Rapid Exocytosis in Single Chromaffin Cells Recorded from Mouse Adrenal Slices

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We report here that brief depolarizations such as action potentials trigger exocytosis in thin mouse adrenal slices. The secretory rates obtained in membrane capacitance recordings from chromaffin cells in slices are faster than those observed in isolated cells. Fast exocytosis in slices is attributable to the rapid release of a small pool of vesicles. The pool recovers from depletion with a time constant of 10 sec. Recruitment of the rapidly released vesicles is strongly hindered by the fast Ca$^{2+}$ chelator BAPTA and much less by the slower chelator EGTA. We suggest that these vesicles are located in close proximity to Ca$^{2+}$ channels. Spatial coupling of Ca$^{2+}$ entry and exocytosis may be sensitive to cell isolation and culture.

Key words: exocytosis; membrane capacitance measurement; chromaffin; adrenal slice; calcium chelators; calcium-secretion coupling; neuroendocrine; calcium current; secretory depression

Exocytosis is triggered by an elevation in cytosolic [Ca$^{2+}$] in both neuroendocrine cells and nerve terminals (Douglas, 1968; Katz, 1969). Furthermore, just as in presynaptic terminals, action potentials (APs) are physiological stimuli for hormone release in neuroendocrine cells (Kidokoro and Ritchie, 1980; Wakade, 1982; Zhou and Misler, 1995). Nevertheless, there are distinct differences between hormone secretion from isolated neuroendocrine cells and release of transmitters from small clear-cored vesicles at neuronal synapses. For example, catecholamine release from isolated chromaffin cells is only loosely coupled to action potentials (Zhou and Misler, 1995), with latencies of several tens of milliseconds, whereas neurotransmission is highly synchronized (Katz and Miledi, 1965). The majority of the time delay of release in isolated chromaffin cells is attributed to the time required for Ca$^{2+}$ to diffuse from a Ca$^{2+}$ channel orifice to the release sites (Chow et al., 1996). On this basis a mean diffusional distance on the order of 300 nm has been estimated (Klingauf and Neher, 1997). Exocytosis of chromaffin granules can occur very quickly if the [Ca$^{2+}$] at the release site is elevated to a sufficiently high level (Heinemann et al., 1994). In fact, isolated chromaffin cells release a small fraction of granules well synchronized with depolarizing stimuli, with latencies of <5 msec (Chow et al., 1994). This more synchronous release has been suggested to result from exocytosis of a small population of vesicles located closer to Ca$^{2+}$ channels (Klingauf and Neher, 1997).

Morphological studies have suggested a polarized phenotype for adrenal chromaffin cells in situ (Carmichael, 1986). Synaptic inputs occur at the neural pole, and exocytosis may take place preferentially at the capillary pole (Carmichael et al., 1989). We were interested in whether chromaffin cells in situ show substantial synchronous secretion, as would be expected if distances for Ca$^{2+}$ diffusion were short in a specialized region of the cell.

We performed patch-clamp measurements of membrane capacitance ($C_{m}$) (Neher and Marty, 1982) in mouse chromaffin cells in thin slices of adrenal glands and in primary culture. We report here that kinetics of exocytosis is more neuron-like in mouse chromaffin cells in slices than in culture. Cells in our slice preparation typically responded to individual APs with sizable exocytotic capacitance changes ($\Delta C_{exo}$), whereas only one of seven isolated cells showed comparable responses to single APs. Cells in slices secreted more than isolated cells for equivalent Ca$^{2+}$ current integrals during short step depolarizations. Two different protocols, both evoking secretory depression, were used to demonstrate the existence of a small, rapidly secreted pool of vesicles in slices. The rapid kinetics of secretion in chromaffin cells in slices is suggested to result from close spatial coupling of release sites and Ca$^{2+}$ channels.

MATERIALS AND METHODS

Adrenal slice preparation and whole-cell recordings. NMRI mice (4- to 10-week-old females) were killed by decapitation. After adrenal glands were removed, they were embedded in a 3% agar solution. The hardened agar block was then glued with cyanocrylate onto the stage of a slicing chamber. The chamber contained ice-cold bicarbonate-buffered saline (BBS, solution 2). Slices of 100–200 µm thickness were sectioned on a vibrating tissue slicer (Campden Instruments, Cambridge, UK) at a frequency of 6 Hz. After the slices were sectioned, they were immediately transferred into a holding chamber containing oxygenated BBS (solution 1, continuously bubbled with 95% O$_2$ and 5% CO$_2$). Slices were kept at 37°C for 15 min and thereafter at room temperature.

For recording, slices were fixed in the recording chamber by means of a grid of nylon threads. After the slices were mounted onto the stage of an upright microscope (Axioskop, Zeiss), the chamber was perfused with bubbled BBS (95% O$_2$ and 5% CO$_2$ solution 1) at a flow rate of 1–2 ml/min. Usually a cleaning pipette was used to remove loose material from the cell surface. Conventional whole-cell recordings (Hamill et al., 1981) were performed with 3–4 MΩ pipettes, and an EPC-9 patch-clamp amplifier together with Pulse software (HEKA, Lambrecht, Germany) were used. Usually, gigahm seals were formed on the nucleus-containing...
Evoked Ca\(^{2+}\) currents were measured under conditions in which potassium currents were blocked by intracellular Cs\(^{+}\) and extracellular d-tubocurarine (Park, 1994). We did not use tetrodotoxin to block sodium channels because it prolongs a transient nonsecretory capacitance change (\(\Delta C_m\)) (Horrigan and Bookman, 1993), which is observed after depolarization of chromaffin cells. Instead, when the whole-cell current was integrated for estimation of the Ca\(^{2+}\) charge (\(Q_{\text{Ca}}\)), the current during the first 1.9 msec was neglected for both preparations. Thus, our \(Q_{\text{Ca}}\) estimate presumably missed the activating phase of the Ca\(^{2+}\) current. For 2 msec pulses, therefore, \(Q_{\text{Ca}}\) represents mainly the Ca\(^{2+}\) tail current (\(Q_{\text{Ca}}\) was measured until 1 msec after the end of the depolarization). No leak correction was applied; instead, cells with resting currents of more than 30 pA were discarded from analysis. Experiments on both preparations were carried out at room temperature.

Isolated chromaffin cell preparation and whole-cell recordings. After they were removed, 8–12 mouse adrenal glands were minced in cold calcium-free saline (Locke’s buffer). The tissue was then incubated with collagenase (Collagenase A, Boehringer Mannheim, Mannheim, Germany; catalytic activity 0.97 U/mg) in Locke’s buffer at a concentration of 3 mg/ml, in a shaking bath at 37°C for 15, 10, and 5 min. The tissue was triturated between two plastic wares. The collagenase was then washed, and the suspended material was filtered through a nylon mesh. After centrifugation, cells were resuspended in culture medium (M199 medium, Biochrom KG, Berlin, Germany) supplemented with penicillin/streptomycin, 10% FCS, and 1 mg/ml bovine serum albumin, plated on poly-L-lysine-coated coverslips, and incubated at 37°C with 5% CO\(_2\).

Cells were used for experiments starting 16 hr after isolation up to day 2 of culture. Chromaffin cells could easily be discriminated from cortical cells and fibroblasts by their round and smooth appearance. Experiments were carried out on an inverted microscope (Zeiss Axiovert 100). An EPC-9 patch-clamp amplifier was used together with PULSE software (Heka, Lambrecht, Germany). The access resistance ranged from 4 to 10 MΩ.

Whole-cell capacitance measurements. After the whole-cell configuration was established, the membrane capacitance was compensated by means of the “\(C_{\text{slow}}\)” compensation feature of the EPC-9. Capacitance measurements were performed using the Lindner–Neher technique implemented as the “\(C_{\text{slow}}+C\)” mode of the “software lock-in” extension of PULSE software. A 1 kHz, 70 mV peak-to-peak sinusoidal stimulus was applied at a DC holding potential of ~80 mV.

Estimation of \(\Delta C_m\). Capacitance changes obtained in mouse chromaffin cells in response to depolarizations show, in addition to stable

\[
\Delta C_m \approx Q_{\text{Ca}} \frac{V_{\text{hold}}}{C_{\text{m,initial}}} - \Delta C_{\text{m,transient}}
\]

is the capacitive transient component that results from the activation of cation channels during the depolarization.

\[
\Delta C_{\text{m,transient}} = Q_{\text{Ca}} \frac{V_{\text{hold}}}{C_{\text{m,initial}}}
\]

where \(C_{\text{m,initial}}\) is the initial capacitance before the depolarization and \(Q_{\text{Ca}}\) is the charge of the Ca\(^{2+}\) current. The capacitance change \(\Delta C_{\text{m,transient}}\) is determined by integrating the transient current over the duration of the depolarization. The change in capacitance \(\Delta C_m\) is then calculated by subtracting the transient capacitance change \(\Delta C_{\text{m,transient}}\) from the total capacitance change.

\[
\Delta C_m = \Delta C_{\text{m,stable}} + \Delta C_{\text{m,transient}}
\]

RESULTS

Mouse chromaffin cells in slices secrete in response to single APs

Patch-clamped mouse chromaffin cells in slices were stimulated with AP-like voltage commands. The AP voltage template used was similar to the average shape of mouse chromaffin cell APs (n = 80) recorded from three cells in slices in the current-clamp mode (Fig. 1A). To monitor the secretory response, we measured \(C_{\text{m}}\) before and after each stimulus (\(C_{\text{m}}\) cannot be measured during the depolarization). In each of the seven cells we studied, AP stimulation caused a stable \(C_{\text{m}}\) increment after a rapidly decaying \(C_{\text{m}}\) transient (for an example, see Fig. 1B). Although the stable \(C_{\text{m}}\) increments most likely represent exocytotic \(C_{\text{m}}\) changes (\(\Delta C_{\text{exo}}\)), the initial \(C_{\text{m}}\) transient (\(\Delta C_{\text{t}}\)) is probably attributable to a nonsecretory capacitance change caused by gating charge movement of sodium channels (Horrigan and Bookman, 1994). Thus, we could still observe \(\Delta C_{\text{t}}\) after rundown of the secretory response as well as after reduction of the voltage-gated Ca\(^{2+}\) entry (see Materials and Methods). Like Horrigan and Bookman (1994), we
could abolish 
DCt by incubation with dibucaine (200 mM, data not shown), which blocks gating charge movement in squid axons (Gilly and Armstrong, 1980). DCt in mouse chromaffin cells in slices on average decays with a time constant of \( \tau \approx 230 \) msec. During this study we used three different approaches to separate exocytotic capacitance changes (DCexo) from DCt, which result in similar estimates for DCexo (see Materials and Methods and Figs. 2A, 3C).

To quantify the exocytotic response to individual APs, we applied five to seven stimuli at intervals of 30–45 sec to seven cells starting 30–60 sec after the beginning of whole-cell recording. After subtraction of the prepulse capacitance, the DCm traces of a cell were averaged, and DCexo was estimated as the asymptote of an exponential fit to DCm (representing the part of DCm remaining after decline of DCm; approach 1 in Materials and Methods). For the pooled data, an average DCexo of 16.6 ± 3.6 fF (n = 7) was determined.

Analogous experiments were performed in isolated mouse chromaffin cells. We used 10 mM extracellular [Ca\(^{2+}\)], instead of the 2 mM [Ca\(^{2+}\)] in the experiments in slices, to compensate for the reduction of calcium current caused by the cell isolation (see “Comparison with isolated mouse chromaffin cells” below). Nevertheless, only one of seven cells showed comparable exocytotic responses to single APs, although all of them secreted in response to longer depolarizations (data not shown). The average DCexo of the seven isolated cells was 4.4 ± 2.6 fF.

For comparison of the observed AP-induced capacitance changes with the results of an amperometric study on AP-stimulated catecholamine secretion from isolated rat chromaffin cells by Zhou and Misler (1995), capacitance units must be converted to numbers of granules. The size of chromaffin granules varies (diameters range from 50 to 500 nM) (for review, see Carmichael, 1986), and thus variation is also observed for membrane capacitance increments attributable to fusion of individual chromaffin granules (Neher and Marty, 1982). The mean capacitance of individual chromaffin granules has been measured to be 2.5 fF in bovine chromaffin cells (Neher and Marty, 1982; Chow et al., 1996). Therefore, isolated mouse chromaffin cells would secrete less than two granules per single AP if one assumes an analogous mean capacitance for mouse chromaffin granules. Zhou and Misler (1995) detected less than one release event/AP at low AP frequency (0.2–1.0 Hz). Considering that the efficiency of their amperometric detector was only \( \approx 25% \) (Zhou and Misler, 1995), whereas all fusion events are revealed by Cm measurements performed in the present study, the numbers obtained in isolated mouse and rat chromaffin cells seem compatible. On the other hand, mouse chromaffin cells in slices did respond to individual APs with larger capacitance changes, which on average would correspond to approximately six to seven granules/AP (if a mean capacitance of 2.5 fF is assumed for chromaffin granules).

Unfortunately, it was not technically possible to confirm by amperometry that the secretory capacitance changes in response to individual APs were accompanied by a release of granules. For comparison of the observed AP-induced capacitance changes with the results of an amperometric study on AP-stimulated catecholamine secretion from isolated rat chromaffin cells by Zhou and Misler (1995), capacitance units must be converted to numbers of granules. The size of chromaffin granules varies (diameters range from 50 to 500 nM) (for review, see Carmichael, 1986), and thus variation is also observed for membrane capacitance increments attributable to fusion of individual chromaffin granules (Neher and Marty, 1982). The mean capacitance of individual chromaffin granules has been measured to be 2.5 fF in bovine chromaffin cells (Neher and Marty, 1982; Chow et al., 1996). Therefore, isolated mouse chromaffin cells would secrete less than two granules per single AP if one assumes an analogous mean capacitance for mouse chromaffin granules. Zhou and Misler (1995) detected less than one release event/AP at low AP frequency (0.2–1.0 Hz). Considering that the efficiency of their amperometric detector was only \( \approx 25% \) (Zhou and Misler, 1995), whereas all fusion events are revealed by Cm measurements performed in the present study, the numbers obtained in isolated mouse and rat chromaffin cells seem compatible. On the other hand, mouse chromaffin cells in slices did respond to individual APs with larger capacitance changes, which on average would correspond to approximately six to seven granules/AP (if a mean capacitance of 2.5 fF is assumed for chromaffin granules).

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Figure 1. Single AP-like voltage commands cause exocytotic Cm changes in mouse chromaffin cells in slices. A (top), Shape of a representative mouse chromaffin cell AP measured in current clamp with a potassium-based pipette solution (dashed line). The solid line shows the simulated AP-like voltage command used to study secretory responses to single APs. A (bottom), A typical current response to the AP-like command, with K+ currents blocked by a Cs+-containing pipette solution (solution A) and d-tubocurarine in the extracellular saline (solution 1). B, Representative Cm measurement before and after application of a simulated chromaffin cell AP from a holding potential of −80 mV. The top trace displays the AP-induced Cm change. After an initial decay (ΔCt) (for more detail, see Results), a stable Cm increment (ΔCexo) remains. The asymptote of the exponential fit to ΔCm was used to quantify the ΔCexo evoked by the individual APs (approach 1 in Materials and Methods). Middle and bottom traces, Membrane conductance (Gm) and series resistance (Gs) are shown to illustrate that there was no major cross-talk among Cm, Gm, and Gs estimates.

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to APs observed in slices represent fusion of chromaffin granules. In particular, there was a large amperometric background current, probably resulting from catecholamines set free from damaged cells (data not shown). From fluctuation analysis of capacitance changes in response to repetitive, brief (10 msec) depolarizations in slices, we have estimated a mean capacitance contribution of single vesicles compatible with values expected for chromaffin granules (T. Moser and E. Neher, unpublished observations). Thus, it is likely that the bulk of the 16.6 pF capacitance change measured in slices in response to individual APs is attributable to exocytosis of chromaffin granules instead of small synaptic-like microvesicles that have been observed in neuroendocrine cells (for review, see Thomas-Reetz and De Camilli, 1994).

High secretory rates in mouse chromaffin cells in slices result from rapid release of a small pool of vesicles

The rate of secretion is commonly modeled as the product of the number of release-ready vesicles and the Ca$^{2+}$-dependent rate constant of secretion (Thomas et al., 1993; Heinemann et al., 1994). Thus, high secretory rates can be obtained by a large pool of fusion-competent vesicles and/or by fast release kinetics. The existence of a distinct depletable pool of vesicles is suggested by the observation that the secretory rate drops despite continued existence of a depletable pool of vesicles (von Rüden and Neher, 1993). The holding potential was depleted of a pool of fusion-competent vesicles, which cannot be refilled sufficiently fast to maintain rapid secretion. We used two protocols designed to deplete pools to characterize the kinetic components of depolarization-induced secretion in mouse chromaffin cells in slices.

**Protocol 1: secretory responses to Ca$^{2+}$ current injections of different duration**

Ideally, the study of release kinetics would include measurements of the secretory rate throughout the time of stimulation. Then, in principle, increasing the duration of the stimulation (depolarization-induced Ca$^{2+}$ current injection) should lead to a drop in secretory rate, indicating pool depletion. However, voltage-dependent conductances make C_m measurements unreliable during the depolarization. In addition, amperometric measurements of catecholamine release, which can be performed during a depolarization, are problematic in slices (see above).

Instead, we reconstructed the relation between secretory response and duration of Ca$^{2+}$ current injection by measuring the ΔC_m response to step depolarizations (to 0 mV) of different durations. This protocol has been used previously to study secretion in bipolar terminals (von Gersdorff and Matthews, 1994), isolated rat chromaffin cells (Horrigan and Bookman, 1994), bovine chromaffin cells (Gillis et al., 1996), and nerve terminals in thin slices of the neurohypophysis (Hsu and Jackson, 1996).

Pulses of different duration were applied in random order. Thirty seconds were allowed for recovery between the depolar-
In the course of the experiments, decline of ΔC_{exo} and Ca^{2+} currents was observed for all pulse durations, which we interpreted as run-down (Augustine and Neher, 1992; Burgoyne, 1995). Interpretation of recent results has suggested that after decline of ΔC_{exo} attributable to run-down, secretion in a different kinetic mode may persist (threshold-type secretory mode) (Seward and Nowycky, 1996).

Figure 2. A and B, shows representative ΔC_{t}-corrected responses to pulses of different length and demonstrates that for short stimuli, the exocytotic capacitance change occurred only during the stimulation. Therefore, even when ΔC_{exo} was measured at different times after short depolarizations (see Materials and Methods and Figs. 2A, 3C), it could be interpreted as the exocytotic capacitance change during the time of depolarization.

The highest secretory rate (calculated as ΔC_{exo}/pulse duration) was observed for the shortest (2 msec) depolarizations, indicating a very short delay of secretion to the onset of the stimulus. On average, 2 msec depolarizations resulted in a ΔC_{exo} of 14.7 ± 3.7 fF (nine depolarizations in three cells), which corresponds to a maximal secretory rate of ~7300 fF/sec.

The top of Figure 3A (filled circles) shows the biphasic rise of ΔC_{exo}, with increasing pulse duration in mouse chromaffin cells in slices (pooled data from 10 cells). The fast component of a double exponential fit to the data had a time constant (τ_{fast}) of ~7 msec (n = 10 cells, 112 depolarizations). The observed drop in the secretory rate could be attributable to depletion of fusion-competent vesicles or result from lessening of the stimulus intensity (for example, because of Ca^{2+} current inactivation).
after the first pulse. 

Figure 4. Secretory depression of slice cells is obtained with a pair of 20 msec depolarizations. This figure shows a representative $\Delta C_m$ trace in response to a pair of 20 msec depolarizations (to $-6$ and 0 mV respectively; see top panel for illustration of the voltage-clamp protocol). Pipette solution B and external solution 1 were used. The sum response ($S$) to both stimuli was measured as the asymptote of an exponential fit (solid line) to $\Delta C_m$ after the second depolarization. The nonexocytotic $\Delta C_1$ makes direct separate measurement of the exocytotic responses to the first and second depolarization ($\Delta C_{exo1}$ and $\Delta C_{exo2}$) difficult. Here, $\Delta C_{exo2}$ was estimated as the difference of $\Delta C_{m}$ averages over the initial 300 msec after the second and the first depolarization, respectively. $\Delta C_{exo1}$ was then calculated as $S - \Delta C_{exo2}$. This analysis relies on the assumption that the nonsecretory transient ($\Delta C_1$) is the same after the first and the second depolarization. This is reasonable, because $\Delta C_1$ saturates for depolarizations as short as 5 msec (Horrigan and Bookman, 1994). For illustration, the exponential fit to the $\Delta C_m$ trace after the second depolarization in addition was overlayed onto the $\Delta C_m$ segment after the first pulse (dashed line).

The slow secretory component apparent in the $\Delta C_m$ versus pulse duration plot could be fitted equally well by a line or a slow exponential. The slope of this plot, which is the average calcium current, does not decline for short pulses even if durations are much longer than $\tau_{fast}$. Thus, the observed drop of the secretory rate is most likely attributable to depletion of available vesicles rather than to $Ca^{2+}$ current inactivation. Therefore, the fast component is interpreted as secretion from a limited pool of vesicles, with $\tau_{fast}$ being the time constant for pool depletion (7 msec) and its amplitude of $\sim$42 fF representing the pool size. Similar results were obtained in five mouse chromaffin cells in slices that were dialyzed with pipette solution A, which contained 200 µM free EGTA without added $Ca^{2+}$ (data not shown).

The slow secretory component apparent in the $\Delta C_m$ versus pulse duration plot could be fitted equally well by a line or a slow exponential. The slope of the best fit line between 20 and 300 msec is $\sim$270 fF/sec. Interpretation of this component is complicated by two observations. Secretion often persists after the end of long depolarizations (Fig. 2B), such that for longer pulses the rise of the $\Delta C_{exo}$ estimates with increasing pulse duration cannot be easily interpreted as secretory rate. Also, the bottom of Figure 3A shows that $Ca^{2+}$ current inactivation is significant for long pulses.

**Comparison with isolated mouse chromaffin cells**

Figure 3 also presents data obtained from 20 isolated mouse chromaffin cells (empty squares) in experiments analogous to those performed in slices. Again, 10 mM extracellular $[Ca^{2+}]$ was used for isolated cells. In most of the isolated cells, short depolarizations (<50 msec) caused much less secretion than in slices; however, longer stimuli (>100 msec) gave similar $\Delta C_{exo}$ values for the two preparations. The highest secretory rate was observed for 5 msec depolarizations, which on average caused $\Delta C_{exo}$ of 7.6 ± 2.52 fF ($n = 46$ responses from 20 cells), corresponding to a secretory rate of $\sim$1300 fF/sec. Responses of isolated mouse chromaffin cells to short depolarizations were similar to those obtained from isolated rat chromaffin cells (diamonds in Fig. 3A, taken from Horrigan and Bookman, 1994) or from isolated bovine chromaffin cells (Gillis et al., 1996).

The bottom of Figure 3A shows that isolated mouse chromaffin cells, on average, have smaller $Ca^{2+}$ current estimates despite the higher extracellular $[Ca^{2+}]$ (10 vs 2 mM). Nevertheless, a plot of $\Delta C_{exo}$ versus $Q_{Ca}$ (Fig. 3B) depicts that cells in slices still secrete more for a given amount of $Ca^{2+}$ influx for small $Q_{Ca}$ values. The difference between mean $\Delta C_{exo}$ values of slice and isolated cells was statistically significant at $Q_{Ca}$ values of 0.9 pC and 2.2 pC ($p < 0.01$ and $p < 0.02$, respectively). At higher $Q_{Ca}$, $\Delta C_{exo}$ values from both preparations were hard to distinguish statistically.

**Protocol 2: secretory responses to pairs of depolarizing pulses**

To obtain a second estimate for the number of rapidly releasable vesicles (pool size), we further studied secretory depression using a paired-pulse protocol. We applied short depolarizations (20 msec, $\sim 3 \times \tau_{fast}$, see above; interval between both stimuli: 300
msec) to preferentially recruit vesicles with fast-release kinetics. Starting 30–60 sec after whole-cell recording, dual pulses were applied with intervals of at least 30 sec to allow replenishment of fusion-competent vesicles. 

The actual pool size would be given exactly by Equation 1 if both stimuli released the same fraction of the pool of releasable vesicles. Here, the depolarizing potentials were adjusted to match the \( Q_{\text{Ca}} \) values of both pulses. Using potentials of \(-6 \text{ mV} \) and 0 mV for the first and second pulses, respectively, the ratio \( Q_{\text{Ca}2}/Q_{\text{Ca}1} \) was 1.05 ± 0.04 (21 double pulses in seven cells). The second Ca\(^{2+}\) injection of the dual pulse, however, presumably caused a higher and spatially more extended rise of [Ca\(^{2+}\)] than the first pulse, because of residual Ca\(^{2+}\) and submembrane "buffer depletion" (or saturation) remaining 300 msec after the first stimulus. In terms of the kinetic pattern revealed by the \( \Delta C_{\text{exo}} \) versus pulse duration plot, we assume that the second depolarization recruited not only vesicles involved in the fast secretory component but also those comprising the slower phase of secretion. This assumption is supported by the finding that a second pulse several seconds after the first actually gave a smaller response than a pulse that followed the first depolarization by only 300 msec (Fig. 6). It is therefore likely that \( B_{\text{max}} \) overestimates the actual pool size (hence the "max" notation). This may not be the case if shorter pulses are used in the dual-pulse protocol. Here, \( B_{\text{max}} \) is taken as an upper boundary for the size of the pool. \( \Delta C_{\text{exo}1} \), on the other hand, is a reasonable lower boundary.

In all seven cells analyzed, secretory depression (\( R < 1 \)) was observed in the majority of the pulses (\( R = 0.60 ± 0.06; n = 21 \) dual pulses from seven cells). The mean \( \Delta C_{\text{exo}1} \) was 31.7 ± 4.1 fF, and the mean \( \Delta C_{\text{exo}2} \) was 17.6 ± 2.4 fF. Three of twenty-one dual pulses were excluded from the \( B_{\text{max}} \) estimation because their \( R \) was >0.8. The average \( B_{\text{max}} \) was then 73.1 ± 10.7 fF. Thus, the dual-pulse analysis (in this set of experiments) indicated a size of the pool of rapidly releasable vesicles between 31.7 and 73.1 fF.

**The fast secretory component in slices is more sensitive to BAPTA than to EGTA**

In the case of close spatial coupling of Ca\(^{2+}\) channels and release sites, secretion should be more sensitive to intracellular application of fast Ca\(^{2+}\) chelators like BAPTA than to slow Ca\(^{2+}\) chelators like EGTA (Adler et al., 1991). The rapid-release kinetics in mouse chromaffin cells in slices prompted us to test the effects of 1 mM free BAPTA and EGTA on the fast secretory component. Ten cells were investigated under each condition, using the same protocol as depicted in Figures 2 and 3.

Both the amplitude and the time constant of the fast exponential component were slightly altered by EGTA (\(-28 \text{ fF} \) and 8 msec, respectively, as compared with 42 fF and 7 msec at low buffering conditions). In contrast, the fast secretory component was hardly detectable in cells dialyzed with BAPTA (Fig. 5A). The delayed rise of \( \Delta C_{\text{exo}} \) in cells dialyzed with BAPTA most likely represents recruitment of vesicles after the chelator became locally increasingly saturated by the incoming Ca\(^{2+}\). Responses to longer depolarizations were comparable between EGTA- and BAPTA-buffered cells but smaller than those observed at low buffering conditions, indicating that both buffers similarly suppress the slow secretory component. A plot of \( \Delta C_{\text{exo}} \) versus \( Q_{\text{Ca}} \)...
Recovery of the fast secretory component in slices (pool refilling)

We investigated the recovery time course of the fast secretory component by comparing \( \Delta C_{\text{ex1}} \) for two "pool-depleting" depolarizations (20 msec) separated by different intervals. Figure 6 shows the time course of recovery with 300 nM [Ca\(^{2+}\)]\(_{\text{ics}}\) in the pipette. The symbols represent ratios of second over first \( \Delta C_{\text{ex1}} \) responses. Secretion was elicited either by separated individual depolarizations (triangles) or by separated dual pulses (circles; the dual pulses had the same configuration as depicted in Fig. 4). In the latter case, ratios were calculated for the first depolarizations (\( \Delta C_{\text{ex1}} \); see Fig. 4) of the two separated dual pulses. Pool refilling measured in both ways is quite similar and well fitted by a single exponential with a time constant of \( \sim 10 \) sec (pooled data from 13 cells, seven preparations). Back-extrapolation to time 0 gave a ratio of 0.2 (empty square in Fig. 6), indicating 80% pool depletion for the first stimulus.

If a first-order kinetic scheme can be assumed for the pool refilling, as indicated by the mono-exponential time course, then the maximal refilling rate is given by the product of the recovery rate constant (1/\( \tau \)) and the pool size. Applying the dual-pulse analysis (see above) to each first of the separated dual pulses (which recruited the completely filled pool in these experiments), we estimated the lower (\( \Delta C_{\text{ex1}} \)) and upper pool size bounds (\( B_{\text{max}} \)) to be 25.4 \( \pm \) 2.35 fF and 55.7 \( \pm \) 4.8 fF, respectively (\( n = 26 \) pulses from eight cells). Taking \( \Delta C_{\text{ex1}}, B_{\text{max}} \) and the refilling rate constant, we calculated lower and upper bounds for the maximal refilling rate as 2.5 and 5.6 fF/sec, respectively.

DISCUSSION

Chromaffin cells in situ can secrete in response to individual APs. We demonstrated that this rapid exocytosis is from a small pool of vesicles that probably experience a very high [Ca\(^{2+}\)] during the stimulation. Furthermore, the characterization of secretion kinetics for wild-type mouse adrenal chromaffin cells lays the foundations for future comparison with transgenic mice.

Kinetic components of depolarization-induced secretion in mouse chromaffin cells in slices

Fast secretory component

When voltage step depolarizations of varying duration are applied to mouse chromaffin cells in slices, a rapid secretory component is observed that can be well separated from a slower secretory component. The fast component most likely represents a pool of vesicles similar in size (42 fF) to rapidly recruited pools found in isolated rat chromaffin cells (33.9 fF) (Horrigan and Bookman, 1994), isolated bovine chromaffin cells (34 fF) (Gillis et al., 1996), and peptidergic nerve terminals in slices of the rat posterior pituitary (40 fF) (Hsu and Jackson, 1996). The size of our fast secretory component also falls well into the range for the pool size derived from the dual-pulse analysis (25–73 fF) in our slice preparation.

The recovery time constant of the rapidly recruited pool in our slice preparation (10 sec) is in good agreement with time constants of pool refilling in other preparations. Thus, Stevens and Tsujimoto (1995) obtained a time constant of 10 sec for synapses of cultured hippocampal neurons, and a time constant of 8 sec was measured in bipolar terminals by von Gersdorff and Matthews (1997). The time needed for complete recovery (\( \sim 3r \)) of the fast secretory component in our slice preparation (with [Ca\(^{2+}\)]\(_{\text{ics}}\) buffered to 300 nM) is slightly shorter than that required for complete pool refilling in bovine adrenal chromaffin cells (60 sec, without addition of Ca\(^{2+}\)-loaded buffers to the pipette solution) (von Rüden and Neher, 1993). This difference could well be caused by the slightly elevated [Ca\(^{2+}\)]\(_{\text{ics}}\) in our experiments, because refilling is Ca\(^{2+}\)-dependent (von Rüden and Neher, 1993).

Slow secretory component

We did not explore the slow secretory component in detail. Although we cannot exclude that it represents a fast delivery or priming process, we favor the idea that longer Ca\(^{2+}\) injections lead to a spatially more extended rise in submembrane [Ca\(^{2+}\)] and thereby recruit fusion-competent vesicles located at greater distances from the Ca\(^{2+}\) channels. The rate of pool recovery (upper boundary: 5.6 fF/sec) is almost 50 times smaller than the slope of the slow \( C_m \) rise (270 fF/sec). Even though [Ca\(^{2+}\)] is higher during a pulse (when the slow secretory component is measured) than between pulses (as in the recovery experiments), it seems unlikely to us that the supply rate could increase fiftyfold because of an increased [Ca\(^{2+}\)]. Thus, it was concluded from experiments...
in which catecholamine secretion was triggered by dialysis with high [Ca\(^{2+}\)] that the vesicle delivery rate may be half-maximal already at a [Ca\(^{2+}\)], of 1.2 \(\mu\)M (Heinemann et al., 1993).

The concept of a “releasable pool” implies a set of vesicles in the same state of fusion competence; however, spatial heterogeneity of the Ca\(^{2+}\) signal during membrane depolarization can divide a pool of vesicles with homogeneous fusion competence into kinetically distinct subpools (Horrigan and Bookman, 1994). The fractions of vesicles comprising the fast and slow secretory components described here might represent subpools of a large readily releasable pool. This large pool was presumably only marginally depleted by our maximal stimuli.

Thus, at least two kinetic components might ensure catecholamine release over a wide range of splanchnic nerve activities. Release at low chromaffin cell AP frequencies will be mediated by the small fast pool, whereas the large slow secretory component is probably recruited during stronger stimulation.

The fast kinetics of secretion in mouse chromaffin cells in slices suggests close spatial coupling of release sites and Ca\(^{2+}\) channels

The average initial secretory rate for mouse chromaffin cells in slices (7300 \(fF/sec\)) is much faster than that obtained for isolated mouse (1300 \(fF/sec\)) (Horrigan and Bookman, 1994), and bovine (860 \(fF/sec\)) (Chow et al., 1994) chromaffin cells. We conclude that a fast release kinetics rather than a large pool underlies the high secretory rate in mouse chromaffin cells in slices, because their fast secretory component compares well in size with rapidly secreted pools described previously for isolated chromaffin cells (see above). High average secretory rates have also been obtained from peptidergic nerve terminals in slices of the posterior pituitary (Hsu and Jackson, 1996). The different kinetics in mouse chromaffin cells in slices and in primary culture are not simply attributable to the larger Ca\(^{2+}\) currents in slices, because cells in slices show more secretion for a given amount of Ca\(^{2+}\) entry when the responses to short depolarizations are compared (Fig. 3B). Possible explanations for the faster release kinetics in chromaffin cells in slices include (1) higher [Ca] at the release sites because of particular spatial arrangements of Ca\(^{2+}\) channels and release sites, (2) higher [Ca] at the release sites in slices caused by contribution of fast calcium-induced calcium release (CICR) present only in the slice preparation, and (3) different Ca\(^{2+}\) dependencies of secretion in slice and isolated cells.

Possibilities (2) and (3) are unlikely. When we intracellularly applied ruthenium red, an inhibitor of CICR (Miyamoto and Racker, 1982), rapid exocytosis remained unaffected (50 \(\mu\)M in the presence of 1 mM free EGTA; data not shown), ruling out a major contribution of CICR. Regarding possibility (3), it seems important to note that the amount of secretion in slice and isolated cells was clearly distinguishable only for small amounts of Ca\(^{2+}\) influx. Therefore, one would have to postulate that only the fast release component is more sensitive to [Ca\(^{2+}\)] in slices, whereas the slow component shares the same (low) Ca\(^{2+}\) sensitivity with the isolated cells.

We favor the interpretation that the Ca\(^{2+}\) sensors of a fraction of release sites in chromaffin cells in slices experience a very high [Ca\(^{2+}\)] because of their close spatial relation to the Ca\(^{2+}\) channels [possibility (1)]. A global elevation of [Ca\(^{2+}\)], of 40–80 \(\mu\)M by flash photolysis in bovine chromaffin cells (Heinemann et al., 1994) provides a vesicle release rate constant comparable to the average value in slices (140 sec\(^{-1}\)). Buffering [Ca\(^{2+}\)], with BAPTA strongly decreased the fast secretory component. The different effects of equimolar concentrations of BAPTA and EGTA on the fast secretory component in mouse chromaffin cells in slices suggest that Ca\(^{2+}\) is trapped by the Ca\(^{2+}\) sensor of the release site before Ca\(^{2+}\) binding to the chelators has reached an equilibrium. This is taken as additional support for short diffusional distances between Ca\(^{2+}\) channels and release sites.

Possible explanations for a high [Ca\(^{2+}\)] at the release sites with Ca\(^{2+}\) originating solely from Ca\(^{2+}\) entry include clustering of Ca\(^{2+}\) channels with or without co-clustering of release sites and molecular coupling of release sites and Ca\(^{2+}\) channels. The polarized phenotype of chromaffin cells in situ (Carmichael, 1986) certainly motivates speculation of a co-clustering of “exocytotic” Ca\(^{2+}\) channels and release sites at the capillary pole (Michelena et al., 1995), which then would favor directional catecholamine release into the capillaries. Robinson et al. (1995) reported overlap of hotspots of submembrane [Ca\(^{2+}\)] and of secretion in isolated bovine chromaffin cells and argued for release from active zone-like structures in these cells. On the other hand, Robinson et al. (1995) acknowledged that hotspots of submembrane [Ca\(^{2+}\)] were seen only in a fraction of cells. Furthermore, functional studies on the same preparation indicated that the majority of the release sites is located, on average, several hundreds of nanometers away from the nearest channel (Klingauf and Neher, 1997). The two findings can be accommodated if it is assumed that morphological specializations, which exist in situ, are preserved only to a variable extent in primary culture. The different secretion kinetics observed for slice and isolated cells in the present study support this assumption.

Molecular coupling has been demonstrated for syntaxins with N- and P/Q-type Ca\(^{2+}\) channels (Bennett et al., 1992; Rettig et al., 1996). To our knowledge, however, no data are available showing to what extent Ca\(^{2+}\) influx through different Ca\(^{2+}\) channel types triggers secretion in mouse chromaffin cells in situ or in isolation. Whether the high [Ca\(^{2+}\)] at release sites of chromaffin cells in slices during depolarization is attributable to molecular coupling of release sites to Ca\(^{2+}\) channels or to segregation of Ca\(^{2+}\) channels into specialized regions of the plasma membrane remains to be clarified.

REFERENCES


