**Time course of Ca\(^{2+}\) concentration triggering exocytosis in neuroendocrine cells**

(Robert H. Chow, Jürgen Klingauf, and Erwin Neher*)

Department of Membrane Biophysics, Max Planck Institute for Biophysical Chemistry, Am Fassberg, D-37077 Göttingen, Germany

**Contribution by Erwin Neher, September 20, 1994**

**ABSTRACT** We have used the secretory response of chromaffin cells to estimate the submembrane intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) “seen” by secretory granules during short depolarizations. The rate of secretion during a depolarization was assessed by combining the electrochemical method of capacitometry and electrical capacitance measurements. The rate was then related to [Ca\(^{2+}\)]\(_i\), based on a previous characterization of how Ca\(^{2+}\) affects the dynamics of vesicle priming and fusion in chromaffin cells (Heinemann, C., Chow, R. H., Neher, E. & Zucker, R. S. (1994) Biophys. J. 67, in press). Calculated [Ca\(^{2+}\)]\(_i\) rose during the depolarization to a peak of <10 \(\mu\)M, then decayed over tens of milliseconds. In synapses, vesicles are presumed to be located within nanometers of Ca\(^{2+}\) channels where [Ca\(^{2+}\)]\(_i\) is believed to rise in only microseconds to near steady-state levels of hundreds of micromolar. Channel closure should lead to a decrease in [Ca\(^{2+}\)]\(_i\), also in microseconds. Our findings of the slower time course and the lower peak [Ca\(^{2+}\)]\(_i\) suggest that in chromaffin cells, unlike synapses, Ca\(^{2+}\) channels and vesicles are not strictly colocalized. This idea is consistent with previously published data on dense-core vesicle secretion from diverse cell types.

Although Ca\(^{2+}\) has long been recognized to trigger secretion in neuronal (1) and neuroendocrine cells (2), quantitative measurements of the time course of submembrane intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) at the secretory apparatus are still lacking. In neurons, several indirect approaches have been applied to estimate the time course. First, some investigators have used mathematical models to understand the dynamics of [Ca\(^{2+}\)] in the vicinity of individual channels, where vesicles are presumed to be located (3–7). These models predict that the [Ca\(^{2+}\)] in the channel “microdomains” rises in only microseconds to “steady-state” levels of hundreds of micromolar. There is a steep concentration gradient, falling away from the pore over a few nanometers, that is sustained as long as the channel remains open. Closure of the channel leads to collapse of the gradient in tens of microseconds. Visualizing such rapid and highly local changes in [Ca\(^{2+}\)], is beyond the resolution of diffusible Ca\(^{2+}\) indicators such as fura-2. Thus, special probes have been used to monitor [Ca\(^{2+}\)] levels beneath the membrane or near Ca\(^{2+}\) channels: aequorin, a low-affinity Ca\(^{2+}\)-indicator protein (8); Ca\(^{2+}\)-activated potassium channels, which are colocalized with Ca\(^{2+}\) channels in hair cells (9); and a membrane-bound derivative of fura-2 (10). Although the probes have been severely limited in temporal and/or spatial resolution, the results are compatible with there being much higher Ca\(^{2+}\) levels adjacent to the plasma membrane than can be measured in the bulk cytoplasm with diffusible Ca\(^{2+}\) indicator dyes, and they have generally supported the notion of the Ca\(^{2+}\) channel microdomain.

These diverse approaches have yielded insight into the behavior of [Ca\(^{2+}\)] near Ca\(^{2+}\) channels, but they do not directly measure the time course at the secretory machinery during a depolarization. Further, the model results depend strongly on assumptions regarding Ca\(^{2+}\) buffering and the exact distances separating vesicles and Ca\(^{2+}\) channels.

We demonstrate here that by combining electrical capacitance measurements and the electrochemical method of amperometry we can estimate the peak level and the time course of [Ca\(^{2+}\)] “seen” by the vesicle fusion apparatus in bovine chromaffin cells. The key is using the secretory response itself to back-calculate [Ca\(^{2+}\)] based upon the previous characterization of the kinetics of secretion as a function of [Ca\(^{2+}\)] (11, 12). An assumption underlying our approach is that the rate of secretion has the same dependence on [Ca\(^{2+}\)] for depolarizing pulses and for the spatially uniform elevation of [Ca\(^{2+}\)] induced by flash photolysis of caged Ca\(^{2+}\).

The rate of secretion at any instant depends upon both the level of [Ca\(^{2+}\)], acting at the fusion machinery and the number of vesicles that are available for fusion in the “release-ready” pool. A kinetic scheme summarizing the relationships among functional vesicle pools is as follows (11).

\[
\begin{align*}
A \xrightarrow{k_1(Ca)} A_1 \xrightarrow{k_2} B_0 & \xrightarrow{\gamma} B_1 \xrightarrow{\delta B_2} \xrightarrow{\delta B_3} \xrightarrow{\gamma} C \\
\end{align*}
\]

Scheme I

Vesicles residing in a release-ready pool (B\(_0\)) bind Ca\(^{2+}\) ions in three successive stages (B\(_1\), B\(_2\), B\(_3\)) and then fuse in a final, rate-limiting step (\(\gamma\)) to increment the fused-vesicle pool (C). Pool B\(_0\) is refilled from two other pools of vesicles in series (pools A and A\(_1\)) that are not yet ready for fusion. This kinetic scheme was able to predict the time course of secretion in response to uniform step elevations of [Ca\(^{2+}\)] (11). We perform the reverse operation here, using the rate of secretion from amperometric measurements to calculate the Ca\(^{2+}\) acting at the secretory machinery during short (20-ms) voltage step depolarizations.

We find that [Ca\(^{2+}\)]\(_i\) rises monotonically to a peak of <10 \(\mu\)M at the end of the depolarization, from which it decays over tens of milliseconds. The peak level attained is lower and the time course is slower than would be expected if vesicles and Ca\(^{2+}\) channels were colocalized on a molecular scale in chromaffin cells.

Part of this work has been presented in abstract form (13, 14).

**METHODS**

**Cell Preparation.** Bovine chromaffin cells were prepared as described (15) and were used for experiments on the first and second day after preparation.

**Abbreviation:** [Ca\(^{2+}\)]\(_i\), intracellular Ca\(^{2+}\) concentration.

*To whom reprint requests should be addressed.
Whole-Cell Patch Clamp, [Ca\(^{2+}\)]

The experiments were performed at room temperature (21–26°C) with a bath solution consisting of 140 mM NaCl, 2.8 mM KCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), and 10 mM Hepes (pH 7.2, with NaOH). The whole-cell configuration was achieved by using Sygard-coated borosilicate glass pipettes having electrode resistances of 2–5 MΩ. Access resistance during experiments ranged from 4 to 15 MΩ, with an internal pipette solution containing 145 mM cesium glu-
tamate, 8 mM NaCl, 1 mM MgCl\(_2\), 2 mM MgATP, 0.3 mM GTP, 10 mM Hepes (pH 7.2, with CsOH), and 100 μM fura-2 (Molecular Probes). The cell-averaged [Ca\(^{2+}\)] was determined in single cells by standard microfluorimetric ratio techniques (11, 16). Capacitance measurements were performed with a “software lock-in amplifier” (based on the pulse control programs from J. Herrington and R. Book-
man, University of Miami). The command potential was a 50-mV peak-to-peak sine wave at 1600 Hz.

Amperometry. Carbon-fiber electrodes were prepared with polyethylene insulation (17). During experiments, the electrodes were backfilled with 3 M KCl and mounted on an EPC-7 patch-clamp headstage. A constant voltage of 800 mV (versus an Ag/AgCl bath electrode) was applied to the electrode, whose tip (sensing surface) was pushed gently against the cell surface. The amperometric current was recorded at 1600 Hz. Analysis was performed with programs written in Igor macro language (WaveMetrics, Lake Oswego, OR).

For all experiments reported here, step depolarizations of 20 ms to +10 mV from a holding potential of −70 mV were applied at 0.2 Hz to activate Ca\(^{2+}\) channels and trigger secretion. These short stimuli elicited release of transmitter in quantal packets—usually only one or two events per depolarization—which appeared as well-isolated current spikes in amperometric recordings. Fig. 1 illustrates the important concepts of latency time and latency histogram.

Estimation of the Submembrane [Ca\(^{2+}\)] Time Course at the Vesicle Fusion Sites. The kinetic model of Heinemann et al. (11) was simplified in the case of single short depolarizations by neglecting replenishment of the pool of release-ready vesicles (pool B\(_0\)) from pools A and A\(_1\), since these replenish-
ment steps take place on the scale of half a second to seconds, much longer than the depolarizing pulses. Further, we have neglected an additional step, the short delay between vesicle fusion (which is detected immediately in capacitance measurements) and the detection of the released product at the carbon fiber (13). This delay is likely to be <5 ms in the present conditions (unpublished work). Neglecting this step simplifies calculations and does not affect the estimate of peak [Ca\(^{2+}\)], although it will lead to a slightly altered time course (not shown).

Pool B\(_0\) varies in size between about 200 and 500 fC in flash experiments (11) as well as for rapid trains of depolarizations (18). The dynamics of the pool are described in matrix notation by

\[
\frac{db}{dt} = \begin{pmatrix} -3αX & β & 0 & 0 \\ 3αX & -β - 2αX & 2β & 0 \\ 0 & 2αX & -β - αX & 3β \\ 0 & 0 & αX & -3β - γ \end{pmatrix} \begin{pmatrix} b_1 \\ b_2 \\ b_3 \end{pmatrix} \tag{1}
\]

where X denotes [Ca\(^{2+}\)]; α, β, and γ are the parameters of the kinetic scheme; and b = (B\(_0\) B\(_1\) B\(_2\) B\(_3\))^T is the vector of the pool sizes of vesicles that have bound zero, one, two, or three Ca\(^{2+}\) ions, as denoted by the subscripts.

Given an initial guess of the [Ca\(^{2+}\)] vector x with components X\(_i\) at each discrete time i, a set of predicted values Y\(_i^{\text{pred}}\) of the observable variable Y\(_i^{\text{obs}}\) (rate of secretion) can be calculated according to

\[
Y_i^{\text{obs}}(b_i(x)) = (0 \ 0 \ 0 \ y_i) b_i 
\]

where b\(_i\) is computed by integrating Eq. 1 with a four-step Runge-Kutta routine. Since the step size for numerical integration had to be smaller than the binwidth of the data, the underlying process X was assumed to be piecewise linear between the data points i.

Now the “unknown” vector x can be determined by minimizing some measure of the deviations of the predicted Y\(_i^{\text{pred}}\) from the measured Y\(_i^{\text{obs}}\). We chose to minimize

\[
χ^2 = \sum_{i} \frac{(Y_i^{\text{obs}} - Y_i^{\text{pred}(b_i(x)})^2}{Y_i^{\text{obs}}} \tag{3}
\]

by using the iterative Levenberg–Marquardt algorithm [note that in the denominator of χ\(^2\) the observed frequencies of secretory events are taken as a measure of the variance, as is appropriate for a Poisson process such as quantal secretion in chromaffin cells (17)]. The fit was accepted when χ\(^2\) differed by <0.1% from one iteration to the next.

RESULTS

Peak [Ca\(^{2+}\)], Attained During a Depolarization. Electrical capacitance measurements monitor the cell membrane surface area, which increases on exocytotic addition of vesicular membrane, giving a cumulative record of secretion (19). The derivative of the capacitance trace at any given time gives the instantaneous rate of secretion (18, 20). Ideally, one would like to measure the slope of the capacitance trace during a depolarization to obtain the rate of secretion, and then relate this to [Ca\(^{2+}\)], by using the data obtained previously from
caged-Ca\(^{2+}\) experiments. Unfortunately, during a depolarization, capacitance measurements are invalid, owing to the large (nonlinear) membrane conductance change associated with ionic channel activation (19, 21). In addition, there is a non-secretion-related capacitance transient (\(\Delta C\)) immediately after termination of a depolarization, which has been attributed to Na\(^+\) channel gating transitions (22). In rat chromaffin cells \(\Delta C\) has an amplitude of about 8 fF per nA of Na\(^+\) current (The Na\(^+\) current in our bovine chromaffin cells averaged about 1.23 ± 0.6 nA, mean ± SD, \(n = 8\)) and decays approximately exponentially with a time constant of <100 ms.

Amperometric detection of released catecholamines is not compromised by such sources of error. The time course of secretion is represented by latency histograms such as that of Fig. 1, which give the frequency of occurrence of release events as a function of time during and after a depolarization. This is proportional to the release rate and its integral should be proportional to the "ideal" capacitance record (i.e., a capacitance record without the above-mentioned problems). Unfortunately, comparison of the two kinds of measurements is not straightforward, since the amperometric electrode detects only a fraction of the release events. The problem can be overcome when capacitance and amperometric events are measured simultaneously. Then, the integral of the latency histogram can be scaled by an empirical factor such that the total amplitude of the two curves agrees. This factor includes the conversion from release event to capacitance (2.5 fF per vesicle; ref. 21) and the fraction of events detected. Fig. 2A illustrates the average capacitance record from 531 depolarizations from the same nine cells as in Fig. 1. Superimposed is the integrated latency histogram (compiled from 790 amperometric events acquired in parallel with the capacitance records). The integrated histogram was scaled to match its amplitude with that of the capacitance record at 180 ms after the end of the 20-ms depolarization, when artifacts are expected to have subsided (22). The required scaling factor allows one to calculate the fraction of events detected. For the case of Fig. 2 a value of 9% is obtained, in reasonable agreement with the fraction of the surface area covered by the carbon fiber (17, 23, 24).

The maximal slope of the integrated latency histogram occurred at the end of the depolarization (tangent line) and, in units of capacitance, was about 860 fF/s. In the caged-Ca\(^{2+}\) experiments, such a maximum rate of secretion was observed with steps in [Ca\(^{2+}\)] (to about 5 \(\mu M\) (Fig. 2B). This preliminary estimate of the peak [Ca\(^{2+}\)] will be refined below.

Our analysis makes the assumption that the release-ready pool of vesicles is approximately the same size in both the flash and the depolarization experiments. This assumption is reasonable, since the pool size depends on the basal [Ca\(^{2+}\)] (18), which was about the same in both sets of experiments (about 500 nM; see Fig. 3C and ref. 11). Endocytosis, which becomes more pronounced at late times, can lead to an underestimate of the amount of secretion as monitored by capacitance, and thus to underscaling of the latency histograms. Due to the third- or fourth-power (11, 18) dependence of the secretory rate on [Ca\(^{2+}\)], however, even a 2-fold underestimate of the rate of secretion would lead to only about a 1.25-fold underestimate of the peak [Ca\(^{2+}\)].

Time course of [Ca\(^{2+}\)]. At the Secretory Machinery. It should be possible to use the kinetic scheme to calculate the [Ca\(^{2+}\)] time course at the secretory machinery during and after a depolarizing stimulus, if one has the starting vesicle pool sizes and the time course of the rate of secretion for a single depolarization (see Methods). The time course of the rate of secretion was obtained by scaling the histogram in Fig. 1 by the empirical scaling factor as determined in Fig. 2A. The histogram is shown in Fig. 3B.

**DISCUSSION**

We have shown that the secretory process itself can provide a readout of the submembrane [Ca\(^{2+}\)] during short depolarizing stimuli. Two approaches yielded apparent peak [Ca\(^{2+}\)] of <10 \(\mu M\) for 20-ms depolarizations. The estimated time course peaked at the end of the depolarization and decayed over tens of milliseconds. These findings differ significantly from model predictions of the [Ca\(^{2+}\)] time course at short distances (10–50 nm) from a channel pore (3–7), suggesting
that in chromaffin cells, vesicles are on average further separated from channels. This possibility is supported by the absence of morphological features resembling active zones (26), the structures where vesicles and channels are localized in synapses (27).

In squid giant synapse and bipolar neurons, the Ca²⁺ buffer EGTA at 10 mM has no effect on secretion (28, 29). These findings have been used to argue that the secretory vesicles are adjacent to channels, since EGTA at this concentration should be ineffective at buffering Ca²⁺ in the immediate vicinity (10–40 nm) of a Ca²⁺ channel pore (5, 7). In contrast, in bovine chromaffin cells EGTA at only 1–5 mM significantly reduces or blocks depolarization-induced secretion (20, 21), supporting the view that vesicles are positioned further from Ca²⁺ channels.

The idea that vesicles are not molecularly coupled to Ca²⁺ channels does not rule out the possibility that some structural feature ensures focal transmitter release. Although chromaffin cells do not form synapses onto other cells, secretion from individual cells may be polarized in the direction of blood vessels (24, 26), for example, by restricting the zones of Ca²⁺ entry. Indeed, in chromaffin cells Ca²⁺ channels appear to be clustered (30). Such clustering apparently results in "hot spots" of secretion, as has been reported by Schroeder et al. (24), using carbon-fiber electrodes having tip diameters of about 2 μm. It is possible also that only certain types of Ca²⁺ channels are clustered, giving rise to differential sensitivity of secretion to selective Ca²⁺ channel blockers (31).

Such complications are not directly addressed in our approach. In fact, our model assumes a single class of functionally identical vesicles, which all experience the same [Ca²⁺]i time course. Nevertheless, the resulting error in our [Ca²⁺]i estimate should be small. Consider, for instance, the case that vesicles are uniformly distributed and Ca²⁺ channels are clustered such that only 12% of the pool of releasable vesicles experience rapid changes in [Ca²⁺]i. This would lead at first approximation to a factor-of-8 underestimate of the secretion rates (per vesicle) and a factor-of-about-2 underestimate of the [Ca²⁺]i. The peak level, then, would still be on the order of only 10 μM, still much less than predicted if vesicles were located in the microdomain of a single Ca²⁺ channel (3–7).

Fig. 4A contrasts schematically two hot spots—one (Upper) with “molecular coupling” of Ca²⁺ channels and vesicles at a small, fixed distance (20–30 nm) and the other (Lower) with random positioning of vesicles. The first case applies to synapses, whereas the second, we propose, applies to neuroendocrine cells such as chromaffin cells. The fusion machinery will be subjected to very different [Ca²⁺]i time courses in the two cases. In the first case (Fig. 4B Upper), upon channel opening, [Ca²⁺]i rises in only microseconds to tens or hundreds of micromolar and, upon channel closing, decays away just as rapidly as predicted by microdomain models of Ca²⁺ diffusion (3–7). The slower rising and falling and lower peak [Ca²⁺], of the second case (Fig. 4B Lower) occur because of the greater diffusional delay and more significant Ca²⁺ buffering effects faced by Ca²⁺ ions traveling.
We gratefully acknowledge Christian Heinemann for participation in experiments, M. Pilot and F. Friedlein for expert technical assistance, and Antonio Artalejo, Kevin Gillis, and Anant Parekh for critical feedback on the manuscript. We also thank Dr. Richard Bookman for providing the 1024 programs for the software lock-in and high-speed multichannel data acquisition.