CALCIUM GRADIENTS AND BUFFERS IN BOVINE CHROMAFFIN CELLS

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SUMMARY

1. Digital imaging and photometry were used in conjunction with the fluorescent Ca$^{2+}$ indicator, Fura-2, to examine intracellular Ca$^{2+}$ signals produced by depolarization of single adrenal chromaffin cells.

2. Depolarization with a patch pipette produced radial gradients of Ca$^{2+}$ within the cell, with Ca$^{2+}$ concentration highest in the vicinity of the plasma membrane. These gradients dissipated within a few hundred milliseconds when the voltage-gated Ca$^{2+}$ channels were closed.

3. Dialysis of Fura-2 into the chromaffin cell caused concentration-dependent changes in the depolarization-induced Ca$^{2+}$ signal, decreasing its magnitude and slowing its recovery time course. These changes were used to estimate the properties of the endogenous cytoplasmic Ca$^{2+}$ buffer with which Fura-2 competes for Ca$^{2+}$.

4. The spatially averaged Fura-2 signal was well described by a model assuming fast competition between Fura-2 and an endogenous buffer on a millisecond time scale. Retrieval of calcium by pumps and slow buffers occurs on a seconds-long time scale. No temporal changes indicative of buffers with intermediate kinetics could be detected.

5. Two independent estimates of the capacity of the fast endogenous Ca$^{2+}$ buffer suggest that 98–99 % of the Ca$^{2+}$ entering the cell normally is taken up by this buffer. This buffer appears to be immobile, because it does not wash out of the cell during dialysis. It has a low affinity for Ca$^{2+}$ ions, because it does not saturate with 1 μM-Ca$^{2+}$ inside the cell.

6. The low capacity, affinity and mobility of the endogenous Ca$^{2+}$ buffer makes it possible for relatively small amounts of exogenous Ca$^{2+}$ buffers, such as Fura-2, to exert a significant influence on the characteristics of the Ca$^{2+}$ concentration signal as measured by fluorescence ratios. On the other hand, even at moderate Fura-2 concentrations (0.4 mM) Fura-2 will dominate over the endogenous buffers. Under these conditions ratiometric Ca$^{2+}$ concentration signals are largely attenuated, but absolute fluorescence changes (at 390 nm) accurately reflect calcium fluxes.
INTRODUCTION

Transient rises in intracellular Ca\(^{2+}\) concentration, [Ca\(^{2+}\)], are signals for exocytosis in many secretory cells (Douglas, 1968; Katz, 1969; Parnas, Dudel & Parnas, 1983; Augustine, Charlton & Smith, 1987; Penner & Neher, 1988). The magnitude and spatiotemporal distribution of these [Ca\(^{2+}\)]\(_{i}\) signals are a consequence of the interplay between cellular Ca\(^{2+}\) sources and Ca\(^{2+}\) removal mechanisms (Zucker, 1989; Sala & Hernandez-Cruz, 1990). In adrenal chromaffin cells, depolarization-induced [Ca\(^{2+}\)]\(_{i}\) signals are due to Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) channels (Febewick, Marty & Neher, 1982; Knight & Kesteven, 1983; O’Sullivan, Cheek, Moreton, Berridge & Burgoyn, 1989) and perhaps additional release of Ca\(^{2+}\) from internal stores (O’Sullivan et al. 1989). Cells remove Ca\(^{2+}\) via a variety of mechanisms, including transmembrane Na\(^{+}-\)Ca\(^{2+}\) exchange (Pocock, 1983) and Ca\(^{2+}\) sequestration into organelles via ATP-driven pumps (Burgoyn, Cheek, Morgan, O’Sullivan, Moreton, Berridge, Mata, Colyer, Lee & East, 1989). By analogy with other cells (Blinley, 1978), it is likely that chromaffin cells also possess cytoplasmic Ca\(^{2+}\) buffers but these have not yet been characterized.

The previous paper quantified the Ca\(^{2+}\) requirements for secretion in chromaffin cells and showed that depolarization produces substantial changes in [Ca\(^{2+}\)]\(_{i}\) at secretory sites (Augustine & Neher, 1992). The discrepancy between the magnitudes of the [Ca\(^{2+}\)]\(_{i}\) levels deduced to be present at the secretory sites and actual measurements of spatially averaged [Ca\(^{2+}\)]\(_{i}\) signals suggested that spatial gradients occur during stimulation. In this paper we use the fluorescence Ca\(^{2+}\) indicator, Fura-2 (Grynkiewicz, Poenie & Tsien, 1985), and digital microscopy to examine more directly the spatiotemporal properties of the [Ca\(^{2+}\)]\(_{i}\) signal evoked by depolarization. We demonstrate that [Ca\(^{2+}\)]\(_{i}\) gradients are present during stimulation. We also examine the properties of the endogenous Ca\(^{2+}\) buffer that shapes the [Ca\(^{2+}\)]\(_{i}\) signal and find that this buffer normally binds most of the Ca\(^{2+}\) entering the cell. However, because this buffer is immobile and does not saturate at [Ca\(^{2+}\)]\(_{i}\) levels as high as 1 \(\mu\)M, the addition of exogenous buffers, such as Fura-2, can alter the form of the [Ca\(^{2+}\)]\(_{i}\) signal measured during depolarization. A preliminary report of some of this work has appeared (Augustine & Neher, 1990).

METHODS

The methods used for patch clamp recording from single chromaffin cells are described in the previous paper (Augustine & Neher, 1991). The standard internal dialysis solution contained (in mM): 140 sodium glutamate, 8 NaCl, 1 MgCl\(_2\), 2 Mg-ATP, 0.3 GTP, 10 Na-HEPES (pH 7.2) and a variable amount of K\(_2\)-EGTA or K\(_2\)-Fura-2 (Molecular Probes, Eugene, OR, USA). In imaging experiments, 0.5 mM EGTA was used in conjuction with 0.1-0.5 mM Fura-2. Other experiments used an EGTA and 0.05-0.4 mM Fura-2. The external solution contained (in mM): 120 NaCl, 2.5 CaCl\(_2\), 20 tetraethylammonium-chloride, 2 MgCl\(_2\), 50 glucose, 10 Na-HEPES (pH 7.2), and 1 \(\mu\)M-tetrodotoxin (TTX).

Images of [Ca\(^{2+}\)]\(_{i}\) signals

Digital imaging methods were used in combination with Fura-2, to examine spatial gradients in [Ca\(^{2+}\)]\(_{i}\) in single chromaffin cells during depolarization. Conventional ratiometric measurements of [Ca\(^{2+}\)]\(_{i}\) with Fura-2 require alternation of the excitation light source between two wavelengths (Tsien & Poenie, 1986). However, the temporal resolution of this method is limited, because of the time required for switching the wavelength of the excitation light and for acquiring images at each of the two wavelengths. In order to minimize the time resolution of our measurements, we used a single-excitation wavelength ratio method (Smith, Osses & Augustine, 1988). In this method, the Fura-2 is excited at a single, Ca\(^{2+}\)-sensitive wavelength, in our case 350 nm (10 nm bandwidth), and the changes in Fura-2 fluorescence emission (at 506-530 nm, \(\Delta F(0)\)) are measured following a stimulus that elevates [Ca\(^{2+}\)]. To correct for non-uniformities in path length and dye concentration, \(\Delta F(0)\) is divided by the pre-stimulus fluorescence, \(F(0)\). Thus, the ratio is:

\[
\frac{\Delta F(t)/F(0)}{= \frac{F(t) - F(0)}{F(0)}}
\]

where \(F(t)\) is the fluorescence at any time later than the time at which \(F(0)\) was measured. Such a ratio is a unique function of the stimulus-induced change in [Ca\(^{2+}\)]\(_{i}\), (see eqn (3), below) as long as the path length and Fura-2 concentration do not change during the measurement. In the experiments reported here, there were no obvious stimulus-dependent changes in path length (measured at excitation at 386 nm, the insensitive (Ca\(^{2+}\)-independent) wavelength of Fura-2). Dye concentration was kept constant both by waiting until dialysis of Fura-2 into the cells had reached a maximum (typically 60-120 s after establishing continuity between the pipette and cytoplasm) and by avoiding bleaching of the dye through the use of a quartz neutral density filter (10-50% transmission) and a Unibrite (Rochester, NY, USA) electronic shutter in the fluorescence excitation optical path to minimize illumination of the Fura-2.

The apparatus used to obtain digital images of [Ca\(^{2+}\)]\(_{i}\) changes was similar to that described in Smith et al. (1988) and Kasai & Augustine (1990). In brief, an inverted microscope (Zeiss IM-35) fitted with a 100 x oil immersion objective (Zeiss Plan 100, numerical aperture = 1.25) was used to excite the intracellular Fura-2 and collect the resultant fluorescence signals. The size of the illumination beam was kept to a minimum (ca 30 \(\mu\)m diameter) with a diaphragm, in order to minimize the depth of field of the fluorescence measurements (Hiraoka, Sedat & Agard, 1990). Fluorescence signals measured with Fura-2 in the cells, as well as background signals measured before Fura-2 was dialysed into the cells, were acquired with an STV video camera (Cohu, model 5000) and the video images were stored on an analog, optical disc storage device (Panasonic TQ-250F). These images were then digitized off-line, using a Matrox MVP-AT frame grabber in an AST Premium 286 computer, and manipulated with computer programs written in C language by Stephen J. Smith. Eight-bit pseudocouloour look-up tables were used to encode the magnitude of the...
& Adams, 1990). The peak change in [Ca\textsuperscript{2+}] is orders of magnitude lower than would be expected if all the Ca\textsuperscript{2+} entering the cell was free and distributed evenly in the cell volume (McBurney & Neering, 1985). Thus, there must be fast Ca\textsuperscript{2+} buffers in addition to the slow uptake and removal systems which govern the recovery time course. Since the two processes are kinetically well

$$\frac{\Delta F(t)}{F(0)}$$ \text{ signals within each element of the 480} \times 512 \text{ pixel array (see Fig. 4). The resultant pseudocolor maps were then masked to exclude extracellular fluorescence signals. The boundaries of the mask were determined by an intensity threshold, with the value of this threshold adjusted until the non-masked area corresponded to the area occupied by the cell, as determined from bright-field images.}

The effective spatial and temporal resolutions of the entire optical system were assessed independently. Spatial resolution was estimated from the profile of fluorescence in a cell loaded with Fura-2. The fluorescence within the cell was not uniform (Fig. 1A), due to the spherical geometry of the cell and the tendency of Fura-2 signals to be brightest in the nuclear region of the cell (see Results). In addition, the fluorescence signal extended beyond the boundaries of the cell (determined from examination under bright-field illumination), due to blurring. The lateral resolution of the system, measured from the distance required for this extracellular fluorescence to decay to half of its maximum, was approximately 2–3 μm (Fig. 1A). The temporal resolution of the system was determined by using a green light-emitting diode in the optical pathway and recording the response to light emitted by a current pulse 500 ms in duration. The time required for the recorded light signal to rise from 10–90% of its maximum was in the order of 100 ms (Fig. 1B), with similar values also measured during the decay of the light signal. Similar response times were observed when Fura-2-loaded cells were briefly illuminated by transiently opening the electronic shutter. The temporal response of the system presumably is limited by the SIT video camera (Inoue, 1985).

In single-wavelength measurements with Fura-2 at 390 nm, if the dye concentration, path length and intensity of excitation light are constant, then the fluorescence signal will lie between two extremes – $F_{\text{max}}$, the fluorescence of the dye when completely bound to Ca\textsuperscript{2+}, and $F_{\text{min}}$, the fluorescence when completely unbound to Ca\textsuperscript{2+}. The relationship between Ca\textsuperscript{2+} concentration, [Ca\textsuperscript{2+}], and fluorescence intensity ($F$) is:

$$[\text{Ca}^{2+}] = K_F (F_{\text{max}} - F) / (F - F_{\text{min}})$$

where $K_F$ is the dissociation constant for Ca\textsuperscript{2+} binding to Fura-2. Combining eqns (1) and (2) yields the following expression for the fluorescence change ratio:

$$\frac{\Delta F(t)}{F(0)} = K_F (F_{\text{max}} - [\text{Ca}^{2+}]_0) / (F_{\text{min}} - [\text{Ca}^{2+}]_0)$$

where $[\text{Ca}^{2+}]_0$ is the initial (resting) value of [Ca\textsuperscript{2+}], $[\text{Ca}^{2+}]_a$ is the value of [Ca\textsuperscript{2+}] during a response, and $K_F = K_F (F_{\text{max}} - F_{\text{min}})$.

This relationship permits changes in fluorescence ratio to be converted into absolute [Ca\textsuperscript{2+}] levels by solving for [Ca\textsuperscript{2+}]. This requires knowledge of [Ca\textsuperscript{2+}]_a which was determined experimentally by alternating exciting the cell with 390 and 360 nm light and calculating the ratio of emission produced by the two wavelengths. This ratio was converted into [Ca\textsuperscript{2+}] using the calibration procedure described in Neher (1989).

We directly tested the validity of this strategy by measuring the changes in fluorescence produced by depolarization of cells dialysed with 100 μM-Fura-2. These cells were alternately excited at either 390 or 360 nm, using the filter wheel described in Neher (1989), and 2-wavelength ratios were calculated from the resultant fluorescence signals. This was then used to calculate [Ca\textsuperscript{2+}]_a as described by Neher (1989) and Augustine & Neher (1992). These signals were then compared to single-wavelength ratios of $\Delta F(t)/F(0)$, measured from the 390 nm signals produced during the same trials used to calculate [Ca\textsuperscript{2+}]_a with the 3-wavelength method. The relationship between [Ca\textsuperscript{2+}]_a and $\Delta F(t)/F(0)$ is shown for two different cells in Fig. 2. One cell had a [Ca\textsuperscript{2+}]_a of 100 nM, and the other had a resting [Ca\textsuperscript{2+}]_a of 270 nM. The predictions of eqn (3) are also plotted, for [Ca\textsuperscript{2+}]_a of 100 or 270 nM (smooth lines). The close correspondence of the experimental values to the predictions of the equation verifies the calibration scheme for the single-wavelength ratios.

### Measurement of the Ca\textsuperscript{2+} binding properties of cytoplasm

**Fura-2 signals under whole-cell patch clamp conditions**. In many neurone-like cells, [Ca\textsuperscript{2+}] rapidly increases during depolarization (in the 10–100 ms time range) and then recovers over a much slower time scale (typically in the range of tens of seconds) (Thayer & Miller, 1990; Hernandez-Cruz, Sala

$$\frac{d([\text{Ca}^{2+}]_a)}{dt} + d([\text{Ca}^{2+}]_a)/dt + d([\text{Ca}^{2+}]_a)/dt = (j_n - j)/v$$

where $[\text{Ca}^{2+}]_a$ is the concentration of a mobile buffer (such as Fura-2) in its Ca-bound form, [SCa] is the concentration of fixed (endogenous) Ca\textsuperscript{2+} buffer in the calcium-bound form, and $v$ is the accessible volume of the cell. For the purpose of this discussion we will consider B and SCa as the exogenously added buffer and $\beta$ and SCa as the endogenous buffer, which for the moment is assumed to be non-mobile. The quantity $j_n$ in eqn (6) is the flux of Ca\textsuperscript{2+} from the pipette (mainly that of BCa) and is given by (see eqn (4) of Mathias et al. (1990)):

$$j_n = ([\text{BCa}] - [\text{Ca}^{2+}]_a)D_B/\rho_0$$

where $R_p$ is the pipette resistance, $\rho$ is the specific resistance of the pipette filling solution and $D_B$ is the diffusion coefficient of the exogenous buffer (assumed to be the same in the Ca\textsuperscript{2+}-bound and -free form). The subscript P denotes pipette-related quantities.
The quantity \( f \) in eqn (6) is the flux across membranes (both across the plasma membrane and across storage organelles). For simplicity, this is assumed to be proportional to the deviation in [Ca\(^{2+}\)] from a steady state value [Ca\(^{2+}\)]\(_{ss}\):

\[ f = \gamma([Ca^{2+}] - [Ca^{2+}]_{ss}). \]  
(7)

Fig. 3. Model used to analyze Ca\(^{2+}\) fluxes in a pipette-cell assembly. It is assumed that the indicator dye (B, free form; BCa, Ca\(^{2+}\)-bound form) exchanges slowly by diffusion between pipette and cell. Inside the cell it competes with a fast, endogenous buffer S; calcium also is slowly removed by uptake mechanisms into intracellular stores and pumped across the plasma membrane (for details, see text).

where \( \gamma \) and [Ca\(^{2+}\)]\(_{ss}\) are constants reflecting the combined action of pumps, exchange carriers and membrane conductances. For the case of impulse-like depolarizations, and corresponding pulse-like influxes of calcium through voltage-activated channels, eqn (7) has to be extended:

\[ f = \gamma([Ca^{2+}] - [Ca^{2+}]_{ss}) - C_{new} \delta(t-t_s). \]  
(8)

where \( \delta(t-t_s) \) is the delta function and \( C_{new} \) is the integral calcium influx during the pulse.

In this analysis the cell is assumed to be in spatial equilibrium. This should be valid for times greater than 1 s, because spatial gradients dissipate within 100-300 ms in chromaffin cells of approximately 15 \( \mu \)m diameter (see below). Combining eqns (6), (8) and (8) yields:

\[ \frac{d[Ca^{2+}]}{dt} = \frac{(dS)}{dt} = \frac{(\gamma([Ca^{2+}]) - (C_{new} \delta(t-t_s)))}{(1 + \epsilon_0 + \epsilon_s)} \]

\[ + \gamma([Ca^{2+}]_{ss} - [Ca^{2+}]) + C_{new} \delta(t-t_s)/\gamma, \]  
(9)

where the calcium binding capacities of B and S respectively have been introduced as

\[ \epsilon_0 = \frac{d[B]}{d[Ca^{2+}]} \quad \text{and} \quad \epsilon_s = \frac{d[S]}{d[Ca^{2+}]}, \]  
(10)

and [BCa] has been replaced by its equilibrium value:

\[ [BCa] = [Ca^{2+}]_B/[B]_0/(1 + [Ca^{2+}]_K_B). \]  
(11)

Here, \([B]_0 \) is the total concentration of buffer B in the cell and \( K_B \) is its binding constant for calcium, while \([S]_0 \) and \( K_S \) refer to the same parameters for buffer S. Combining eqns (10) and (11) we obtain:

\[ \epsilon_s = K_B/[B]_0/(1 + [Ca^{2+}]_K_B)^3. \]  
(12)

\([B]_0 \) changes with time as buffer is dialysed into the cell. These changes in \([B]_0 \) can be used to measure the impact of \([B]_0 \) on cell buffering and deduce the relative contribution of endogenous Ca\(^{2+}\) buffers to the total amount of buffering within the cell. Below we describe two different approaches to estimating endogenous Ca\(^{2+}\) binding capacity; one based on the kinetic changes in the Ca\(^{2+}\) signal produced by adding Purin-2 and a second based on the amount of Ca\(^{2+}\) bound to the added Purin-2.

Method 1 - Analysis of the kinetics of the Ca\(^{2+}\) signal. By analogy to eqns (5) and (6), the changes in Purin-2 concentration within the cell, \([B]_0 \), occurring as the dye diffuses between pipette and cytoplasm can be described by a kinetic equation that reflects conservation of B:

\[ d[BCa]/dt = ([BCa]_0 - [BCa])D_B/B_p/R_p \varphi, \]  
(13)

with \([B]_0 \) the total concentration of B in the pipette. Here the simplifying assumption has been made that the diffusion coefficient of B is the same in the Ca\(^{2+}\)-bound and -free forms. By this simplification, eqn (13) can be solved independently of eqn (9). Eqn (13) predicts that \([B]_0 \) will exponentially approach \([BCa]_0 \) with a loading time constant \( \tau_B \) of:

\[ \tau_B = D_B/B_p/\varphi. \]  
(14)

This loading time constant can be measured when observing the fluorescence signal at 360 nm, the wavelength where Purin-2 fluorescence is independent of its Ca\(^{2+}\) binding state.

After \([B]_0 \) has reached its maximum level, the only time-dependent quantity on the right-hand side of eqn (6) is [Ca\(^{2+}\)]. For small deviations from steady state, eqn (6) can be linearized with respect to [Ca\(^{2+}\)], yielding exponential relaxations towards the steady state with the time constant, \( \tau \):

\[ \tau = \frac{1 + \epsilon_0 + \epsilon_s}{\gamma + D_B/B_p/\varphi}. \]  
(15)

Equation (15) is best discussed in terms of a corrected time constant, \( \tau' \), which takes into account diffusion of buffer between cell and patch pipette. \( \tau' \) is defined as:

\[ \tau' = \frac{1}{(1-\epsilon_0-\epsilon_s)} \]

(16)

which, together with eqns (14) and (15), yields

\[ \tau' = \frac{1 + \epsilon_0 + \epsilon_s}{\gamma + (D_B/B_p)/\varphi}. \]  
(17)

This quantity is linear with respect to \( \epsilon_0 \). Thus eqn (17) predicts a straight line if \( \tau' \) values are plotted against \( \epsilon_0 \). Experimentally, \( \epsilon_0 \) can be varied either by adding different amounts of Ca\(^{2+}\) buffer to the pipette or by varying [Ca\(^{2+}\)] (eqn (12)). The negative y-axis intercept of such a plot is \( 1 + \epsilon_0 \), yielding the endogenous Ca\(^{2+}\)-binding capacity of the cell. It should be pointed out, however, that \( \tau' \) depends critically on \( \gamma \) (the second term in the denominator of eqn (17) very often is only a small correction). \( \gamma \), according to eqn (7), reflects the combined action of pumps and other calcium removal mechanisms, which may depend on [Ca\(^{2+}\)], on the cell history, and on many other factors. Extrapolating such plots, therefore, may not be very reliable.

Method 2 - Analysis of the amount of Ca\(^{2+}\) bound to Purin-2. A more straightforward estimate of the endogenous binding capacity can be obtained by examining increments in [BCa] during short pulses of Ca\(^{2+}\) influx. Integrating eqn (9) over the short period of influx yields:

\[ \Delta[Ca^{2+}] + \Delta[BCa] + \Delta[SCa] = C_{new}/\gamma, \]  
(18)

where all but one term on the right side of eqn (9) have been neglected, assuming that the integration time is brief relative to \( \tau \), the time constant for the exponential removal of Ca\(^{2+}\) from the cell.
Measurement of fluorescence decrements and calcium current integrals

The ratio \( f \), as defined in eqn (24), requires measurement of two parameters during depolarizing pulses: the fluorescence decrement at 390 nm and the Ca\(^{2+} \) current integral. For this purpose fluorescence at both 300 and 390 nm was sampled every 500 ms as described in the companion paper (Augustine & Neher, 1992). Linear regression was used to fit straight lines to segments of the 300 nm trace (each 4–10 sample points in length) both before and immediately following depolarizing pulses. Both lines were extrapolated to the time of the stimulus, and the difference between the two values was taken as the fluorescence decrement.

Ca\(^{2+} \) currents in response to depolarizing stimuli were measured, employing a P/4 technique (Armstrong & Bezanilla, 1977). In some cases there were residual Na\(^{+} \) currents not eliminated by the TTX in the external saline (see Fig. 7 for a worst-case example). In these cases Ca\(^{2+} \) current integral was estimated as the product of the plateau inward current and the length of the depolarizing pulse.

Determination of \( K_{p} \), the binding constant of Fura-2

Both methods for determining \( \kappa_{p} \) require calculation of \( \kappa_{p} \), which in turn relies on knowledge of \( K_{\text{p}} \), the calcium dissociation constant of Fura-2. In this case, dual excitation-wavelength ratio measurements require one of the excitation wavelengths used to measure \( R \) at the isosbestic point for Fura-2, as described below. For determination of \( K_{\text{p}} \), the effective calcium dissociation constant for Fura-2, was already defined in eqn (4) in terms of fluorescence parameters measured at a single calcium-sensitive wavelength \( [Ca^{2+}]_{\text{free}}/[Ca^{2+}]_{\text{total}} \). When the other excitation wavelength is at the isosbestic point (\( \sim 360 \) nm), the fluorescence intensities of the free and bound forms of Fura-2, \( F_{\text{free}} \) and \( F_{\text{bound}} \), are equal. Multiplying both the numerator and denominator of eqn (4) by this value yields:

\[ K_{\text{p}} = K_{\text{p}} \left( \frac{F_{\text{free}}}{F_{\text{bound}}} \right) \]

Because \( R_{\text{min}} = F_{\text{free}}/F_{\text{bound}} \) and \( R_{\text{max}} = F_{\text{free}}/F_{\text{bound}} \), then:

\[ K_{\text{p}} = K_{\text{p}} \left( R_{\text{min}}/R_{\text{max}} \right) \]

and \( K_{\text{p}} \), the inverse of \( K_{\text{p}} \), is:

\[ K_{\text{p}} = R_{\text{max}}/R_{\text{min}} \]

Thus, the calcium binding constant of Fura-2 in cytoplasm can readily be calculated from the three constants determined during calibration of dual-wavelength ratio measurements.

The numerical value of \( f_{\text{max}} \)

Estimation of \( \kappa_{p} \) as in Method 2 also requires determination of \( f_{\text{max}} \), the fraction of incoming calcium captured by Fura-2 at saturating concentrations. Whereas only relative numbers, \( f/f_{\text{max}} \), are required for evaluation of \( \kappa_{p} \), it is also important to know whether the absolute value of \( f_{\text{max}} \) is compatible with the expectation that, at high dye concentration, Fura-2 should capture all of the calcium entering the cell. If \( S_{\text{app}}, S_{\text{c}} \), and \( S_{\text{r}} \) are specific fluorescence intensities for the free (f) and bound (b) subscripts) forms of Fura-2 measured at excitation wavelengths 2 and 1, as defined by Grynkiewicz et al. (1985), then \( R_{\text{max}} \) and \( R_{\text{min}} \) are given by \( S_{\text{app}}/S_{\text{b}} \) and \( S_{\text{c}}/S_{\text{b}} \), respectively. When excitation wavelength 1 is at the isosbestic point, a simple relationship can be obtained between the relative fluorescence change during a stimulus (\( \Delta F_{\text{free}}/F_{\text{free}} \)) and the integral of the calcium current. In this case, the ratio \( \Delta F_{\text{free}}/F_{\text{free}} \) is:

\[ \frac{\Delta F_{\text{free}}}{F_{\text{free}}} = \frac{\Delta (\text{BOCs})}{[\text{BOCs}]} \frac{[\text{Ca}^{2+}]_{\text{free}}}{[\text{Ca}^{2+}]_{\text{total}}} = \frac{\Delta (\text{BOCs})}{[\text{BOCs}]} \left( \frac{R_{\text{max}} - R_{\text{min}}}{R_{\text{max}} - R_{\text{min}}} \right) \]

where use again has been made of \( S_{\text{app}} = S_{\text{b}} \).
If the concentration of Fura-2 is sufficiently high, then all of the Ca^{2+}-influx will be taken up by Fura-2 and \( \Delta [Ca] \) will be proportional to the calcium current integral according to:

\[
\Delta [Ca] = \int -I_{Ca} dt / (2FV) 
\]

(37)

where \( F \) is the Faraday constant and \( V \) is the cell volume. Thus, the value of the ratio \( f_{max} \) (eqns (24) and (25)) should be given by:

\[
f_{max} = \frac{\Delta R_{max}}{I_{Ca} \int dt} = \frac{E_{max}(R_{max} - R_{min})}{2FV[B_2]R_{max}R_{max}}.
\]

(38)

Here, \( E_{max} \) and \( \Delta R_{max} \) should be measured in the same (but otherwise arbitrary) units, \( v \) is the accessible volume of the cell and \( [B_2] \), after complete loading, is assumed to be equal to the Fura-2 concentration in the pipette.

**Results**

We first use digital imaging methods to examine spatial gradients of [Ca^{2+}] during depolarization and then examine the properties of the intrinsic buffers that influence the depolarization-induced [Ca^{2+}] signal. In the Discussion we evaluate the impact of these buffers, as well as of the Fura-2 added to the cytoplasm, on the [Ca^{2+}] signal produced by depolarization.

**Imaging spatial gradients of Ca^{2+}**

Video imaging allowed us to visualize [Ca^{2+}] gradients within single chromaffin cells. In these experiments, the microscope was focused on the central plane of the spherical (or cylindrical) cell, to attempt to measure [Ca^{2+}] changes with minimal contributions from the top and bottom regions of the cell. Although the optical sectioning properties of the microscope were not ideal (Hiraoka et al. 1990), fluorescence signals were different at different levels of focus within the depth of the cell. This indicates that the fluorescence signals measured in central focal planes should have reduced contributions from the other, out of focus, regions of the cell.

The resting fluorescence of Fura-2 was often brightest in the central region of the cell (e.g. Fig. 1A). Although some of this non-uniformity was probably due to the spherical geometry of the cell, it often appeared that the nucleus was more fluorescent than the cell cytoplasm. Accumulation of fluorescent Ca^{2+} indicators in the nuclear region has been found for other cells (e.g. Hernandez-Cruz et al. 1990) and could be caused by nuclear accumulation of dye or exclusion of dye from the non-nuclear portion of the cytoplasm (for example, by dye being excluded by chromaffin granules). Because the ratio of fluorescence emitted when cells were alternatively excited by 360 and 390 nm was constant throughout the cell (not shown), these gradients do not represent differences in [Ca^{2+}] within the nucleus, as has been reported for other cells (Williams, Fogarty, Tsien & Fay, 1985; Hernandez-Cruz et al. 1990). Whatever the cause of this non-uniformity, it is expected that it would not distort the measurements of [Ca^{2+}], because of the use of the ratio method. The spatial uniformity of the resting [Ca^{2+}] signal indicates that this is the case.

Depolarization caused an influx of Ca^{2+}, as measured by a transient Ca^{2+} current (Penwick et al. 1982; Clapham & Neher, 1984; Hoshi, Rothlein & Smith, 1984; Kim & Neher, 1987; Augustine & Neher, 1992), and a rise in [Ca^{2+}], (Knight & Kasten, 1983; O’Sullivan et al. 1989; Augustine & Neher, 1992). Examples of [Ca^{2+}] signals produced by 500-ms-long depolarization to +5 mV are shown in Fig. 4 at the beginning of the depolarization, the changes in Fura-2 fluorescence were restricted to the edge of the cell (Fig. 4A). At later times during the depolarization, these changes were present throughout the cell but still usually were largest near the membrane.

![Fig. 4. Imaging of [Ca^{2+}], changes in a single chromaffin cell responding to a 500-ms-long depolarization to +5 mV. Changes in relative fluorescence intensity (390 nm excitation) have been encoded into the pseudocolor scale shown at the lower left. A–C, Images obtained during the depolarization, at 100 ms, 300 ms and 800 ms after the beginning of the depolarization respectively. D and E, fluorescence maps at 1 and 10 s following the end of the depolarization. F, schematic illustration showing the position of the patch pipette relative to the outline of the cell. During the experiment the microscope was focused on a plane through the centre of the cell and the fluorescence of the patch pipette was not visible.](image-url)
Augustine & Neher, 1992). In these experiments we saw no indication of the large and localized release of Ca$^{2+}$ from internal stores reported in intact chromaffin cells depolarized with high external K$^+$ or acetylcholine (O'Sullivan et al. 1989).

**Endogenous buffers of the chromaffin cell**

The characteristics of the fluorescence changes described above reflect the influence of both endogenous buffers and those added by the pipette (Fura-2 and EGTA). In order to reveal the true characteristics of the [Ca$^{2+}$]$_i$ signal resulting from depolarization it is necessary to define the contribution of endogenous and exogenous buffers to the measured fluorescence changes. While the buffering properties of exogenous Ca$^{2+}$ buffers ($\kappa_5$ in eqn (10)) are readily definable, little is known about the properties of the endogenous buffers ($\kappa_5$ in eqn (10)). In this section we use spatially averaged, photomultiplier measurements of Fura-2 fluorescence intensities at two wavelengths to deduce several properties of the endogenous buffers.

Two approaches for estimating cytoplasmic Ca$^{2+}$-binding capacity were presented in the Methods section. Both rely on a kinetic separation between fast buffering mechanisms and slower removal mechanisms. Furthermore they assume spatial uniformity of [Ca$^{2+}$]$_i$ at early times following a depolarizing pulse, such that fluorescence readings can be taken before calcium is removed. Figure 6 shows that...
[Ca^{2+}] is uniform at the end of a long depolarizing pulse and that removal is, indeed, slow with respect to the time scale of spatial re-equilibration within the cell. Figure 7 further demonstrates the kinetic separation with a spatially averaged Fura-2 signal recorded from a chromaffin cell by means of a photomultiplier. Figure 7A shows the changes in fluorescence (390 nm excitation wavelength) produced by a depolarizing stimulus of 20 ms duration. In this figure, fluorescence is displayed with a negative baseline, so that an upward deflection represents a decrease in fluorescence and an increase [Ca^{2+}]. The depolarizing stimulus elicited the Ca^{2+} current illustrated in the expanded section. [Ca^{2+}] increased during the stimulus, and for 10 to 20 ms following the stimulus. The increase that follows the stimulus may either reflect Fura-2 binding kinetics or spatial redistribution within the cell. Similar delayed increases have been described in Aplysia neurones (Tillotson & Nasai, 1988) and rat sympathetic neurones (Thayer & Miller, 1990). In these cells the delays are longer than in chromaffin cells, presumably because the larger diameters of these cells increases the time required for spatial re-equilibration. After the delayed increase, the fluorescence signal slowly decayed back to the resting [Ca^{2+}] level over a period of 10–30 s. Similar gradual returns to baseline [Ca^{2+}] have been seen in many cell types following depolarization (e.g. Gorman & Thomas, 1978; Smith & Zucker, 1980; McBurney & Neering, 1983; Ahmed & Connor, 1988; Thayer & Miller, 1990) and presumably reflect the slow removal of Ca^{2+} from the cytoplasm. Analysing the rate of this slow decline as a function of added calcium buffer was used in Method 1 to estimate cytoplasmic Ca^{2+}-binding capacity.

Figure 7B shows the same Fura-2 signal as in A, but on a more compressed time scale. A straight line has been fitted by eye to part of the record in order to show that the decay is linear on such a time scale, although this decay actually represents the start of a more or less exponential return to baseline. The important point is that there is no indication of additional kinetic components, indicative of buffering or removal mechanisms of intermediate components. This warrants the simple model of Fig. 3. It also illustrates that the amplitude of the depolarization-induced increment in fluorescence can be conveniently measured by extrapolating the slow relaxation back to the time of the stimulus, and comparing this to the pre-stimulus baseline. Such a measurement was necessary for Method 2, the second means of estimating cytoplasmic Ca^{2+}-binding capacity.

### Capacity of the endogenous Ca^{2+} buffer

In both procedures used for estimating the cytoplasmic Ca^{2+} buffer capacity, \( \kappa_B \), Fura-2 is the only exogenous calcium buffer and is loaded into the cell by dialysis from a patch pipette. Figure 8A illustrates an experiment where a relatively thin-tipped pipette was used to slowly deliver Fura-2 to the cytoplasm of a chromaffin cell. A high concentration of Fura-2 (0.4 mM) was used in this experiment to deliberately emphasize the Ca^{2+}-buffering effects of Fura-2. The uppermost trace represents fluorescence at 360 nm. It displays the loading time course of Fura-2 and was well fitted by an exponential with a time constant of 190 s. The centre trace is fluorescence at 390 nm, which shows step-like decrements whenever depolarizing stimuli (to +10 mV test potential from a holding potential of -70 mV) were given. The bottom trace in Fig. 8A displays \([\text{Ca}^{2+}]_i\) as calculated from the fluorescence ratio (Gryniewicz et al. 1985). The amplitudes of the depolarization-induced changes in \([\text{Ca}^{2+}]_i\), decreased as Fura-2 diffused into the cell. This reflects the buffering effect of the added Fura-2, which progressively takes up a higher proportion of the incoming calcium. At the same time, recovery of the \([\text{Ca}^{2+}]_i\) changes became slower, which is also due to the buffer capacity added by Fura-2 (eqn 15).

Both procedures used to estimate \( \kappa_B \) also require knowledge of \( \kappa_B \), the Ca^{2+}-binding capacity of the Fura-2 added to the cytoplasm of a cell. This was calculated from...
[B₄], the cellular concentration of total Fura-2, and Kᵦ, the Ca²⁺-binding constant of Fura-2 in the cellular environment (eqn (12)). [B₄] was estimated from the 390 nm fluorescence signal, assuming that the steady state level of the 390 nm signal represents fully equilibrated Fura-2 concentration (400 μM in the case of the experiment of Fig. 8A). Kᵦ was calculated from the Fura-2 calibration constants (eqn (35)) and was 6.6 x 10⁶ M⁻¹.

**Estimate based on the time constant of [Ca²⁺] signals**

The theory presented above indicates that the endogenous Ca²⁺-binding capacity of the chromaffin cell can be estimated from the time constants of recovery of [Ca²⁺] after small perturbations (Method 1). Intuitively this can be understood by considering that, for a given increment in [Ca²⁺], the load of calcium to be removed from the cell is increased with added binding capacity. There are a number of limitations to such an approach. First, the time constants depend on numerous cellular parameters (eqn (17)), particularly those relating to the poorly defined slow mechanisms for Ca²⁺ removal. Second, the estimate should apply only to small deviations from equilibrium (small with respect to the dissociation constant of Fura-2), whereas experimentally induced deviations usually were substantial to provide adequate resolution for measurement. Finally, the analysis should strictly apply only to the steady state, after the loading process has come to completion.

Despite these limitations, exponents could be fitted to the decay of many [Ca²⁺] signals (see also Thayer & Miller, 1990). As evident in Fig. 8A, the time course of decay became slower as the intracellular concentration of Fura-2 was elevated. Figure 9 presents a plot of τ' as defined by eqn (18), versus κₑ, the Ca²⁺-binding capacity of intracellular Fura-2. According to eqn (17), τ' should increase linearly with κₑ. In this figure the data from eighteen experiments, including values obtained at steady state and others measured during slow increases in Fura-2 concentration, are used. Values at any binding capacity scatter by about a factor of 2 to 3 in different cells. However, a clear trend is apparent in the data and a linear regression fitted to all data points yielded a negative z-intercept of 89, which is an estimate of κₑ, the Ca²⁺-binding capacity of the endogenous buffer (eqn (17)). This indicates that.
in the absence of Fura-2, only 1/90 of the calcium entering the cell appears as free calcium. The y-intercept of Fig. 9 indicates the recovery time constant expected in the absence of Fura-2. The range of values encountered was 5–12 s in different experiments, with a value of 7.2 s derived from a linear regression fit to all points.

**Estimate based on the fraction of calcium bound by Fura-2**

Method 2 is based on analysing the amplitudes of fluorescence changes which occur during depolarizing stimuli. While changes in \([\text{Ca}^{2+}]\), as measured by fluorescence ratios, decrease during loading of a cell with excess amounts of Fura-2, the fraction of calcium entering that is captured by Fura-2 increases because Fura-2 competes more favourably with the endogenous buffer. It was shown above that the fraction \(f\), defined as the ratio of \(\Delta F_{390}\) over the calcium current integral, should increase monotonically with an increasing concentration of Fura-2. It reaches a limiting value, \(f_{\text{max}}\), when all of the incoming calcium is taken up by Fura-2. In this case, \(\Delta F_{390}\) is an accurate measure of \(\text{Ca}^{2+}\) flux. In the example of Fig. 8A, six depolarizing stimuli were given while Fura-2 diffused into the cell. The first five pulses were each 50 ms in duration. They resulted in progressively larger \(f\)-values, as shown in Fig. 10C, where they are plotted as ◆. The rightmost round symbol in Fig. 10C represents the sixth response of Fig. 8A, which was elicited by a 200-ms-long pulse. Its \(\text{Ca}^{2+}\) current integral was 360 times larger than that of the preceding pulse and the magnitude of the resulting fluorescence decrement was larger by the same factor (within 2%), such that \(f\)-values were almost identical. This sequence of responses illustrates both expectations of the theory: (i) \(f\) eventually reaches a maximum during Fura-2 loading and, (ii) once at this maximum, the fluorescence signal is proportional to the amount of \(\text{Ca}^{2+}\) entry.

The relationship between \(f\) and \(\kappa_{\text{b}}\) is shown for two different cells in Fig. 10A and C. Both sets of data illustrate the saturation of \(f\) as a function of \(\kappa_{\text{b}}\) that was predicted from theory (eqn 28). Figures 10B and D are double reciprocal plots of data in A and C, respectively. Close inspection reveals that there is some upwards curvature in the plots. This is a consistent trend in all the cells analysed this way \((n = 8)\). In order to obtain estimates for \(f_{\text{max}}\), straight lines were fitted to the double reciprocal plots, restricting the regression range to nine values closest to the y-axis. These lines are shown in Fig. 10B and D. Between cells, \(f_{\text{max}}\) varied with a relative standard deviation of 10%. \(\kappa_{\text{b}}\) was estimated according to eqns (20) or (32) (see Methods section). The mean value \((\pm \text{S.E.M.})\) from nine individual measurements originating from six different cells was 75 ± 7. This value is definitely more accurate than the one derived through Method 1. It means that, in the absence of Fura-2, out of seventy-six parts of calcium entering the cell, seventy-five parts are bound to endogenous buffer and one part appears as free calcium.

Once \(f_{\text{max}}\) has been determined on a set-up, the integral \(\text{Ca}^{2+}\)-flux can be determined by inversion of eqn (24):

\[
\int \text{Ca} \, df = \frac{\Delta F_{390}}{f_{\text{max}}},
\]  

(39)

provided that the concentration of Fura-2 is high enough to achieve saturating levels of \(\kappa_{\text{b}}\). In evaluating eqn (30) it is important to note that the physical units of \(\Delta F_{390}\)

**Calcium in Chromaffin Cells**

should be the same as those used during determination of \(f_{\text{max}}\). Also, photomultiplier sensitivity and illumination conditions have to be absolutely constant. Some of these influences can be eliminated by relating all fluorescence values to a fluorescent standard. In this context, fluorescent beads (Fluoresbrite TM Carboxylate BB beads

**Fig. 10.** The ‘fraction’ \(f\), the ratio of \(\Delta F_{390}\) over calcium current integral, as a function of calcium-binding capacity of Fura-2 \((\kappa_{\text{b}})\) during loading (●) and unloading (■). \(\Delta F_{390}\) is the difference in fluorescence readings at 390 nm before and after a stimulus (see Methods section). A and C, two different experiments similar to those of Fig. 8B and D, double reciprocal plots of the data in A and C, respectively. The units on the ordinate are fluorescence units/Coulomb. No dimensions are given because fluorescence units are arbitrary. Ca\(^{2+}\)-binding capacity of Fura-2 was estimated according to eqn (31). Straight lines in B and D are linear regression fits, restricted to nine data points closest to the ordinate.

4.3 μm diameter, from Polyscience, Northampton, UK) proved very convenient because they have fluorescence intensities similar to chromaffin cells under the conditions of our measurement. Beads were added to the chamber at low density such that their fluorescence readings could be taken in between measurements or cells.

After a cell has been loaded to the extent that all of the incoming calcium is taken up by Fura-2, the magnitude of depolarization-induced fluorescence changes should be directly proportional to the integral of calcium current. Equation (38), in theory, would allow the calculation of the proportionality constant. This equation assumes that the dye has equilibrated diffusionally, such that the total Fura-2 concentration \((B_T)\) is the same as in the pipette. In addition, this calculation requires the
calibration constants $R_{\text{max}}$ and $R_{\text{min}}$, measurement of absolute fluorescence $F_{\text{max}}$, and of cell volume. Most of these parameters are known quite accurately. Cell volume, however, presents a problem, since only volume accessible to Fura-2 should actually be considered. Since this was not known, we used eqn (38) to calculate accessible volume from measured $f_{\text{max}}$ values. We also determined total cell volume by measuring cell diameter and assuming that the cells were perfect spheres. By dividing accessible volume by total volume, an accessible volume fraction can be calculated. In six measurements of this kind, a value of $0.35 \pm 0.09$ (mean $\pm$ s.e.m.) was found. It thus appears that about 15% of the volume of the cell excludes dye. It should be stressed that this is only an order-of-magnitude estimate because of the many assumptions involved. The fact that the value is reasonable, on the other hand, shows the internal consistency of all the assumptions made and indicates that fluorescence changes under these conditions do indeed reflect the calcium entering the cell, as opposed to calcium released from intracellular stores.

Mobility of the endogenous Ca$^{2+}$ buffer

If the endogenous buffer is diffusible, it will be lost from the cell as it is washed out of the cytoplasm and diluted by the much larger volume of the patch pipette. In principle, this could be tested by evaluating $\kappa_B$, the endogenous calcium-binding capacity (eqn (26)), at different times following patch rupture. However, the estimate of $\kappa_B$ based on $f$-values requires accurate determination of $f_{\text{max}}$. This is only possible when $f$ approaches $f_{\text{max}}$, which normally takes place after about 1 min of loading. Under these conditions, however, small errors in $f$ lead to larger errors in determining $\kappa_B$, as implied by the specific form of eqn (26). Therefore $\kappa_B$ has to be reduced again, if $\kappa_B$ is to be measured at later times.

To achieve this, we sequentially loaded and unloaded cells using two pipettes. Figures 8A and B present such an experiment. The artifact in the fluorescence traces, near the end of the recording in Fig. 8A, marks the time when the first pipette was slowly removed from the cell. Three minutes later another whole-cell recording was established on the same cell, using a second pipette. The new pipette was filled with only 50 μM-Fura-2, as opposed to 400 μM for the case of the first pipette. The recordings of Fig. 8B shows the ensuing fluorescence signals. The fluorescence at 360 nm slowly returned to low levels, as Fura-2 concentration in the cell decreased. [Ca$^{2+}$] started out relatively high because the cell was slightly damaged during breakthrough with the second pipette. However, changes in the fluorescence signal due to voltage pulses could still be well resolved. Depolarization-induced increments in [Ca$^{2+}$] increased, and the time constants of recovery shortened, as Fura-2 diffused out of the cell. An analysis of $f$-values was performed; the corresponding data points are plotted in Fig. 10 as square symbols, while circles indicate measurements made during Fura-2 loading. Both sets of data points are quite well described by the same curve. Correspondingly, determination of $\kappa_B$ on four cells (according to eqn (26)) gave similar mean values for loading and unloading runs, as shown in Table 1. Although there is substantial scatter in the values, it is clear that there is no tendency for $\kappa_B$ to decrease during the 10-15 min duration of the whole-cell recording. Thus, it appears that most of the endogenous cytoplasmic Ca$^{2+}$ buffer is not mobile.

<table>
<thead>
<tr>
<th>$\kappa_B$-loading</th>
<th>$\kappa_B$-unloading</th>
</tr>
</thead>
<tbody>
<tr>
<td>Loading time constant (s)</td>
<td>Dialysis time constant (s)</td>
</tr>
<tr>
<td>93</td>
<td>120</td>
</tr>
<tr>
<td>63±21 (2)</td>
<td>192</td>
</tr>
<tr>
<td>62</td>
<td>74</td>
</tr>
</tbody>
</table>

Each line represents one cell; values given are means $\pm$ s.d. (n) of those data points where $f$ was smaller than $f_{\text{max}}$ by at least 20%. Dialysis time is the total time for which the cell had been in contact, with a patch pipette up to the mean time of the unloading measurement.

Fig. 11. Effect of high [Ca$^{2+}$], on the calcium-binding capacity of Fura-2. A is an experiment very similar to that of Fig. 8, except that at the time of the asterisk a rapid train of depolarizing stimuli was given, which increased [Ca$^{2+}$] to almost 1 μM. The ‘fraction $f$’ was calculated and plotted in B both during the loading process (○) and for individual stimuli during or after the pulse train (●) as in Fig. 10.
Saturability of the endogenous buffer

\[ [Ca^{++}]_i \] often varied between 0.2 and 0.5 \( \mu \text{M} \) as a result of the manipulations performed in some experiments (e.g., Fig. 8B). If the endogenous \( Ca^{++} \) buffer changes its degree of saturation during such changes in \([Ca^{++}]_i\), then \( k_s \) would also change. For example, if the endogenous buffer had the same affinity as FurA-2, then \( k_s \) would change 5- to 4-fold over this range of \([Ca^{++}]_i\) (eqn (12)). Thus, changes in \([Ca^{++}]_i\) can be used to provide some information on the degree of saturation of the endogenous buffer.

\([Ca^{++}]_i\) can readily be changed during an experiment by giving trains of depolarizations, as illustrated in Fig. 11A. Initially five well-separated depolarizations were applied to determine values of \( f \) and \( f_{o_{max}} \) at relatively low \([Ca^{++}]_i\) (0.16-0.22 \( \mu \text{M} \)). These were followed by a rapid train of pulses that increased \([Ca^{++}]_i\) to a mean value of 0.71 \( \mu \text{M} \). Figure 11B demonstrates that values of \( f \) obtained at low \([Ca^{++}]_i\) ( ) almost superimposed on those obtained during pulsing ( ). Correspondingly, the mean value of the endogenous Ca\(^{++}\)-binding capacity, \( k_s \), at high \([Ca^{++}]_i\), is hardly distinguishable from that measured at low \([Ca^{++}]_i\), during dye loading (Table 2). Subsequently, several trains of pulses were given at different times. The values of \( k_s \) calculated during these trains support the notion that \( k_s \) is not strongly dependent on \([Ca^{++}]_i\), or on the length of time that the cell has been dialyzed. These results imply that the endogenous buffer did not saturate when \([Ca^{++}]_i\) was raised up to 1 \( \mu \text{M} \) and, thus, that this buffer has a relatively low affinity for \( Ca^{++} \).

**DISCUSSION**

In this paper we have examined two interrelated aspects of \( Ca^{++}\) signalling in single chromaffin cells: calcium gradients and calcium buffering. Digital imaging methods have directly demonstrated gradients in \([Ca^{++}]_i\) during brief depolarizing pulses. These gradients are closely linked to the influx of \( Ca^{++}\) through voltage-gated \( Ca^{++}\) channels, because they rapidly dissipate when the channels are closed by repolarization or inactivation. Quantitative information from imaging has to be used with caution, however, since the indicator dye, which also is a mobile calcium buffer, may greatly influence the result (Sala & Hernandez-Crus, 1980). Therefore FurA-2 was used as an exogenous \( Ca^{++}\) buffer to estimate some of the properties of the endogenous cytoplasmic \( Ca^{++}\) buffer of chromaffin cells. These estimates reveal that the endogenous buffer normally binds most of the \( Ca^{++}\) that enters through the \( Ca^{++}\) channels. However, because this buffer is immobile and has a relatively low affinity for \( Ca^{++}\), the addition of a mobile, high-affinity buffer, such as FurA-2, allows the exogenous buffer to out-compete this endogenous buffer and greatly alter the nature of the depolarization-induced \([Ca^{++}]_i\) signal.

**Properties of the endogenous \( Ca^{++}\) buffer**

Two different methods based on competition between FurA-2 and the endogenous buffer for \( Ca^{++}\) have been used to evaluate the properties of the endogenous \( Ca^{++}\) buffer of chromaffin cells. Method 1, using measurements of the slowing of the time course of \([Ca^{++}]_i\) removal following a stimulus, indicated that \( k_s \), the endogenous \( Ca^{++}\)-binding capacity (bound calcium over free calcium), was 90. Method 2, estimating the fraction of the incoming \( Ca^{++}\) which is bound to FurA-2, yielded a \( k_s \) value of 75. The latter value is definitely more accurate, since it rests on fewer assumptions, and has less scatter, experimentally. Although each method has limitations, the good correspondence between the two estimates supports the approach taken. The values indicate that 98-99% of all \( Ca^{++}\) ions entering the chromaflagin cells are rapidly bound to the endogenous cytoplasmic \( Ca^{++}\) buffer. Studies of other neural-line cells also indicate that cytoplasmic buffering exerts a substantial influence on the magnitude of the \([Ca^{++}]_i\) signal (Gorman & Thomas, 1980; Smith & Zucker, 1980; McBurney & Neering, 1985; Ahmed & Connor, 1988; Thayer & Miller, 1989). Unfortunately, numerical values for binding capacity cannot easily be compared, because previous estimates were either obtained in a different \( Ca^{++}\) concentration range (Ahmed & Connor, 1988) or else the measurements were performed on a much slower time scale, such that slow buffering mechanisms contribute (Baker & Schlaepfer, 1978). However, our value is close to that (100) used by Smith & Zucker (1980) to fit fast Arensazmi III signals in 

**CALCIUM IN CHROMAFFIN CELLS**
Unlike in the case of neutrophils (von Tscharner, Deranleau & Baggioini, 1986) the endogenous Ca\(^{2+}\) buffer in chromaffin cells does not allow any signs of saturation for [Ca\(^{2+}\)] up to 1 \(\mu\)M (Table 2). This suggests that the buffer has a low affinity for Ca\(^{2+}\) ions. Our value of \(K_s\) is compatible with 325 \(\mu\)M of buffer at 5 \(\mu\)M affinity, or 750 \(\mu\)M at 10 \(\mu\)M affinity. This excludes Ca\(^{2+}\)-binding proteins such as parvalbumins, which have Ca\(^{2+}\) affinities in the submicromolar range (Ebashi & Ogawa, 1988), and is marginally compatible with calmodulin. More likely would be molecules such as annexins, which are present in chromaffin cells and have Ca\(^{2+}\) affinities as low as

| TABLE 2. Endogenous calcium binding capacities at different [Ca\(^{2+}\)] values and times |
|---|---|---|---|---|
| Time (s) | Loading 1 | Loading 2 | Loading 3 | Loading 4 |
| [Ca\(^{2+}\)] (\(\mu\)M) | Mean | Mean | Mean | Mean |
| 0.18 | 70 | 67 ± 8 | 51 ± 6 | 90 ± 13 | 89 ± 12 |

All values are from one cell. Time is the mean time after patch rupture for the pulses of a given train. Mean calcium is the mean of mean values in [Ca\(^{2+}\)], during steps. Errors, where given, are S.E.M.'s.

Hundreds of micromolar (Pollard, Burns & Rojas, 1988; Creutz, Drust, Martin, Kambouris, Snyder & Hamman, 1988). Also the troponin-C-like Ca\(^{2+}\)-binding protein isolated from adrenal medulla (Kuo & Coffee, 1976) has an affinity compatible with our findings, but, according to its abundance, it represents only a minor fraction of the total calcium binding capacity.

**Implications for measurement of [Ca\(^{2+}\)] signals**

The observation that Fura-2 can compete effectively with endogenous Ca\(^{2+}\) buffers sets some limits on the use of this dye as a cytoplasmic Ca\(^{2+}\) indicator. If the goal is to measure changes in free calcium concentration undisturbed by the presence of the indicator dye, then the method of choice is the ratio method as described by Grynkiewicz et al. (1986) and the calcium binding capacity of Fura-2 should be smaller than that of the endogenous buffer. If, however, the goal is to measure Ca\(^{2+}\) fluxes into the cytoplasmic compartment at fixed [Ca\(^{2+}\)], then the intracellular concentration of Fura-2 should be high enough so that all of the Ca\(^{2+}\) load is taken up by the Fura-2. Under such conditions, the magnitude of the Ca\(^{2+}\) flux is proportional to the absolute fluorescence changes at the Ca\(^{2+}\)-sensitive wavelength (eqn (39)). The two measurements are complementary in the same way as are current clamp and voltage clamp measurements. What should be avoided is an intermediate situation, where fluorescence signals will give only qualitative hints. In the case of chromaffin cells, 400 \(\mu\)M-Fura-2 seems to be adequate for measurement of Ca\(^{2+}\) fluxes elicited by brief depolarizations (Fig. 8A). In the case of [Ca\(^{2+}\)] measurements, the magnitude of the signal is inversely related to the sum of \(K_s\) and \(K_p\) (eqn (21)), so that \(K_s\) should be no more than 10% of \(K_p\) if the [Ca\(^{2+}\)] signal is to be within 90% of its normal value. For chromaffin cells, with a \(K_s\) of about 75, then \(K_s\) should be no more than 7-5, which would require a Fura-2 concentration of no more than 75 \(\mu\)M for a [Ca\(^{2+}\)] signal of 1 \(\mu\)M (eqn (12)). For smaller Ca\(^{2+}\) signals the requirement is more stringent.

For instance, for Ca\(^{2+}\) signals comparable in magnitude to the \(K_p\) of Fura-2 (which was found to be 0.15 \(\mu\)M in chromaffin cell cytoplasm), the limiting Fura-2 concentration would be approximately 4 \(\mu\)M, and 38 \(\mu\)M-Fura-2 would double the endogenous calcium-binding capacity. This result is very similar to that of Timmerman & Ashley (1986) who found that 30 \(\mu\)M-Fura-2 almost doubles the relaxation time of force in striated muscle.

Spatially resolved measurements require even more stringent criteria. For a faithful report on the spatiotemporal pattern of Ca\(^{2+}\) changes, the indicator should not only not bind significant amounts of calcium, but also not contribute to its redistribution. In the absence of mobile endogenous buffers, the only diffusing calcium atoms are those free and those bound to Fura-2. The flux of calcium \(J_{Ca}\) through any small volume element of length \(dx\) can then be written as:

\[
J_{Ca} = -\frac{D_{Ca}}{\Delta x} \left( \frac{\Delta [Ca^{2+}]}{\Delta x} - D_{BCa} \left( \frac{\Delta [BCa]}{\Delta x} \right) \right),
\]

where \(D_{Ca}\) and \(D_{BCa}\) are diffusion coefficients of calcium and BCa (the calcium loaded form of exogenous buffer), respectively. Free calcium is known to diffuse slowly in cytoplasm (Hodgkin & Keynes, 1957), such that \(D_{Ca}\) and \(D_{BCa}\) can be considered roughly equal. Then, the contributions of the two terms in eqn (40) to total calcium flux are proportional to the local gradients of the respective chemical species. Assuming [Ca\(^{2+}\)] values of 1 \(\mu\)M near the membrane and 0.1 \(\mu\)M in the centre, and a total Fura-2 concentration of 10 \(\mu\)M, one would expect approximately 9 \(\mu\)M of Ca\(^{2+}\)-bound Fura-2 near the membrane and 4-5 \(\mu\)M in the centre of the cell (after Fura-2-Ca\(^{2+}\) equilibration, which is rapid). Thus, even this minute amount of Fura-2 would dominate the redistribution process. This effect of a mobile chelator also has been demonstrated in the model calculations of Sala & Hernandez-Cruz (1990).

The above discussion assumes a complete absence of mobile endogenous buffers, as suggested by our measurements. The measurements cannot totally exclude, however, the presence of some amounts of mobile buffers, since the present protocol only allows reliable measurements of \(K_s\) after some Fura-2 has diffused into the cell. A small amount of highly mobile buffer which would escape in that time span might have been overlooked.

The considerations discussed in the previous paragraphs suggest that an extremely low concentration of Fura-2 is needed to accurately depict the spatial profile of the [Ca\(^{2+}\)] changes occurring during depolarization. However, our imaging experiments required substantially higher concentrations of Fura-2 (100-500 \(\mu\)M) in order to have sufficient fluorescence for measurement with the video camera. At these concentrations, it is likely that the Fura-2 is distorting the spatial gradients and is causing them to collapse more rapidly than in an unperturbed cell. The limited temporal and spatial resolution of the imaging method (Fig. 1) would lead to a further underestimation of the steepness of these gradients. For example, although depolarization is thought to raise [Ca\(^{2+}\)] levels at the release sites to 10-100 \(\mu\)M (Augustine & Neher, 1992), the highest levels measurable with the imaging method are in the order of 1 \(\mu\)M (e.g. Fig. 4). This presumably is due to the fact that the [Ca\(^{2+}\)] gradients at the release sites drop off over dimensions of a fraction of
Release of calcium from intracellular stores

Several investigators have observed release of calcium from intracellular stores (Lipscombe et al. 1988; O'Sullivan et al. 1989; Hernandez-Cruz et al. 1990) as part of the depolarization-induced calcium signal. The close quantitative agreement between calcium influx, as measured by current, and that detected by Fura-2 fluorescence reported here leaves little room for such release in our study (see also Thayer & Miller, 1990). Further, our imaging experiments suggest that the depolarization induced elevation of [Ca\(^{2+}\)] is originated in the vicinity of the plasma membrane (Fig. 4), while calcium released from intracellular stores often appears first in the interior of the cell (e.g. O'Sullivan et al. 1989). It might be argued that we had to buffer calcium quite strongly in all the experiments aimed at quantitative flux measurements and, thus, prevented 'calcium-induced calcium release'. However, the agreement between theory and experiments also extends to responses during the loading and unloading episodes (Figs 8 and 10) where exogenous buffer had much less buffering power. If there had been any significant contributions from intracellular release, it should have shown up in the form of marked deviations from the binding curves displayed in Fig. 10, or as humps in the time courses of recovery. Such humps were, indeed, seen occasionally following excessive stimulation with long-pulse trains (not shown); but single pulses up to 2 s in duration never produced such a response. Release of intracellular calcium may, thus, occur with intense long-term stimulation, such as with K\(^+\)-depolarization, but it is probably not associated with single action potentials in bovine chromaffin cells.
CALCIUM IN CHROMAFFIN CELLS


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