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Multiple Calcium-Dependent Processes Related to Secretion in Bovine Chromaffin Cells

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Summary

We have used the caged calcium compound DM-nitrophen to investigate the kinetics of calcium-dependent secretion in bovine chromaffin cells. Perfusion with partially calcium-loaded nitrophen often caused a loading transient—slow secretion for up to 1 min due to displacement of Ca^{2+} by cytoplasmic Mg^{2+}. Flash photolysis elicited 100 μM $[Ca^{2+}]_i$ steps that evoked intense secretion, lasting a few seconds. In cells experiencing a loading transient, $[Ca^{2+}]_i$ steps evoked an especially fast secretion. A persistent, slow secretion often followed these fast phases. Distinct kinetic components may reflect secretion from pools that are differentially capable of release. Both secretion and movement of vesicles between pools appear to be $[Ca^{2+}]_i$ sensitive. Later $[Ca^{2+}]_i$ steps sometimes evoked a rapid capacitance decrease, indicating a fast, $[Ca^{2+}]_i$-dependent phase of endocytosis.

Introduction

Calcium is the link between depolarization and secretion in many cells, including chromaffin cells (Douglas, 1968; Katz, 1969). However, the mechanisms by which calcium acts remain unknown. The role of intracellular calcium concentration ($[Ca^{2+}]_i$) in secretion in chromaffin cells has been studied in three ways: the effect of elevated $[Ca^{2+}]_i$ on secretion rate has been studied in permeabilized cells (Knight and Baker, 1982); the average $[Ca^{2+}]_i$ has been measured during secretion triggered by depolarization or release of calcium from internal stores (Neher and Marty, 1982; Knight and Kesteven, 1983; Cheek et al., 1989; Augustine and Neher, 1992); and the secretion stimulated by perfusion of cells with calcium buffers through a whole-cell patch pipette has been measured (Augustine and Neher, 1992).

With dialysis and perfusion of calcium buffers, the rate of secretion achieved for a given $[Ca^{2+}]_i$ was substantially less than that observed in response to brief depolarizations. This was taken to indicate that depolarizing pulses opened calcium channels that elevated $[Ca^{2+}]_i$ locally near release sites to substantially higher levels than the average $[Ca^{2+}]_i$ recorded photometrically. Furthermore, even at very high cytoplasmic $[Ca^{2+}]_i$ (10–100 μM), secretion rates during dialysis always remained substantially lower than those obtained during voltage pulses. Several other means of rapidly elevating $[Ca^{2+}]_i$ also yielded higher secretion rates. The total amount of secretion, on the other hand, was always much larger with calcium dialysis than with rapid calcium elevation. This behavior was interpreted to reflect the presence of two pools of hormone, a relatively small, rapidly releasable pool and a larger reserve pool. However, the calcium dependence of movement between pools, if there is any, has not been studied. Following heavy secretion, reductions in cell surface area measured electrically were sometimes observed and taken to indicate endocytotic recovery of vesicular membrane (Neher and Marty, 1982), but it is not known whether this process is calcium dependent.

Each of the methods used until now is subject to serious limitations: $[Ca^{2+}]_i$ can be changed by known amounts, but only slowly, in permeabilized or internally dialyzed cells. Thus releasable vesicles might be depleted before the target $[Ca^{2+}]_i$ is reached. Depolarization can change $[Ca^{2+}]_i$ quickly by opening voltage-sensitive calcium channels, but then sharp spatial nonuniformities result, invalidating average $[Ca^{2+}]_i$ as a measure of the calcium concentration triggering release. A method is needed to change $[Ca^{2+}]_i$ rapidly and uniformly in a known fashion.

For this purpose, we have exploited the properties of the caged calcium compound DM-nitrophen (Kaplan and Ellis-Davies, 1988), a photolabile calcium chelator whose affinity for calcium is reduced rapidly on exposure to ultraviolet illumination. We filled cells by perfusion from patch pipettes with mixtures of nitrophen and the calcium-sensitive indicator furaptra (Konishi et al., 1991). Nitrophen was photolyzed rapidly with light flashes from a xenon arc flash lamp, and photometric measures of $[Ca^{2+}]_i$ were correlated with rates of secretion. The latter were measured as changes in the cell surface area as reported by the cell's capacitance.

Results

Properties of DM-Nitrophen

The photolabile chelator DM-nitrophen has several characteristics that are important in the design and interpretation of experiments: First, a large step increase in $[Ca^{2+}]_i$ is achieved only when a proportion of nitrophen roughly equal to the nitrophen not bound to calcium is photolyzed. Second, once this point is reached, the level of $[Ca^{2+}]_i$ generated increases rapidly with little additional photolysis. Third, even before large step rises in $[Ca^{2+}]_i$ are evoked, flashes elicit a short-lived spike in $[Ca^{2+}]_i$ (Kaplan, 1990; Zucker, 1993), reflecting the slow equilibration due to rebinding of calcium released from photolyzed nitrophen with remaining unphotolyzed chelator. Finally, mag-

nesium competes with calcium for the cation-binding site of nitrophen.

The first two characteristics make it difficult to adjust flash intensity to elevate $[Ca^{2+}]_i$ by a precise amount, especially to an intermediate level in the 10^{-5} to 10^{-6} M range. In many experiments, we filled cells with approximately 8–9 mM DM-nitrophen, loaded 33%–37% with calcium (see Experimental Procedures for exact formulation). In such an experiment, a flash from our Rapp flash lamp at one-fourth maximum power, which photolyzes about 55% of the nitrophen, would be expected to raise $[Ca^{2+}]_i$ from an initial value of 3 nM to about 20 nM (Figure 1). Consistent with this prediction, such a flash caused no detectable rise in $[Ca^{2+}]_i$ measured with furaptra, nor did it stimulate secretion measured as an increase in cell capacitance (Figure 2). However, a second flash ought to elicit a large step rise in $[Ca^{2+}]_i$ into the range of 10^{-5} to 10^{-4} M range, and this was consistently observed. Another common filling solution that we used was more heavily loaded with calcium (4.5 mM or sometimes higher), and it often contained an additional calcium buffer (4–6 mM DPTA [1,3-diaminopropane-2-ol-N,N'-tetraacetic acid]) to reduce somewhat the maximum level of $[Ca^{2+}]_i$ achieved by nitrophen photolysis. In such experiments, the first flash was expected to photolyze sufficient nitrophen to cause a step rise of $[Ca^{2+}]_i$ in the 10 μM range (Figure 1). This was also consistently observed, and in this case the first flash always evoked an increase in cell capacitance indicative of secretion (Figure 3).

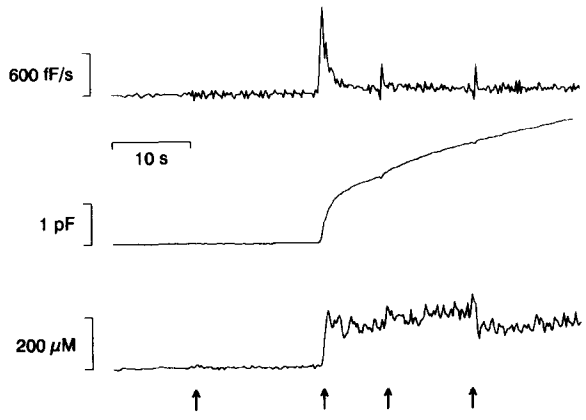

Figure 2. Flash Responses in a Cell Showing Almost No Loading Transient, When the First Flash Elicited No Step Increase in $[Ca^{2+}]_i$

The rate of secretion is shown on the top trace in femtofarads per second of membrane capacitance, which is the time derivative of the middle trace, showing membrane capacitance in picofarads. The bottom trace shows $[Ca^{2+}]_i$ calculated from furaptra fluorescence. The record begins 210 s after establishing the whole-cell configuration and beginning perfusion with 9 mM nitrophen and 3 mM Ca^{2+}. The starting membrane capacitance was 5 pF. The first 85 J flash (at the first arrow on the left) photolyzed approximately 55% of the nitrophen and elicited no detectable step increase in $[Ca^{2+}]_i$ and no secretion. A 340 J flash (next arrow) photolyzed approximately 98% of the nitrophen and elevated $[Ca^{2+}]_i$ to about 150 μM, evoking fast and slow phases of secretion. Later 340 J flashes had little additional effect on either $[Ca^{2+}]_i$ or secretion.

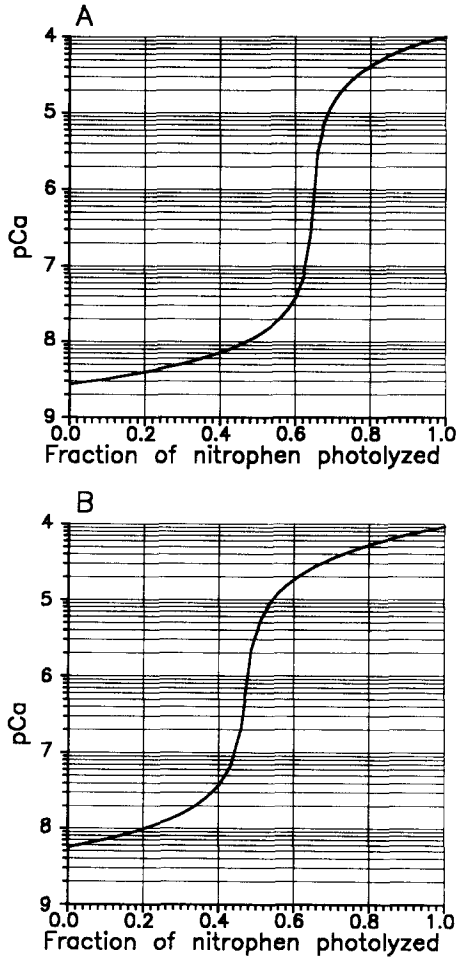

Figure 1. Effect of DM-Nitrophen Photolysis on $[Ca^{2+}]_i$

The expected steady-state effect of photolysis of different fractions of DM-nitrophen on $[Ca^{2+}]_i$ is calculated from a model of nitrophen photolysis and binding of the photolyzed and unphotolyzed chelator to Ca^{2+} (Delaney and Zucker, 1990). In (A), simulations are for 8.5 mM nitrophen and 3 mM Ca^{2+} and include the effects of 0.3 mM furaptra and a similar amount of native buffer (Neher and Augustine, 1992). In (B), simulations are for 8.5 mM nitrophen, 4.5 mM Ca^{2+}, and 4 mM DPTA.

The third characteristic of nitrophen means that each of a series of 85 J flashes, even the first one that elevates $[Ca^{2+}]_i$ to only 20 nM, triggers a spike of $[Ca^{2+}]_i$ to a peak level of about 150–200 μM lasting about 2 ms (Zucker, 1992a). Our calcium-measuring apparatus is not sufficiently fast to resolve this calcium spike. The result that the first such flash never increased the capacitance of chromaffin cells in the absence of a step rise in $[Ca^{2+}]_i$ (Figure 2; Figure 4) indicates that such a brief, though intense, $[Ca^{2+}]_i$ spike is unable to evoke significant amounts of secretion.

The fourth characteristic of nitrophen means that the effective affinity of nitrophen for calcium is dependent on the magnesium concentration, just as it is for its parent compound, EDTA. This could be used to advantage, by including a known amount of magne-

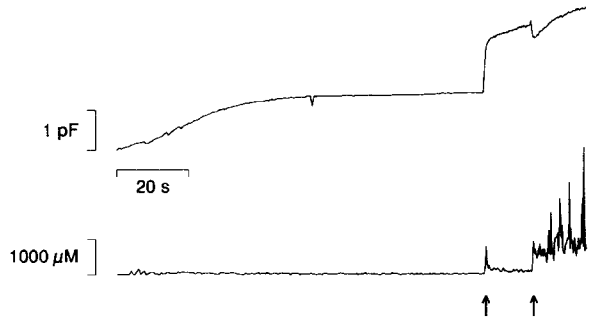

Figure 3. Flash Responses in a Cell with a Loading Transient, Showing an Ultrafast Response and a Reduction in Capacitance

This figure shows membrane capacitance beginning at 7.4 pF (top trace) and $[Ca^{2+}]_i$ (bottom trace) in a cell filled with 9 mM nitrophen and 5 mM Ca^{2+}. The record starts a few seconds after establishing the whole-cell configuration. A loading transient consisting of a 1.3 pF slow capacitance increase and an $[Ca^{2+}]_i$ reaching about 30 μM occurs early in the trace. Responses to two flashes are shown (arrows), in which the first photolyzes about 80% of the nitrophen, and the second about 96%. The first flash elevates $[Ca^{2+}]_i$ to approximately 80 μM and evokes an ultrafast response followed by a slow phase of secretion. The second flash elevates $[Ca^{2+}]_i$ to about 600 μM and evokes a reduction in membrane capacitance. The ultrafast response was so fast that its rising phase could not be discriminated by differentiating the capacitance trace, so the derivative is not shown.

sium in perfusion solutions to regulate the size of $[Ca^{2+}]_i$ steps achieved by photolysis of nitrophen. However, magnesium would then also be released from the nitrophen when it is photolyzed, which could conceivably have an effect on secretion and would also confound our measurement of $[Ca^{2+}]_i$ with furaptra (whose fluorescence depends on both $[Ca^{2+}]$ and $[Mg^{2+}]$; Raju et al., 1989). Therefore, we omitted magnesium from our perfusion solutions. Nevertheless, magnesium influences the early part of the measurement. Shortly after the whole-cell configuration is established by disrupting the membrane in the patch pipette, nitrophen loaded with calcium enters the cell at low concentration from the pipette and confronts high magnesium concentration in the cytoplasm. This mixture displaces calcium from the nitrophen for a period of approximately 30 s, until the magnesium is washed out of the cell and the calcium-nitrophen mixture approaches its final level. This is observed as an increase in $[Ca^{2+}]_i$ in the range of 3–30 μM. The size of the $[Ca^{2+}]_i$ transient, measured from the furaptra fluorescence ratio as furaptra entered the cell, depended strongly on the degree of calcium loading of the nitrophen in the pipette. This rise in calcium was invariably accompanied by a gradual increase in capacitance of 19.6 ± 11 fF/s (mean ± SD). We call this phenomenon a loading transient. An example is shown in Figure 3.

One problem with the interpretation of the measurement of $[Ca^{2+}]_i$ during the loading transient is that some of the furaptra signal as it enters the cell should be due to binding of Mg^{2+} as it washes out of the cell. To estimate the magnitude of this interference, 5 cells were loaded with a calcium-free mixture of nitrophen and furaptra. A fluorescence signal corresponding to $[Ca^{2+}]_i$ around 2.5 μM was measured. Since this was substantially less than the signal typically recorded when nitrophen was loaded with calcium, we believe the furaptra fluorescence during loading was responding primarily to calcium displaced from nitrophen by magnesium.

Effects of $[Ca^{2+}]_i$ Steps on Secretion

The most remarkable result of our study was the variety and variability of the effects of $[Ca^{2+}]_i$ elevation on cell capacitance. We have classified our responses into four types:

–Fast secretion: These responses are characterized by a sharply rising rate of capacitance increase (dC/dt), which reaches its peak within a second or two and subsequently decays with a time constant of 1–7 s. An example of such a response is shown in Figure 2. Both the amplitude and rate of decay of this type of response depended weakly on the magnitude of the $[Ca^{2+}]_i$ step. The total capacitance change fell in the range of 0.2–1.9 pF (0.6 ± 0.4 pF, mean ± SD). Such responses usually were observed for flashes that elevated $[Ca^{2+}]_i$ to above 80 μM. In 14 such cases, in which single exponentials could be reasonably fitted to the records of secretion rate (such as the top trace in Figure 2), a time constant of 2.9 ± 1.9 s was evaluated. This time constant may be thought of as providing a

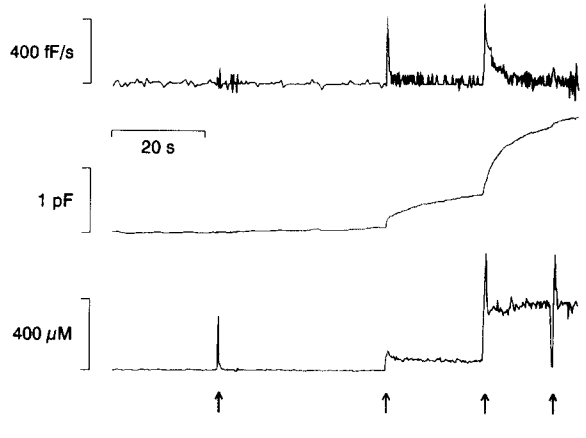

Figure 4. Flash Responses in a Cell with No Loading Transient, Showing an Ultrafast Response to the First Flash That Evoked Secretion

Rate of change of capacitance, total membrane capacitance beginning at 5.2 pF, and $[Ca^{2+}]_i$ are shown beginning 150 s after establishing the whole-cell configuration in a cell filled with 9 mM nitrophen and 3 mM Ca^{2+}. The first two 85 J flashes photolyzed approximately 55% and 80%, respectively, of the nitrophen. The first flash failed to elevate $[Ca^{2+}]_i$ into the detectable micromolar range (the spike on the $[Ca^{2+}]_i$ trace is a flash artifact) and evoked no secretion. The second flash raised $[Ca^{2+}]_i$ to about 70 μM and evoked ultrafast and slow responses. The ultrafast response was unusual in cells showing no loading transient. The third 340 J flash photolyzed 99% of the nitrophen and evoked additional fast and slow responses.

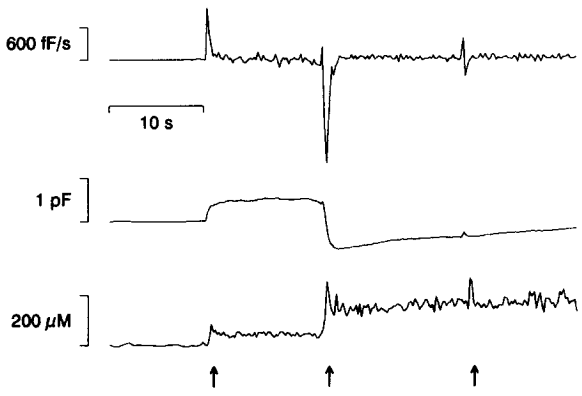

Figure 5. Flash Responses in a Cell with a Loading Transient, Showing an Ultrafast Secretion and Membrane Retrieval to a Later Flash

Rate of change of capacitance, membrane capacitance beginning at 7.2 pF, and $[Ca^{2+}]_i$ are shown beginning 150 s after establishing the whole-cell configuration in a cell filled with 8 mM nitrophen, 4.5 mM Ca^{2+}, and 4 mM DPTA. This cell showed a 1.5 pF loading transient before the trace began. The first 85 J flash photolyzed approximately 55% of the nitrophen, elevated $[Ca^{2+}]_i$ to about 30 μM, and evoked an ultrafast response followed by a small slow phase. The next 340 J flash photolyzed 98% of the nitrophen, raised $[Ca^{2+}]_i$ to about 140 μM, and evoked a sudden membrane retrieval. The third 340 J flash had little additional effect.

measure of the rate at which a releasable store of hormone is secreted. Rates of secretion at elevated $[Ca^{2+}]_i$ did not drop to zero following such fast secretion; rather slow secretion (see below) continued for time periods up to 1 min (Figure 2). Regression analysis of time constants showed that fast secretion is accelerating rather than slowing down at higher $[Ca^{2+}]_i$. Contrary to this trend, we observed ultrafast secretion at lower $[Ca^{2+}]_i$ as described below.

–Ultrafast secretion: Very often we observed a small amount of capacitance increase at the first flash that elevated $[Ca^{2+}]_i$ noticeably. These responses, on the level of our time resolution, appeared as step-like changes of 0.05–1.6 pF amplitude (0.5 ± 0.5, mean ± SD) as shown in Figures 3–5. When the flash elevated $[Ca^{2+}]_i$ to values between 10 and 50 μM, these ultrafast responses appeared alone. In the range of 50–100 μM, they were either alone or on top of a fast secretion event. For values above 100 μM, only mixed events or single fast events were observed. Figure 6 shows a bar graph of decay time constants for the fast and ultrafast responses. We classified as ultrafast responses falling in the sharp peak with time constants less than 0.5 s.

–Slow secretion: A nearly steady rate of secretion often occurred in the late phase following a large step in $[Ca^{2+}]_i$. It lasted at least 10 s and often 30 s or more before gradually declining to zero over the course of a minute or two. This process may be seen in the responses of Figures 2–4. In 17 cases in which this rate was analyzed as a pedestal to an exponentially decaying fast secretion event, it was found to be 41 ± 27 fF/s (mean ± SD). In these cases $[Ca^{2+}]_i$ was 163 ± 99 μM. The total extent of this secretion is several picofarads and thus of the same order of magnitude as total secretion observed by Augustine and Neher (1992) in cells dialyzed with high $[Ca^{2+}]_i$. We cannot give accurate numbers on this quantity, because cells very often developed abrupt leaks before slow secretion came to completion.

–A calcium-dependent reduction in cell capacitance was also often observed (see Figures 3 and 5). This was the most variable effect seen. Usually the response was rapid and short lived, decaying within 1–2 s. Occasionally, long-lasting decreases in capacitance were observed as reported earlier (Neher and Marty, 1982). Large scale capacitance drops were seen only in cells from 2 animals, suggesting that individual variation was important in determining the magnitude of this behavior. As a rule, however, decreases in capacitance were observed only in cells that had undergone substantial prior secretion, in response to either prior nitrophen photolysis or a substantial loading transient. We therefore interpret these responses as representing recovery or retrieval of vesicular membrane that had previously fused with the plasma membrane during secretion of vesicles.

Effect of Loading Transient on Subsequent Responses

We suspected that ultrafast responses might represent the release of vesicles already "docked" or attached to the plasma membrane and ready for secretion, whereas fast secretion was due to fusion of "nearly docked" vesicles. Slow secretion might be rate limited by the need to first move "undocked" vesicles into position at the membrane. If both secretion and the

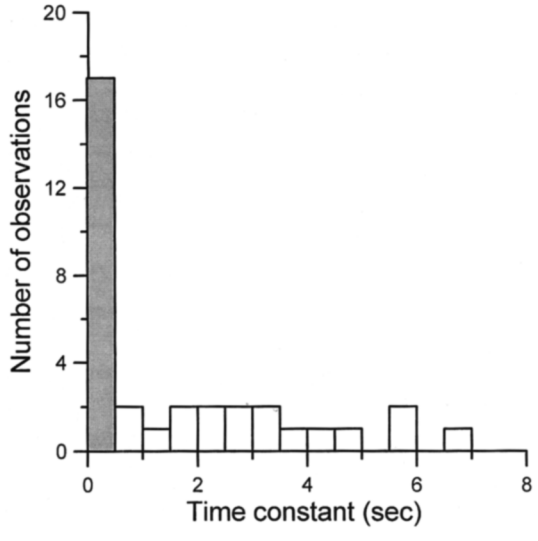

Figure 6. Decay Rates of Fast and Ultrafast Responses to Nitrophen Photolysis

Bar graphs of time constants of decay of calcium-activated capacitance increases that were well described by an exponential. Responses falling in the left-most bin (<0.5 ms) were classified as ultrafast; the others were considered fast.

movement of vesicles to docking sites are calcium dependent, then prior exposure of cells to a modest $[Ca^{2+}]_i$, as occurs during loading transients, would influence the probability that vesicles are docked and hence the subsequent occurrence of ultrafast release to nitrophen photolysis. To prevent a loading transient, we predipped patch pipettes for 15–30 s in a solution of 3 mM EGTA, 67% loaded with calcium (calculated free $[Ca^{2+}]$ about 150 nM) before back-filling the pipette with the usual perfusion solution. This procedure produced an approximately 1 mm column of calcium buffer between the filling solution and the cytoplasm, which should dissipate within about 10 min. This barrier prevented nitrophen loaded with calcium from entering the cell before most of the magnesium had washed out and effectively eliminated the loading transient in most cells. However, these cells contained some EGTA and probably were filled with somewhat lower concentrations of nitrophen. This accounts for the finding that brighter light flashes were required to elicit steps of $[Ca^{2+}]_i$ comparable to those achieved in cells not subjected to the predipping procedure.

Cells treated in this fashion generally behaved similarly to cells that experienced a loading transient, with some quantitative differences. These cells showed less tendency to display capacitance drops, which we interpret as membrane retrieval: although small reductions were often observed to later flashes, the large drops seen in some cells were not observed, but this may be a reflection of variability dependent on source of cells.

A more meaningful difference seems to be an effect of prior exposure to elevated $[Ca^{2+}]_i$ on the likelihood that a test flash raising $[Ca^{2+}]_i$ to a given level will evoke an ultrafast secretion. We looked at responses to light flashes that elevated $[Ca^{2+}]_i$ to between 10 and 40 μM in 29 cells. Eighteen of these cells displayed a loading transient of at least 0.25 pF: 78% of these 18 cells responded to the test flash (which elevated $[Ca^{2+}]_i$ to 25.5 ± 1.4 μM, mean ± SEM) with an ultrafast secretion. Of the remaining 11 cells having no such prior exposure to elevated $[Ca^{2+}]_i$ during loading, only 36% gave an ultrafast secretion to the test flash (which elevated $[Ca^{2+}]_i$ to 24.3 ± 2.1 μM). These data indicate that a prior exposure to elevated $[Ca^{2+}]_i$ significantly increases the probability that a subsequent criterion rise in $[Ca^{2+}]_i$ will evoke an ultrafast response (chi square test, $P < 0.05$).

Another way to test for an effect of previously elevated $[Ca^{2+}]_i$ on the response to a flash is simply to measure the normalized peak rate of secretion (dC/dt divided by the total change in capacitance) in response to $[Ca^{2+}]_i$ steps in the criterion range of 10–40 μM. In the same 29 cells analyzed above, the average peak rate of capacitance increase was 48 ± 15 fF/s (mean ± SEM) per pF of overall capacitance increase in cells that had not been exposed to a prior rise in $[Ca^{2+}]_i$, and 160 ± 33 fF/s per pF in cells that had been so exposed. This difference is significant ($P < 0.02$, Mann-Whitney test).

Discussion

Comparison with Previous Results

Our results indicate that a rise in $[Ca^{2+}]_i$ can trigger secretion at a wide range of rates. We have observed peak capacitance increases of up to 1000 fF/s per pF of overall capacitance change, or 1500–2000 fF/s in several cells, to $[Ca^{2+}]_i$ steps to 200 μM or less. Ultrafast responses were so short lived that we could not always resolve their peaks or decay rates, whereas fast responses seemed to represent the emptying of a store of hormone within about 2 s. These large rates of secretion are substantially greater than those observed by Augustine and Neher (1992) when $[Ca^{2+}]_i$ was elevated by perfusion of calcium buffers or by release from intracellular stores. Such methods of changing $[Ca^{2+}]_i$ slowly would be unable to elicit these short-lasting modes of secretion. Depolarization that raises average cell $[Ca^{2+}]_i$ to a few micromolar can evoke release rates as high as those observed (Augustine and Neher, 1992), suggesting that depolarization rapidly elevates $[Ca^{2+}]_i$ at release sites near calcium channels to the 50–200 μM range that we find is able to activate fast and ultrafast secretion.

Following rapid secretion, we often see a sustained, slower rise of capacitance, to around 40 fF/s. We also observe loading transients of about half that rate while filling cells with nitrophen, which usually elevates $[Ca^{2+}]_i$ to about 15 μM. These release rates are quite close to, but slightly less than, those seen by Augustine and Neher (1992) when they changed $[Ca^{2+}]_i$ slowly and to similar levels. We are not certain whether our lower secretion rates represent a significant difference, perhaps resulting from a side effect of DM-nitrophen, or are due to variability in secretion potency of different batches of chromaffin cells. We suspect some side effects of DM-nitrophen or other components of our internal filling solution, since secretion required higher $[Ca^{2+}]_i$ values as compared with previous work.

We initially thought that the ultrafast responses were evoked by calcium spikes on partially photolyzing nitrophen (Kaplan, 1990; Zucker, 1993). However, in many experiments, the first flash failed to trigger a sustained rise in $[Ca^{2+}]_i$, but would still have caused a calcium spike. However, such stimuli never evoked ultrafast responses, or any other secretion. This was surprising, since a similar calcium spike evokes particularly powerful transmitter release at the squid giant synapse (Delaney and Zucker, 1990; Zucker et al., 1991). This might be taken to mean that $[Ca^{2+}]_i$ must remain elevated for more than a few milliseconds in order to trigger exocytosis in chromaffin cells. However, if secretion saturates at the highest rates we have observed (2000 fF/s), then a calcium spike of about 2 ms might cause a rise in capacitance of only 4 fF, which we would not have been able to detect.

One surprising result is the rather weak correlation between rate of secretion and magnitude of $[Ca^{2+}]_i$ step for most of the response types. Certainly one cause of this result is the large variability among chro-

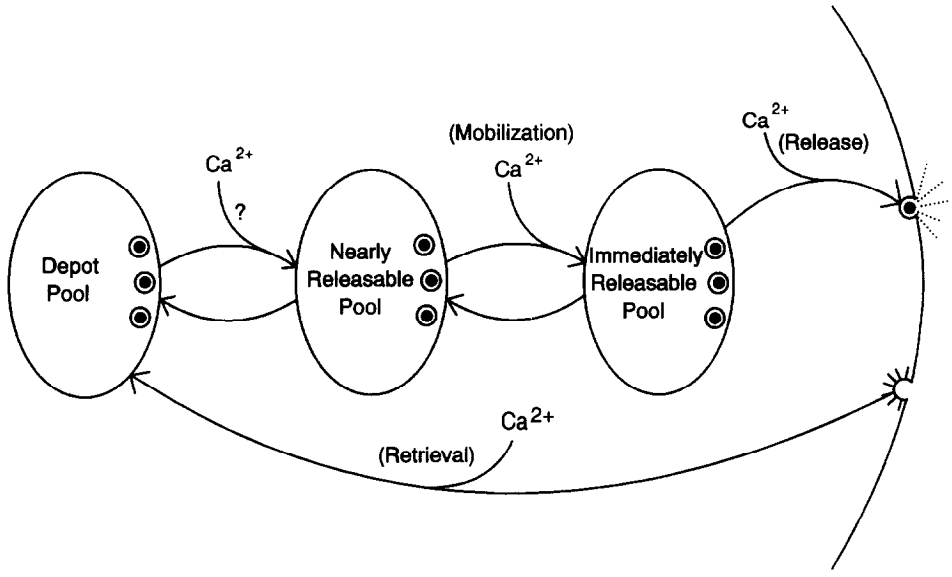

Figure 7. Hypothetical Scheme for the Origin of Different Responses to Flash Photolysis of DM-Nitrophen in Chromaffin Cells

Ultrafast responses are proposed to arise from vesicles in an immediately releasable pool, perhaps docked at release sites; fast responses, from vesicles in a nearly releasable pool, perhaps near release sites but not docked; and slow secretion, from vesicles in a depot pool that must first move through the nearly releasable pool to the immediately releasable pool. Calcium evokes release from the immediately releasable pool, mobilizes vesicles into the immediately releasable pool and perhaps also into the nearly releasable pool, and activates a membrane retrieval process that recovers vesicular membrane previously fused with the plasma membrane. The retrieval process may involve additional intermediate pools (data not shown).

maffin cells, especially cells from different animals. For fast and ultrafast components of release, another source of variability is likely to be the amount of recent release, especially during the loading transient, which may have depleted the pools supplying these release modes (see below) to varying degrees. Another problem is that rapid capacitance decreases and increases may occur nearly simultaneously, confounding the quantitative measure of either. Finally, the slow release process may have been saturated at the high levels of $[Ca^{2+}]_i$ following nitrophen photolysis, as also appears to be the case in the data of Augustine and Neher (1992).

Origin of the Different Modes of Release

The present results add a new degree of complexity to calcium action in secretory processes. Previous evidence demonstrating that a rise in $[Ca^{2+}]_i$ results in secretion has been confirmed. We have now shown that calcium plays a number of more or less distinct roles. Figure 7 provides a schematic of what we propose as the sites of calcium action and the origin of the different components of capacitance change that a rise in $[Ca^{2+}]_i$ can evoke.

We imagine that secretable hormone, contained in vesicles, is in various states of releasability. Some vesicles are docked or attached to release sites on the plasma membrane: these are available for immediate release and result in a very rapid, but short-lived capacitance transient (our ultrafast release) in response to a step rise in $[Ca^{2+}]_i$. But under most circumstances, few vesicles are in this state. Most are at lesser or greater distances from the plasma membrane, or are more or less eligible for immediate release. Some of these vesicles are close to release sites, or are in a nearly releasable state, and can be released within seconds of a rise in $[Ca^{2+}]_i$. These vesicles contribute to the fast release in response to a $[Ca^{2+}]_i$ step, especially that portion of the fast release that occurs more than 1 s after $[Ca^{2+}]_i$ rises. The fact that a prior rise in $[Ca^{2+}]_i$ can bias the response toward an ultrafast release suggests that the movement of vesicles from the nearly releasable state to the immediately releasable state is also calcium dependent. Finally, after the immediately releasable and nearly releasable vesicles undergo exocytosis, a steady, slow rate of secretion persists for as long as $[Ca^{2+}]_i$ remains elevated, until the full complement of vesicles are secreted. Our results do not indicate whether or not this step, too, is calcium dependent. It may be that vesicles move in a calcium-independent manner from a depot store in the bulk cytoplasm to more nearly releasable positions as soon as the latter become available. It appears, however, that after the releasable stores are emptied, their refilling from the depot store is the rate-limiting step in secretion, even while $[Ca^{2+}]_i$ remains elevated.

We observe a total amount of slow secretion of several picofarads, up to a maximum of 10 pF. If the fusion of one vesicle contributes about 2.5 fF of capacitance, slow secretion occurs from a pool of up to 4000 vesicles. This is somewhat less than the 10,000 vesicles estimated to be releasable by nicotinic agonists (Burgoyne, 1991). Perhaps secretion is less than optimal

due to washout of relevant factors in the whole-cell configuration, such as Mg-ATP. Fast and ultrafast responses averaged about 0.5 pF, corresponding to 200 vesicles, roughly similar to the number that appear to lie in the exclusion zone beneath the plasma membrane (Burgoyne, 1991). Calcium may act on calpactin (Drust and Creutz, 1988) to dock vesicles in preparation for fusion, resulting in an ultrafast response to a subsequent $[Ca^{2+}]_i$ rise. Alternatively, calcium might act to disassemble the cortical actin network and permit vesicles to reach docking sites (Aunis and Bader, 1988).

Finally, and most surprisingly, we have found that after most or all calcium-dependent secretion has been exhausted, a later rise in $[Ca^{2+}]_i$ often leads to a decrease in capacitance, which is sometimes quite large and quite rapid. On occasion, we have seen a $[Ca^{2+}]_i$ step to 50–200 μM lead to the recovery within just 2 s of more than 50% of the capacitance increase of prior secretion. It is unclear at this point whether this calcium-dependent membrane retrieval is due to the simultaneous endocytosis of large numbers of vesicles or the rapid formation of a large vacuole, whose surface area may approach half that of the original cell area. Visual inspection of chromaffin cells following capacitance drops revealed no such structure, but it may have gone undetected.

One alternative explanation for the reduction in capacitance we have observed is that it represents the appearance of some sort of structural change that reduces the electrical access to some part of the membrane surface, rather that an actual pinching off of surface membrane. However, the change in complex admittance caused by such an increase in access resistance would show up as an increase in series resistance to a portion of the membrane. Since we monitor series resistance during all experiments and observe no change concurrent with capacitance reduction, this explanation can be rejected.

It must be acknowledged, however, that the picture offered in Figure 7 is not the only possible interpretation of our results. In particular, we have no real evidence that the various observed rates of secretion arise from groups of vesicles in pools linked in a strictly serial fashion. It is possible that more complex rules govern the rate of secretion, or the movement of vesicles between various pools.

Experimental Procedures

Preparation of Cells and Measurement of Secretion

Chromaffin cells from bovine adrenal glands were cultured as described previously (Marty and Neher, 1985). Cells were used 1–4 days after dissociation. The external bathing solution contained 140 mM NaCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES-NaOH (pH 7.2), and 2 mg/ml glucose. Membrane potential was held at −68 mV after correction for a junction potential of −8 mV. Transmembrane current was measured using conventional whole-cell patch-clamp methods and the EPC-9 patch-clamp system (Heka Elektronik, Lambrecht, Germany). The EPC-9 provides automatic series resistance and membrane capacitance compensation. Secretion was monitored as an increase in membrane capacitance, measured using the high resolution, low noise frequency domain technique described by Lindau and Neher (1988). A sinusoidal voltage (800 Hz, 16 mV rms) was applied across the cell membrane and a two-phase, lock-in amplifier was used to measure AC admittance from the current signal. The real and imaginary parts of the admittance were sampled at 2–5 Hz, together with the DC current at the holding potential. From these three quantities, membrane capacitance, series resistance, and membrane conductance were calculated online. Experiments in which apparent changes in series resistance accompanied apparent changes in membrane capacitance were discarded. At the end of some experiments, membrane capacitance independently measured using the EPC-9 capacitance compensation algorithm was found to be similar to the final value reported by the lock-in amplifier.

Control of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was controlled by use of the caged calcium chelator DM-nitrophen (Kaplan and Ellis-Davies, 1988). Our internal solution usually contained nominally 10 mM nitrophen–30%–60% bound to one calcium ion and the remainder in the Na^+ form. We measured the purity of our nitrophen stocks by titration with $CaCl_2$ while measuring $[Ca^{2+}]$ with calcium-sensitive microelectrodes and obtained estimates of 80%–100%. The internal solution also contained 17 mM NaCl, 42 mM Cs-HEPES, 100 mM cesium glutamate, and 0.3 mM GTP, to resemble normal intracellular Na^+ and Cl^- levels, block K^+ current with cesium, provide good control of intracellular pH, and retain the normal G protein regulation of secretion (Knight et al., 1989). In some solutions, the calcium chelator DPTA was added to 4–6 mM to blunt the large changes in calcium concentration caused by photolysis of DM-nitrophen. We measured the effective calcium dissociation constant of DPTA at pH 7.2 as 81 μM, using calcium-sensitive microelectrodes in a solution of 10 mM DPTA, 5 mM $CaCl_2$ in 100 mM KCl, 20 mM MOPS. The osmotic pressure of all pipette solutions was kept at 320 mosm. The salts in our solutions were obtained from Merck; CsOH used to prepare cesium glutamate was from Aldrich Chemical Co., and other reagents were supplied by Sigma Chemical Co.

$[Ca^{2+}]_i$ was elevated rapidly by photolysis of DM-nitrophen (Calbiochem, La Jolla, CA) using a high energy xenon flash lamp (Gert Rapp Optoelektronik, Hamburg, Germany). This lamp discharges up to 340 J of electrical energy in about 1 ms. The light flash was restricted to the near ultraviolet with a Schott UG11 filter having an infrared reflecting coating. When focused onto a cell with a quartz condenser, a flash at one-fourth maximum power photolyzed about 55% of nitrophen 50% loaded with calcium. This was determined by comparing the measured rise in calcium concentration (see below) to predictions of calcium concentration change from a computational model of the calcium binding reactions of nitrophen, its photoproducts, and DPTA (Figure 1; see Delaney and Zucker, 1990). Consistent results were obtained in cells with and without DPTA. To check that the photoproducts of nitrophen do not evoke secretion, 6 cells filled with nitrophen but no added calcium were subjected to repeated flashes. No change in capacitance was observed. To prevent inadvertent photolysis of DM-nitrophen, cells were viewed only with light filtered to remove wavelengths shorter than 540 nm. Illumination for photometry was reduced to the point at which photolysis of DM-nitrophen was small with respect to its supply through the patch pipette, as judged by constancy of measured $[Ca^{2+}]_i$ during pauses of illumination.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by dual-wavelength ratiometric fluorimetry with the indicator dye furaptra (Konishi et al., 1991) purchased from Molecular Probes (Eugene, OR); the free acid (0.3 mM) was included in the internal perfusion solution. The dye was excited with light alternated at 7 Hz between 350 and 390 nm, and the emitted fluorescence was collected with a Leitz 50× water immersion objective, transmitted through a 425 nm dichroic mirror and 470–545 nm barrier filter, and detected with a Zeiss Photometer. Fluorescent light in response to the two excitation wave-

Table 1. Solutions Used to Calibrate Furaptra in Chromaffin Cells

Solution	Na_4NP	Cs-HEPES	Cs-Glu	NaCl	GTP	Furaptra	$CaCl_2$	K_2-EGTA	K_5-DPTA
A	10	50	120	30	0.3	0.3	-	10	-
B	10	114	120	30	0.3	0.3	14.5	-	25
C	10	50	120	30	0.3	0.3	15	-	-

Chromaffin cells were perfused with one of these solutions from patch pipettes. Furaptra fluorescence changes were measured during progressive photolysis of DM-nitrophen with 85 J flashes, and the results were correlated with calculated $[Ca^{2+}]$ levels in the solutions to derive fluorimetric calibration parameters appropriate for different numbers of flashes. NP, DM-nitrophen. All concentrations are in millimolar.

lengths was collected as described by Neher (1989). The two demultiplexed light signals, membrane potential, transmembrane current, and real and imaginary parts of the complex admittance were acquired simultaneously at a sample rate of 2 or 5 Hz in an Atari personal computer, displayed online, and saved on disc as data files. A rough online conversion of the furaptra fluorescence to a calcium concentration was also provided by the computer. Offline postprocessing included recalibration of calcium records (see below) and differentiation of capacitance records, sometimes with digital smoothing, to produce a measure of the rate of secretion.

Calibration of Furaptra

Furaptra fluorescence is also sensitive to magnesium concentration (Raju et al., 1989), and nitrophen also binds magnesium and releases it when photolyzed (Kaplan and Ellis-Davis, 1988). Therefore, we felt it necessary to keep magnesium out of the internal solution. Since Mg^{2+} is usually provided with ATP as a cofactor, it seemed advisable to do experiments without Mg^{2+}-ATP. However, Baker and Knight (1986) have reported that Mg^{2+}-ATP is required for secretion in permeabilized chromaffin cells. To test whether this requirement extends to intact cells, we compared secretion evoked by exposure to 1.5 mM ionomycin (CalBiochem) with 2 mM $CaCl_2$ in the medium in cells with and without 2 mM Mg^{2+}-ATP in the internal solution. Ionomycin caused $[Ca^{2+}]_i$ to rise to about 1.5 μM (measured with fura-2 as described by Augustine and Neher [1992]) and evoked secretion at rates up to 30 fF/s in both groups of cells (see Augustine and Neher, 1992). Moreover, omission of Mg^{2+}-ATP in cells perfused with calcium buffer has no effect on rate of secretion for the first 2–4 min (C. Heinemann and E. Neher, unpublished data), the usual duration of our experiments. Therefore, Mg^{2+}-ATP was omitted from internal perfusion solutions in this study. This result is in agreement with a recent study by Bittner and Holz (1992), which shows that an ATP requirement develops only several minutes after first stimulating cells with high calcium.

The sensitivity of fluorescent calcium indicators is affected by intracellular environmental factors (Roe et al., 1990), photobleaching (Becker and Fay, 1988), and the presence of photolabile chelators, which may themselves be fluorescent (Zucker, 1992). Therefore, we devised a calibration protocol for furaptra that resembled the actual measurement conditions and provides calibration parameters appropriate for different amounts of exposure to photolytic light.

We assume that after n flashes of 85 J (the dimmest flashes we used), $[Ca^{2+}]$ is related to R, the ratio of fluorescences excited at 350/390 nm, according to

$$[Ca^{2+}] = K_{eff}(n)(R - R_{min}(n))/(R_{max}(n) - R, \quad (1)$$

which is analogous to Equation 5 of Grynkiewicz et al. (1985), but allows K_{eff} (the effective dissociation constant of furaptra), R_{min} (minimum fluorescence ratio at low $[Ca^{2+}]$), and R_{max} (maximum fluorescence ratio at high $[Ca^{2+}]$) to vary as functions of flash number (n), because of changes in the fluorescence properties of the solution mix as photolysis proceeds.

Three sets of whole-cell recordings were performed to determine $R_{min}(n)$, $R_{max}(n)$, and $K_{eff}(n)$ using pipette solutions that were similar to test solutions, but included calcium buffers such that $[Ca^{2+}]$ was either fixed or varied with ultraviolet light flashes in a predictable fashion. For determining R_{min}, solution A (see Table 1), which included 10 mM EGTA and no calcium to fix $[Ca^{2+}]$ at low levels, was used. Following establishment of a whole-cell recording, ten 85 J flashes were applied at times similar to those of flashes during test measurements (150–300 s after break in). Fluorescence values were recorded, and it was found that R_{min} (= 0.082) did not change significantly with the flashes.

For determining K_{eff} and R_{max}, ideally solutions should be used with $[Ca^{2+}]$ fixed at intermediate and very high values, respectively. This is not possible if calibration solutions are to resemble test solutions. To fix $[Ca^{2+}]$ at intermediate levels in the presence of 10 mM DM-nitrophen, excessively high concentrations of calcium buffer would be required. Likewise, to achieve calcium concentrations high with respect to K_{eff} of furaptra, almost isotonic calcium salt solutions would be required. We therefore chose to use calibration solutions that buffer $[Ca^{2+}]$ partially to medium and high values (solutions B and C) and to calculate $[Ca^{2+}]$ in these solutions as a function of flash number, based on the calcium binding constants of the compounds involved and on the photolysis efficiency of our flash lamp. Calculations assumed calcium dissociation constants of 5 nM for nitrophen, 81 μM for DPTA, 0.25 mM for the nitrosoacetophenone-substituted iminodiacetic acid photoproduct of nitrophen photolysis, and 120 mM for glutamate. The latter values were obtained by measuring free $[Ca^{2+}]$ in mixtures of calcium and photolyzed nitrophen or glutamate, using calcium-sensitive electrodes for the former and optical indicators for the latter. Predictions were calculated for several flash numbers, and functions were fitted by least squares to obtain analytical expressions for the intermediate $[Ca^{2+}]$ solution ($[Ca^{2+}]_{med}(n)$) and the high $[Ca^{2+}]$ solution ($[Ca^{2+}]_{high}(n)$), respectively:

$$[Ca^{2+}]_{med}(n) = 27.9 + 13.4n - 0.88n^2 \quad (2)$$

$$[Ca^{2+}]_{high}(n) = 2794 - 432\exp(-0.546n), \quad (3)$$

where $[Ca^{2+}]$ is in micromolar and n is the number of 85 J flashes. Concentration values and fits are shown in Figure 8A.

Likewise, measured values of fluorescence ratios were fitted to yield functions $R_{med}(n)$ and $R_{high}(n)$, respectively:

$$R_{med}(n) = 0.1003(1 + 0.06n - 0.0016n^2) \quad (4)$$

$$R_{high}(n) = 0.586(1 - 0.015n). \quad (5)$$

Pooled data from four calibration experiments are shown in Figure 8B, together with the fits.

Equations 2–5 can be inserted into Equation 1 to yield, for a given number of flashes, two equations for the two unknowns $K_{eff}(n)$ and $R_{max}(n)$, considering $R_{min}(n)$ to be fixed and known (see above). A BASIC routine that evaluated these quantities was written. In the program that displayed digitized experimental data from experiments, $[Ca^{2+}]$ values were calculated according to Equation 1 for a given segment between two flashes, after $K_{eff}(n)$ and $R_{max}(n)$ had been evaluated for the number of flashes preceding that segment.

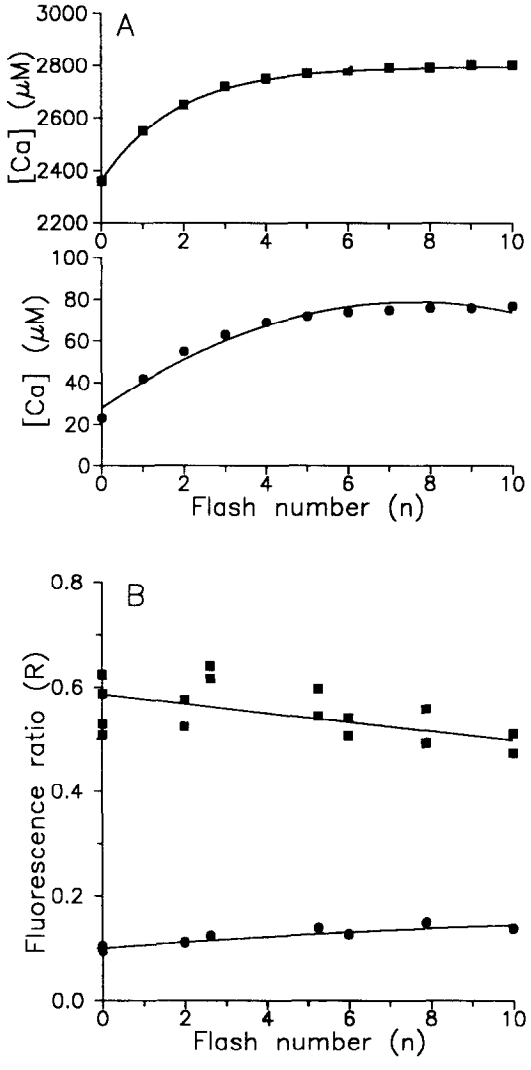

Figure 8. $[Ca^{2+}]$ and Fluorescence Ratios in Calibration Solutions Used to Determine the Calibration Constants in Equation 1

(A) Calculated $[Ca^{2+}]$ in high $[Ca^{2+}]$ (upper) and in low $[Ca^{2+}]$ (lower) calibration solutions, as functions of 85 J flash number. The fitted lines (from the top) are from Equations 3 and 2. (B) Measured fluorescence ratios in the high $[Ca^{2+}]$ (closed squares) and the low $[Ca^{2+}]$ (closed circles) calibration solutions, as a function of flash number. The fitted lines (from the top) are from Equations 5 and 4.

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