs and is close to the time constant of the fitted function $\tau = 41$ μs. Underestimation of $\tau$ is probably due to the limited frequency response of the voltage pulse command. To prevent "clipping" of the headstage amplifier, voltage steps have been filtered with a 30-μs rise time filter at the input into the patch-clamp amplifier. The signal was recorded and played into the computer as above, where it was low pass filtered (10 kHz, −3 dB) and sampled at a rate of 50 kHz.
trace, and Fig. 2 C). In fact, in Fig. 2 A (lower trace), \( C_m \) started to decline from the resting value of 6.2 pF immediately after the start of dialysis. This was followed by a steady rise in \( C_m \), presumably due to an influx of \( \text{Ca}^{2+} \) through a leak or through membrane channels. A similar biphasic response was seen in 3 of 28 cells studied. In

**FIGURE 2.** The effect of cytosol dialysis with different \( \text{Ca}^{2+} \)-containing pipette-filling solutions on the macroscopic changes of membrane capacitance in a single lactotroph cell. Arrows denote the time of patch rupture, with adjacent numbers showing resting \( C_m \). (A) Filled circles are the time when resting capacitance was determined and read from the \( C_r \) potentiometer. Traces were recorded in the "compensated" mode of recording (see Methods). Top trace: \( \text{Ca}^{2+} \)-induced membrane capacitance increase in a cell voltage clamped at \(-66 \text{ mV}\). Filled triangle indicates the time when holding potential was changed to 0 mV. Cytosol was dialyzed with pipette-filling solution containing mainly K-gluconate in which estimated \([\text{Ca}^{2+}]_i\) was 750 nM (0.1 mM EGTA, 0.5 mM Ca-EGTA). The cell was 1 d in culture. (A) Bottom trace: dialysis with low \([\text{Ca}^{2+}]_i\)-containing solution; \([\text{Ca}^{2+}]_i < 30 \text{nM}\) (EGTA, 2.85 mM) induced no increase in \( C_m \), recorded in a different cell. Holding potential was \(-61 \text{ mV}\). Open triangle denotes the time when potential was changed to 0 mV. Note the lower noise in the bottom trace, which is probably due to the lower series resistance, which increased from 13 to 15 M\(\Omega\) during the experiment. Series resistance during top recording increased from 18 to 24 M\(\Omega\). The cell was 7 d in culture. B and C show macroscopic membrane capacitance changes recorded by the "uncompensated" mode of recording (see Methods). \( G_r \) is series conductance, \( G_m \) is parallel combination of leak and membrane conductance, and \( C_m \) is membrane capacitance. Resting \( C_m \) was digitally subtracted from the \( C_m \) traces and is indicated below the arrows. (B) The cell (cultured for 1 d) was held at \(-74 \text{ mV}\) and dialyzed with pipette-filling solution containing 3.5 mM Ca-EGTA and 0.5 mM EGTA ([Ca\(^{2+}\)], \~1,050 \text{nM}). (C) The cell (cultured for 3 d) was held at \(-71 \text{ mV}\) and dialyzed with pipette-filling solution containing 10 mM EGTA ([Ca\(^{2+}\)], \~50 \text{nM}).

most cells \( C_m \) slowly increased (20 cells) (Fig. 2 C), but occasionally decreased (2 cells) or stayed constant (2 cells) (see Fig. 4 B). During an experiment in which cells were dialyzed with low \( \text{Ca}^{2+} \)-containing solutions (0.1, 2.85, or 10 mM EGTA, 30 nM [Ca\(^{2+}\)]), the increase in \( C_m \) was 5 ± 3% \((n = 22, \text{ranging from 1 to 14%})\), which is
significantly smaller ($P < 0.05$, analysis of variance) than in experiments where cells were dialyzed with high Ca\textsuperscript{2+}-containing solutions (750 nM [Ca\textsuperscript{2+}]).

The Ca\textsuperscript{2+} dependence of $C_m$ is more clearly demonstrated in Fig. 3. This relationship suggests that the rise in [Ca\textsuperscript{2+}], required to induce optimal hormone secretion would be $\sim 1 \mu$M, and is consistent with results reported for the Ca\textsuperscript{2+} sensitivity of prolactin secretion by clonal cells (Ronning and Martin, 1986).

The variability of responses recorded at low Ca\textsuperscript{2+} is probably biased by the fact that the recordings were of different lengths, from 100 to 1,170 s. Therefore, the rate of the $C_m$ change was calculated, assuming this was adequately approximated by a linear function (e.g., Fig. 2 A, lower trace; Fig. 2 C; Fig. 9, initial section of the $C_m$ signal). In Fig. 4 B, the rate of change of $C_m$ (expressed relative to resting $C_m$) was plotted versus holding potential. It is apparent that the variability of these responses is not affected either by dialysis of cells with different calcium buffering capacity solutions (0.1–10 mM EGTA), or by the concentration of external Ca\textsuperscript{2+}. It also appears that the variability of responses is not affected by the age of cells studied (see numbers adjacent to the data points, indicating days in culture). However, it seems that holding potential had an effect on these responses, since a faster increase in $C_m$ was more likely if the holding potential was around $-70$ mV than if it was held at around $-80$ mV. At this latter potential the $C_m$ signal was also seen to decrease slightly after membrane rupture.

Voltage dependence of the steady $C_m$ increase can be more clearly demonstrated at
the single cell level. When the cell was depolarized from -61 to 0 mV (see triangle in lower trace, Fig. 2 A), this resulted in an increase in the rate of \( C_m \) increase from 0.002 to 0.008%/s. This may indicate that the steady increase in \( C_m \) was due to the entry of calcium ions into the cytosol through voltage-activated channels rather than through some nonspecific leak (see Discussion). A similar depolarization also induced an increase in the rate of \( C_m \) increase in the top trace (Fig. 2 A). In Fig. 4 A the \( C_m \) signal was recorded by the computer-aided “uncompensated” mode of recording and it is shown that changes in holding potential affected the time course of the \( C_m \) signal.

These results, obtained on single cells, demonstrate that a rise in intracellular \( \text{Ca}^{2+} \) plays an important role in the stimulus–secretion coupling of bovine pituitary cells. Voltage sensitivity of \( C_m \) also suggests a role for voltage-activated channels in these processes. These results are consistent with the effect of addition of high \( K^+ \) on prolactin secretion (Vale et al., 1977).

Microscopic “On” and “Off” Membrane Capacitance Steps

At this point a question arose as to whether the \( \text{Ca}^{2+} \)-induced \( C_m \) increase, as in Fig. 2 A (top) or Fig. 2 B, could be viewed as the sum of many microscopic events, such as exocytic fusions of single vesicles. Capacitance measurements on other secretory cells (Neher and Marty, 1982; Fernandez et al., 1984; Maruyama, 1986; NuBe and Lindau, 1988) have revealed small, discrete \( C_m \) steps with magnitudes in the 2–30-fF range. These have been interpreted as fusions or retrievals of single vesicles to or from the plasma membrane. We have also observed small, discrete \( C_m \) steps (Fig. 5). “On” steps denote an increase in \( C_m \) (upward deflection), consistent with an exocytotic event; “off” steps denote a decrease in \( C_m \), an endocytotic event.

These events were observed either when the \( C_m \) signal was decreasing or slowly increasing as randomly occurring steps at low frequency (~0.01 Hz), or they were

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**Figure 4. (opposite)** (A) The effect of holding potential \((V_m)\) on the time course of the capacitance changes \((C_m)\), recorded by the “uncompensated” method of recording (see Methods). Arrow indicates time of membrane rupture. Resting \( C_m \) of 6.9 pF was digitally subtracted. \( G_s \) denotes series conductance and \( G_m \) parallel combination of leak and membrane conductance. To diminish changes in membrane conductance during membrane depolarization, Na-glutamate replaced K-glucosate in the pipette-filling solution, which also contained 0.1 mM EGTA and 2 mM ATP. The cell (2 d in culture) was bathed in normal recording medium. \( G_m \) was calculated from d.c. membrane current and driving voltage, where the reversal potential was measured to be 0 mV. After membrane rupture the cell was held at -72 mV and a spontaneous increase in \( C_m \) was observed. The change in membrane potential to -17 mV resulted in an increase in \( C_m \) of ~100 fF, which stabilized at a peak of 7.2 pF before a slow decline was observed. Meanwhile, a hyperpolarization to -79 mV apparently speeded the decay after a delay of ~60 s. At the end of the experiment the holding potential was changed to -16 mV, which reversed the trend of the \( C_m \) signal. Similar changes in the time course of \( C_m \), evoked by the changes in the holding potential, were observed in six of seven cells studied. (B) The relationship between the rate of the macroscopic change in \( C_m \) (per second) relative to resting \( C_m \) (%), and the holding potential (millivolts). The rate of \( C_m \) increase was calculated for records of various lengths (110–1,170 s). Cells were dialyzed with different calcium buffering capacity solutions (circles, 0.1; triangles, 2.85; squares, 10 mM EGTA) in bathing medium containing either 1.8 mM (filled symbols) or 5.0 mM \( \text{Ca}^{2+} \) (open symbols). Numbers adjacent to symbols denote culture age in days. Pipette-filling solution as in Fig. 2.
A

\[ C_m \]

\[ V_m \]

\[ G_a \]

\[ G_m \]

B

\[ \% \Delta C_m \]

\[ s \]

\[-90\, \text{mV} \quad -80\, \text{mV} \quad -70\, \text{mV} \quad -60\, \text{mV} \]

FIGURE 4.
resolved during a more noisy appearing, rapidly fluctuating $C_m$ signal. A representative recording of steplike $C_m$ jumps is shown in Fig. 5 (middle), which is an enlargement of the initial portion of the lower trace of Fig. 2A. “On” and “off” steps were present immediately after the start of the dialysis and the number of observed events declined subsequently (see also Fig. 2A, lower trace). In other cells, steplike events were observed during the late portion of the recording. In some recordings there were more “on” than “off” steps; in others, both events were equally frequent. In rare cases an “off” step followed an “on” step (or vice versa) of the same size, as shown on Fig. 5 (bottom, left). In most cases, however, the size of steps did not appear to be related to previous events. These steplike events were observed in the absence of voltage stimulation, or if the intracellular solution was ATP free, but they were difficult to resolve with 10 mM EGTA intracellular buffer. Of 12 cells studied with 10 mM EGTA, only 2 “off” step-like events were observed. However, they were often seen with 2.85 mM or lower internal EGTA.

The amplitude of steplike events was not significantly different in conditions where $[Ca^{2+}]_{i}$ was high or low. “On” step-like events measured in two cells gave amplitudes at high $Ca^{2+}$ of $5.1 \pm 3.2 \mu F (n = 28)$ and were not significantly different to those measured at low $Ca^{2+}$ in a different cell of $6.3 \pm 2.9 \mu F (n = 41)$ (two-tailed $t$ test). The measurements were therefore pooled together.

The amplitude distribution of the steplike events, measured in 12 cells, is shown in Fig. 6. It can be seen that “on” and “off” steps are similarly distributed, with mean values of $5.2 \pm 3.6 \mu F (n = 246)$ and $5.3 \pm 3.4 \mu F (n = 236)$, respectively. To answer
the question of whether these steplike events represent the fusion and retrieval of single vesicles, a distribution of vesicle surface area is shown by the broken line (Fig. 6). Surface area was estimated from morphological data obtained by Ingram et al. (1988, their Fig. 3c) and the specific membrane capacitance of 0.8 μF/cm². There is a good correlation between the three distributions, which supports the view that discrete C_m steps are reflecting interactions of single vesicles at the level of plasma membrane.

![Figure 6. Frequency distribution of size of discrete (microscopic) on (open bars) and off steps (shaded bars) of C_m, recorded by the “compensated” method (see Methods). “On” steps denote an increase in C_m and “off” steps a decrease in C_m. Bin size is 2 fF. Broken line denotes distribution of single granule surface area (estimated from data of Ingram et al., 1988; see their Fig. 3c), with ordinate on the right, Top abscissa shows scaling for vesicle diameters calculated from vesicle surface area.](image)

The Effect of Membrane Depolarization on Membrane Capacitance

Lactotrophs are excitable and among other voltage-activated conductances possess Ca^{2+}-permeable channels. These open upon depolarization, and are thought to be involved in stimulus–secretion coupling (DeRiemer and Sakmann, 1986; Lingle et al., 1986). In Fig. 7 we isolated voltage-activated Ca^{2+} currents by replacing the pipette-filling solution and the extracellular recording medium with a solution consisting mainly of Na-glutamate. The extracellular [Ca^{2+}] ([Ca^{2+}]_o) had to be raised to 10 mM to allow for currents > 10 pA to be recorded; the peak current recorded at 5 mV was 37 pA. With only 5 mM [Ca^{2+}], the peak current was ~12 pA at 10 mV
These results are consistent with the previous report of Cobbett et al. (1987b). We have therefore studied the effect of membrane depolarization on $C_m$.

It was shown that $[\text{Ca}^{2+}]$, required for half-maximal prolactin release is $\sim 0.5$–$0.8 \mu M$ (Fig. 3 of Ronning and Martin, 1986), and that a single depolarization of 2–5 s produces a rise in $[\text{Ca}^{2+}]$, to $\sim 0.5$–$0.6 \mu M$, which decays to resting $[\text{Ca}^{2+}]$ within 10–20 s (Schlegel et al., 1987; Benham, 1989). A sustained train of depolarizing pulses summates voltage-induced $\text{Ca}^{2+}$ transients to a plateau level (Benham, 1989). We therefore expected to evoke a $C_m$ increase by membrane depolarization.

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**Figure 7.** Voltage-activated $\text{Ca}^{2+}$ currents in a single lactotroph, cultured for 2 d. The cell was voltage clamped to $-72$ mV holding potential and steps to various potentials were applied. Series conductance was 97 nS (0–1-μS range) and resting capacitance was 4.8 pF (0–10-pF range). The pipette contained 140 mM Na-glutamate, 10 mM NaCl, 2 mM MgCl$_2$, 10 mM HEPES, and 0.1 mM EGTA, while the bath contained the same solution as the pipette-filling solution, but 10 mM CaCl$_2$ and 5 μM TTX were added instead of 0.1 mM EGTA. In the upper trace the leakage current was measured by steps to $-92$ mV. Voltage-activated currents were recorded 8 min after membrane rupture. In B (leakage currents were not subtracted) the $\text{Ca}^{2+}$ current was maximally activated by a step to 5 mV. (C) The $I-V$ relationship (the peaks of averaged currents ($n = 3$) versus voltage steps) shows that the threshold for the voltage-activated $\text{Ca}^{2+}$ current is $\sim 50$ to $-40$ mV and is maximally activated at potentials positive to 0 mV. Leakage currents were subtracted. Curve was drawn by eye. Traces in A and B were low pass filtered at 3 kHz ($-3$ dB, Bessel) and acquired at a rate of 6.25 kHz. A CED 1401 A/D interface with a PC/AT computer was used to generate voltage steps and for the whole-cell current acquisition. The program was kindly provided by Mr. J. Dempster (University of Strathclyde, Glasgow, Scotland).

In our experiments on $>30$ cells, a single depolarization was inadequate to evoke an increase in $C_m$ (see Fig. 8). Adding ATP (17 cells) to the pipette solution to prevent the "rundown" of voltage-activated $\text{Ca}^{2+}$ currents yielded a similar result. Even after depolarizations of $>10$ s, the $C_m$ level after the pulse was not significantly different to that before the depolarization. These results contrast to those reported for chromaffin cells (Neher and Marty, 1982; Clapham and Neher, 1984; Bookman and Schweizer, 1988; Penner and Neher, 1989).

A sustained train of depolarizing pulses induced a small increase in $C_m$ in 8 of 12 cells studied. A typical experiment is shown in Fig. 8, in which an increase in $C_m$ of


\[ \frac{\sim 70 \text{ fF}}{} \text{was recorded during the sustained train stimulation, taking 80 s to reach the half-maximum value. This increase in } C_m \text{ is probably due to facilitated voltage-sensitive entry of Ca}^{2+} \text{ into the cytosol, as supported by the following evidence: (a) the addition of Co}^{2+}, \text{ a Ca}^{2+} \text{ channel blocker (Hagiwara and Byerly, 1981), into the bathing medium during the sustained train of depolarizations (n = 3, not shown) altered the rate of change of } C_m, \text{ indicating a role for Ca}^{2+}-\text{permeable channels in} \]

\[ \text{stimulus-secretion coupling; and (b) if the concentration of intracellular Ca}^{2+} \text{ buffer was raised from 0.1 to 10 mM EGTA, the cells did not respond to sustained depolarization trains (n = 5).} \]

As expected, the presence of 1 \text{ mM TTX and 5 mM TEA in the bathing medium did not prevent the response (n = 3), indicating the relative unimportance of voltage-activated Na}^{+} \text{ and K}^{+} \text{ channels in stimulus-secretion coupling, in agreement with the results of Cobbett et al. (1987a).} \]

\[ \text{FIGURE 8. The effect of a train of depolarizing pulses (indicated by stimulus artifacts in all traces) on resting capacitance (A) recorded in the “compensated” mode. 80-mV pulses of 210 ms were applied every 2 \text{ s from a holding potential of } -71 \text{ mV. Top trace is an enlarged trace of A, } B \text{ is the lock-in signal to which membrane conductance and series resistance are principally contributing, and } C \text{ is current. Asterisks denote readings from } C_s \text{ potentiometers of the patch-clamp amplifier (8.1 pF, [left] and 8.2 pF [right]; 0–100-pF range), and are close to artifacts used for phase adjustments. Series resistance was 9.2 M}\Omega \text{ at the beginning and end of the trace (1–101-M}\Omega \text{ range).} \]

\[ \text{Dotted line above the bottom trace indicates zero current level. Note the absence of inward current due to the presence of TTX in the bathing medium and to the limited resolution of the chart recorder. Bandwidth of } A \text{ and } B \text{ signals, 25 Hz; bandwidth of bottom trace, 10 Hz (} -3 \text{ dB). The pipette solution included 1 mM ATP, 0.1 mM cAMP, and 0.1 mM EGTA ([Ca}^{2+}] \sim 30 \text{ nM); bathing medium also contained 1 \mu \text{M TTX and 5 mM TEA. Note that the } B \text{ signal is displayed at 10 times lower gain than the } A \text{ signal. Lock-in signal, proportional to the real part of admittance, was usually noisier than the signal proportional to the imaginary component. From inspection of the current trace it is evident that the excess noise of the } B \text{ signal is not due to noise of cell conductance; rather, it is generated by series resistance. This phenomenon was observed in some cells, and a similar observation has been reported by Neher and Marty (1982). The cell was 2 d in culture.} \]

\[ \text{As expected, the presence of 1 \mu \text{M TTX and 5 mM TEA in the bathing medium did not prevent the response (n = 3), indicating the relative unimportance of voltage-activated Na}^{+} \text{ and K}^{+} \text{ channels in stimulus-secretion coupling, in agreement with the results of Cobbett et al. (1987a).} \]
The Effect of Extracellular Co\(^{2+}\) on the Spontaneous \(C_m\) Increase

In the "compensated" mode of \(C_m\) recording, addition of Co\(^{2+}\) into the bathing medium altered the rate of the depolarization-evoked \(C_m\) increase (in six of nine cells studied), and also affected the conductance signal (in eight of nine cells studied), suggesting a decrease in membrane conductance by Co\(^{2+}\). However, it should be mentioned that such a phenomenon could relate to phase setting displacement on the lock-in amplifier, and the results should be considered cautiously. To investigate

Figure 9. Effect of Co\(^{2+}\) on membrane capacitance (\(C_m\)), and parallel combination of leak and membrane conductance (\(G_a\)), recorded by the computer-assisted method ("uncompensated" mode; see Methods). \(G_a\) denotes series conductance; \(C_m\) is membrane capacitance from which resting capacitance (5.2 pF) was subtracted. The initial step indicates time of patch rupture; holding potential was −75 mV. Real and imaginary parts of admittance signals were low pass filtered at 1 Hz and d.c. membrane current at 10 Hz (all −3 dB). The pipette solution contained mainly K-gluconate (see Methods), with 0.1 mM EGTA ([Ca\(^{2+}\) \(~ 30\) nM); bathing solution with 5 mM Ca\(^{2+}\). Arrows denote Co\(^{2+}\) application; each application increased [Co\(^{2+}\)]_o by 2 mM. Bottom trace ([Ca\(^{2+}\)]_i) shows the effect of Co\(^{2+}\) application (2 mM) on resting [Ca\(^{2+}\)]_i recorded with the fura-2 fluorescent dye. The arrow indicates superfusion of Co\(^{2+}\) added to the recording medium with 5 mM [Ca\(^{2+}\)]_o. After ~ 1 min resting [Ca\(^{2+}\)]_i had dropped from ~65 nM to ~40 nM. The time course for this change may be quicker since no account has been taken of the time required for the solution in the chamber to be replaced. This effect is not due to quenching of fura-2, as similar results were obtained by superfusion of Ca\(^{2+}\)-free or bathing media with added EGTA (2 mM). The cell was 2 d in culture.

this problem further, a computer-based method for real-time estimation of \(C_m\), \(G_m\), and \(G_a\) was used (Lindau and Neher, 1988). In these experiments the effect of Co\(^{2+}\) on the spontaneous increase of \(C_m\) was tested. In seven of eight cells where a spontaneous increase in \(C_m\) was observed, this was diminished or reversed upon addition of Co\(^{2+}\) (2–8 mM; Fig. 9). The likely explanation for this is that the addition of Co\(^{2+}\) diminished or blocked the Ca\(^{2+}\) influx through Ca\(^{2+}\)-permeable channels, which was supporting the spontaneous \(C_m\) increase.
Such an influx can be demonstrated by adding Co\(^{2+}\) into the bathing medium while [Ca\(^{2+}\)]\(_i\) is measured by the fura-2 fluorescence method. In Fig. 9 (bottom trace) such treatment resulted in a decrease in resting [Ca\(^{2+}\)]\(_i\) from ~60 to 30 nM. An average resting [Ca\(^{2+}\)]\(_i\) of 63 ± 2 nM (n = 27) was recorded under such conditions and is in agreement with previous reports (Mason et al., 1988a, 1989, 1990). After the addition of Co\(^{2+}\) it decreased to 37 ± 7 nM (n = 27).

In agreement with this, it was also observed that in 9 of 13 cells studied, the addition of Co\(^{2+}\) also reduced G\(_m\) (Fig. 10). G\(_m\) is the sum of the leak and membrane conductances, and because the leak conductance is about the same order of magnitude as the membrane conductance in the cell of Fig. 9, the lack of correlation between C\(_m\) and G\(_m\) is not unexpected.

We have also tested whether the effect of Co\(^{2+}\) on C\(_m\) suppression was due to a direct inhibitory action of Co\(^{2+}\) on the exocytotic apparatus from an extracellular site on the plasma membrane. If Co\(^{2+}\) directly inhibited exocytosis, an inhibitory effect on the Ca\(^{2+}\)-induced increase in C\(_m\) would be expected. Note that the Ca\(^{2+}\)-induced response in C\(_m\) is attained by cytosol dialysis of high Ca\(^{2+}\)-containing pipette solutions. The addition of Co\(^{2+}\) after C\(_m\) reached a steady state did not suppress the Ca\(^{2+}\)-induced C\(_m\) increase in any of six cells studied. Interestingly, in two of six cells a slight increase after Co\(^{2+}\) addition was observed (Fig. 10), presumably due to an alteration of the ratio between the rates of exocytosis and endocytosis via the diminished influx of Ca\(^{2+}\). However, in a few experiments longer exposure to Co\(^{2+}\) in the bathing medium (>15 min) prevented the cell from responding to high [Ca\(^{2+}\)]\(_i\) (two of seven cells tested). This suggests that Co\(^{2+}\) may have a direct inhibitory action on C\(_m\) under these experimental conditions.
DISCUSSION

Microscopic and Macroscopic Capacitance Measurements

We have applied the patch-clamp technique to monitor membrane capacitance (as a measure of plasma membrane surface area in electrical contiguity) in bovine lactotrophs, and demonstrated, at the cellular level, a role for Ca\(^{2+}\) in hormone secretion from adenohypophyseal cells. These cells are ideal for electrophysiological investigation because they are small (low resting conductance) and spherical, and can be adequately represented by a single time-constant electrical circuit (Fig. 1). A good correlation was found between squared cell radius and \(C_m\), so changes in \(C_m\) are thought to reflect changes in cell surface area. Morphological studies (Ingram et al., 1988) have shown that the cytosol of these cells is rich in prolactin-containing secretory granules. Fusion and retrieval of single granules with the plasma membrane has been previously resolved using \(C_m\) measurements in chromaffin cells (Neher and Marty, 1982), mast cells (Fernandez et al., 1984), pancreatic acinar cells (Maruyama, 1986), and neutrophils (Nüse and Lindau, 1988). We have also detected discrete steplike events in \(C_m\) (Fig. 5), which are probably the result of single vesicle fusion and retrieval events. This is supported by the comparison of vesicle sizes estimated from electrophysiological and morphological data (Fig. 6).

It is interesting to note that the size distribution of "on" and "off" steplike events, representing single exocytotic and endocytotic phenomena, respectively, are similar, indicating a common mechanistic determinant for the granule size in both processes and supporting the recently proposed "pore" model for exocytosis (Breckenridge and Almers, 1987a, b; Zimmerberg at al., 1987). A similar observation was made in pancreatic acinar cells (Maruyama, 1986) and in mast cells (Fernandez et al., 1984), but different results were reported for chromaffin cells (Neher and Marty, 1982) where some endocytotic events were larger than the exocytotic events. It is possible that this is due to the different origin of the various granule sizes in these cells, as has been reported for chromaffin cells (Baker and Knight, 1982). However, it is also possible that apparently single, large "off" steps often seem to be composed of small steps both in chromaffin cells (Neher and Marty, 1982) and in bovine lactotrophs (not shown). Composite "off" steps could be easily overlooked because of the limited frequency response of the recording apparatus. Further investigation would yield valuable information about granule formation (Alvarez de Toledo and Fernandez, 1988a, b) and the physico-chemical concepts that might take part in regulatory and constitutive exocytosis and endocytosis.

Comparison of the macroscopic \(C_m\) responses in cells dialyzed with high \(\text{Ca}^{2+}\)-containing pipette solutions (Fig. 2) with the microscopic \(C_m\) changes (Fig. 5) indicates that the slow kinetics of the macroscopic \(C_m\) changes are limited by much slower processes than the granule fusion or retrieval event itself. These are probably occurring away from the fusion and retrieval sites and could involve many biochemical pathways, in particular those that control granule translocation. Thus the macroscopic \(C_m\) measurements seem to be a good experimental approach to investigating the role of the cytoskeleton in hormone responses; the cytoskeleton has been suggested to be an important barrier for granules diffusing to the plasma.
membrane (Cheek and Burgoyne, 1986). On the other hand, the rate-limiting step of the macroscopic \( C_m \) increase could be due to the low probability of a fusion event.

**The Role of \( \text{Ca}^{2+} \) in Stimulus Secretion Coupling in Lactotrophs**

Although \( \text{Ca}^{2+} \) is a pivotal second messenger in many secretory cell types (Penner and Neher, 1988; Rink and Knight, 1988), there is growing evidence from a number of cells that elevated \( [\text{Ca}^{2+}]_i \), alone cannot fully explain the observed secretory response. Secretory exocytosis need not be triggered by a rise in \( [\text{Ca}^{2+}]_i \), as demonstrated in platelets (Rink and Hallam, 1984), juxtaglomerular apparatus (Keeton and Campbell, 1981), parathyroid gland cells (Nemeth and Scarpa, 1987), neutrophils (Nuße and Lindau, 1988), and mast cells (Neher, 1988). Thus, second messengers other than \( \text{Ca}^{2+} \) can play an important role in stimulus-secretion coupling. Among a number of candidates, there is strong evidence for a central role of guanine nucleotide-binding proteins (G proteins). Although this is the case for nonexcitable cells, it still holds for excitable cells that a rise in \( [\text{Ca}^{2+}]_i \), is sufficient to initiate exocytotic secretion, where Ca-independent mechanisms (such as G proteins) only have modulatory influences (Penner and Neher, 1988).

Our experiments provide direct evidence to support the latter hypothesis. Cell dialysis by high \( \text{Ca}^{2+} \)-containing solution is sufficient to trigger exocytosis (Figs. 2 and 3), directly establishing a role for \( [\text{Ca}^{2+}]_i \), in stimulus-secretion coupling of bovine lactotrophs. In addition to this regulatory system, a role for G proteins as modulatory control components has been shown recently in the cells studied here (Sikdar et al., 1989), apparently via marked inhibition of Ca-dependent exocytosis. Involvement of G proteins in stimulus-secretion coupling has been found in other excitable cells (Knight and Baker, 1985; Bittner et al., 1986; Penner and Neher, 1988). Thus, the control of exocytosis in bovine lactotrophs seems to be similar to that of the prototype excitable endocrine cell (Penner and Neher, 1988).

The maximal \( C_m \) increase in chromaffin cells after cytosol dialysis by high \( \text{Ca}^{2+} \)-containing pipette-filling solutions is \( \sim 200-300\% \) (Penner et al., 1986), while in bovine lactotrophs, mouse pancreatic \( \beta \) cells, and rat pars intermedia cells, a typical increase of \( \sim 30-50\% \) is seen (Figs. 2 and 3; Penner and Neher, 1988; Zorec and Kordas, 1989). The differences in these \( C_m \) increases presumably reflect differences in the number of vesicles contained in each cell type, or different ratios of exocytosis to endocytosis.

There appears to be a small difference in the kinetic response of the exocytotic apparatus of lactotrophs compared with that of chromaffin cells. There is evidence for two phases of secretion in chromaffin cells, as short stimulation by depolarizing voltage pulses causes a small \( C_m \) increase within 5–50 ms, whereas longer depolarizations induce additional, larger increases after a delay (Bookman and Schweizer, 1988). These two "modes" may reflect fusion of docked vesicles after localized increases in \( [\text{Ca}^{2+}]_i \), beneath the plasma membrane due to short-lived \( \text{Ca}^{2+} \) influx, and the time-lagged secretion of more remote vesicles that require transport to the plasma membrane. Using patch pipettes filled with a solution known to support exocytosis in chromaffin cells (Neher and Marty, 1982), we were unable to evoke an increase in \( C_m \) in lactotrophs by single depolarizations lasting as long as 10 s.
There could be at least two explanations for the lack of response in these cells: (a) the rise in \([\text{Ca}^{2+}]_i\), which has been demonstrated in other systems (Schlegel et al., 1987; Benham, 1989), was presumably too low to trigger exocytosis; and/or (b) the exocytotic machinery was unable to respond to such fast stimuli.

The rise in \([\text{Ca}^{2+}]_i\), under our experimental conditions could be insufficient to trigger exocytosis due to the desensitization or inactivation of voltage-activated channels because of "rundown" (Cobbett et al., 1987a, b) or "washout" phenomena (Penner et al., 1987). On the other hand, a high concentration of Ca\(^{2+}\) buffers would attenuate \([\text{Ca}^{2+}]_i\) rises, as well as the possible effect of calcium buffers endogenous to the cell. To minimize channel rundown, ATP (1–3 mM) was included in the pipette solution, and the EGTA buffer concentration was kept minimal (0.1 mM). We have also recorded voltage-activated Ca\(^{2+}\) currents (Fig. 7).

Increased \([\text{Ca}^{2+}]_i\) (0.5 to 0.8 \(\mu\text{M}\)) produces an increase in \(C_m\) in slow experiments, and this concentration is achieved by short depolarizations (2–5 s) in clonal cells (Schlegel et al., 1987; Benham 1989). However, we observed that a sustained train of depolarizations was necessary to cause an increase in \(C_m\) (Fig. 8). During these experiments the rise in \([\text{Ca}^{2+}]_i\) was essential in causing the increase in \(C_m\) because: (a) addition of Co\(^{2+}\) to the bathing medium prevented the increase in \(C_m\) induced by a sustained train of depolarizations; and (b) substitution of 10 mM EGTA for 0.1 mM EGTA-containing intracellular buffer also prevented the increase in \(C_m\) induced by a sustained train of depolarizations.

Ideally we would have liked to measure \(C_m\) and \([\text{Ca}^{2+}]_i\), directly, but it has already been reported for clonal cells that during a train of depolarizing pulses \([\text{Ca}^{2+}]_i\) rises to a plateau level of \(~0.4–0.5\ \mu\text{M}\). The similarity of these concentrations to levels occurring during a single short depolarization leads us to conclude that the duration of raised \([\text{Ca}^{2+}]_i\), is very important. The timescale of the changes in \(C_m\) are similar to those occurring when cells were dialyzed by high Ca\(^{2+}\) (Figs. 2 and 10). In separate experiments using direct fura-2 measurement of \([\text{Ca}^{2+}]_i\), the thyrotropin-releasing hormone–stimulated transient rise associated with prolactin release in these cells is characterized by a half-life of 20–30 s (Mason et al., 1989).

The lack of \(C_m\) response to a short depolarization in bovine lactotrophs is therefore probably due to the slow response time of the exocytotic apparatus. This could reflect the absence of the "fast mode" of secretion in these cells (Bookman and Schweizer, 1988). If this is the case, an interesting correlation could be found between the two cell types. Chromaffin cells are adapted to respond quickly upon stimulation, while lactotroph cells are not innervated and are involved in regulation of much slower processes, such as lactation in mammals. Spectrophotometric measurements of \([\text{Ca}^{2+}]_i\), under voltage-clamp conditions should clarify these possibilities.

**The Role of Electrical Activity in Prolactin Secretion**

Adenohypophysial cells are excitable and possess voltage-activated Ca\(^{2+}\) channels (Kidokoro, 1975; Taraskevich and Douglas, 1977). Thus it was logical to suggest that electrical excitability in this group of endocrine cells was a general phenomenon and that control of the firing frequency of Ca\(^{2+}\)-dependent action potentials might play a key role in basal and stimulated secretion (Taraskevich and Douglas, 1984; Ozawa and Sand, 1986). Bovine lactotrophs are excitable (Ingram et al., 1986), and among
other voltage-activated channels possess at least two types of Ca\textsuperscript{2+}-permeable channels (Cobbett et al., 1987b; Fig. 7). However, the role of electrical activity in the control of Ca\textsuperscript{2+} entry and thus in the control of basal and stimulated secretion is uncertain, as these cells are largely devoid of spontaneous electrical activity (Ingram et al., 1986; Cobbett et al., 1987a). Although our experiments provide support for the role of voltage-activated Ca\textsuperscript{2+} permeating channels in stimulus-secretion coupling in lactotrophs (Figs. 4A and 8), we also find some evidence for an alternative mechanism for the regulation of hormone secretion, which may involve the control of the tonic influx of Ca\textsuperscript{2+}, viz.

(a) At resting membrane potential, a spontaneous increase in C\textsubscript{m} was observed in ~70% of cells studied, which is probably supported by a tonic influx of Ca\textsuperscript{2+} through the membrane. In Fig. 9 (bottom trace) it is shown that resting [Ca\textsuperscript{2+}]\textsubscript{i} is determined by such an influx and is revealed by the addition of Co\textsuperscript{2+}.

(b) Channels involved in supporting such an increase in C\textsubscript{m} are most probably Ca\textsuperscript{2+}-permeable channels as the C\textsubscript{m} increase can be diminished or reversed by the addition of the Ca\textsuperscript{2+}-permeable channel blocker Co\textsuperscript{2+} to the bathing medium (Fig. 9).

(c) Depolarization speeds the C\textsubscript{m} increase (Figs. 2A, 4A, and 8), which suggests the involvement of voltage-activated channels.

(d) The addition of Co\textsuperscript{2+} also attenuated the G\textsubscript{m} signal (parallel combination of leak and membrane conductance) in 69% of cells, suggesting a steady influx of Ca\textsuperscript{2+} ions at rest.

(e) Such an influx is consistent with the steady-state inactivation properties of Ba\textsuperscript{2+}-permeant, voltage-activated channels, which predicts a small channel population to be open even at membrane potentials around \(-80\) mV (Cobbett et al., 1987b). Most of our experiments have been performed at a holding potential of \(-70\) mV, and if one takes into account the junction potential of approximately \(-5\) to \(-8\) mV, a small fraction of voltage-activated Ca\textsuperscript{2+} channels would be open. Resting currents of 2–10 pA have been recorded at these potentials. It is estimated that these currents are large enough to carry a sufficient Ca\textsuperscript{2+} flux into the cytosol to produce a significant change in [Ca\textsuperscript{2+}]\textsubscript{i} (see Neher, 1988). Therefore, the spontaneous increase in C\textsubscript{m} could be due to an influx of Ca\textsuperscript{2+} through voltage-activated channels at resting conditions.

(f) Fura-2 imaging experiments (Fig. 9) have revealed resting [Ca\textsuperscript{2+}]\textsubscript{i} to be <100 nM, but recent imaging experiments on bovine lactotrophs show [Ca\textsuperscript{2+}]\textsubscript{i} gradients in the cytosol, with larger Ca\textsuperscript{2+} activity beneath the plasma membrane, indicating a tonic influx of Ca\textsuperscript{2+} at resting conditions (Mason et al., 1988a, 1990).

Finally, dynamic imaging has shown that Ca\textsuperscript{2+} homeostasis in these cells is devoid of periodic transient rises in [Ca\textsuperscript{2+}]\textsubscript{i} (Mason et al., 1989; Akerman, S. N., R. Zorec, T. R. Cheek, R. B. Moreton, W. T. Mason, and M. J. Berridge, unpublished results; see also Fig. 9, bottom trace), which supports previous electrophysiological results (Ingram et al., 1986; Cobbett et al., 1987a) on the absence of spontaneous electrical activity in these cells. All this taken together is in agreement with the view that a tonic influx of Ca\textsuperscript{2+} at rest is sufficient to support exocytosis in bovine lactotrophs in vitro.

This mechanism might participate in the control of basal and stimulated prolactin secretion, which is believed to be regulated via the hypothalamic regulators, dopamine and thyrotropin-releasing hormone, the former being an inhibitor and the
latter a stimulant. These agents are thought to act via the control of \([Ca^{2+}]_i\), partly through the modulation of the influx of \(Ca^{2+}\) (Mason et al., 1988a, 1989). Our experiments demonstrate the existence of such an influx of \(Ca^{2+}\), sufficient to support exocytosis. This \(Ca^{2+}\) influx is probably expressed to a larger extent in our conditions, as the tonic inhibitor of prolactin secretion, dopamine, was absent during cell culture (Ben-Jonathan, 1985). Further experiments are needed to demonstrate at the single cell level the effects of these regulators on \(C_m\), which would unequivocally demonstrate their role in prolactin secretion. Preliminary experiments have shown this to be a difficult task as whole-cell recording conditions, although having many advantages, also pose some unwanted conditions, such as the "wash-out" of the important soluble substances linking biochemical control pathways (Penner et al., 1987).

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