Simultaneous Capacitance and Amperometric Measurements of Exocytosis: A Comparison

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ABSTRACT We measured the exocytotic response induced by flash photolysis of caged compounds in isolated mast cells and chromaffin cells. Vesicle fusion was measured by monitoring the cell membrane capacitance. The release of vesicular contents was followed by amperometry. In response to a GTPγS stimulus we found that the time integral of the amperometric current could be superimposed on the capacitance trace. This shows that the integrated amperometric signal provides an alternative method of measuring the extent and kinetics of the secretory response. Very different results were obtained when photolysis of caged Ca^{2+} (DM-nitrophen) was used to stimulate secretion. In mast cells, there was an immediate, graded increase in membrane capacitance that was followed by step increases (indicative of granule fusion). During the initial phase of the capacitance increases, no release of oxidizable secretory products was detected. In chromaffin cells we also observed a considerable delay between increases in capacitance, triggered by uncaging Ca^{2+}, and the release of oxidizable secretory products. Here we demonstrate that there can be large increases in the membrane capacitance of a secretory cell, triggered by flash photolysis of DM-nitrophen, which indicate events that are not due to the fusion of granules containing oxidizable substances. These results show that increases in capacitance that are not resolved as steps cannot be readily interpreted as secretory events unless they are confirmed independently.

INTRODUCTION

There are currently two types of electrophysiological recordings that are commonly used to monitor exocytosis at the single cell level. These are membrane capacitance and amperometric measurements. Both of these methods have high temporal resolution, which facilitates kinetic studies of the secretory process. Several recent studies have shown that uncaging Ca^{2+} from DM-nitrophen in patch-clamped cells stimulates a rapid increase in cell membrane capacitance. These results suggested that the delay between the stimulation of the cell and the evoked secretory response is very short, on the order of a few milliseconds (Neher and Zucker, 1993; Thomas et al., 1993; von Gersdorff and Matthews, 1994). However, there are certain limitations of membrane capacitance measurements that must be borne in mind in studies such as these. Cell membrane capacitance recordings are, at their simplest, a measurement of cell surface area. However, a basic assumption of these measurements is that the cell can be modeled as a simple, single time constant, electrical equivalent circuit and that the cell membrane capacitance can be approximated by measurements of the cell's admittance at about 1 kHz (Neher and Marty, 1982; Joshi and Fernandez, 1988). Thus, any changes in the electrical equivalent circuit of the cell that are unrelated to secretion will affect the capacitance measurements. For example, changes in the dielectric constant contributed by membrane proteins can produce changes in the specific capacitance (Fernandez et al., 1982; Horrigan and Bookman, 1994). Changes in membrane area that are not related to the fusion of secretory vesicles with the plasma membrane (e.g., cell swelling; Zorec and Tester, 1993) will also be detected.

In peritoneal mast cells and in other cells with large granules, step increases in the cell membrane capacitance observed during exocytosis indicate, unambiguously, the fusion of single secretory vesicles (Fernandez et al., 1984; Breckenridge and Almers, 1987; Scepek and Lindau, 1993). In neuroendocrine cells, step increases in capacitance have been observed, although they are more difficult to see because of the small size of the secretory granules (e.g., Neher and Marty, 1982; Robinson et al., 1995). Membrane capacitance measurements recorded in the whole-cell patch-clamp configuration can only resolve, as step increases, the fusion of individual vesicles that are larger than approximately 200 nm in diameter. Fusion of secretory vesicles smaller than this size would give a graded increase in membrane capacitance. Thus capacitance measurements are incapable of discriminating between the fusion of synaptic vesicles or small dense core secretory granules. Furthermore, in those cases in which fusion events cannot be distinguished as step increases in capacitance, the kinetics of vesicle fusion is complicated by underlying processes of membrane retrieval (Neher and Zucker, 1993; Thomas et al., 1993, 1994; von Gersdorff and Matthews, 1994; Parsons et al., 1994; Burgoyne, 1995).

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An alternative method of measuring secretion is to monitor the release of oxidizable secretory products by placing a carbon fiber microelectrode (held at a constant potential) in close proximity to a single isolated cell (Wightman et al., 1991). Release of secretory granule contents results in the generation of brief current spikes as secretory products are oxidized on the surface of the carbon fiber (Wightman et al., 1991). Combined amperometric and capacitance measurements have shown that the detection of a single amperometric spike corresponds to the fusion of a single granule with the plasma membrane (observed as a stepwise increase in membrane capacitance). Thus each amperometric spike represents the release of a single granule's contents (Alvarez de Toledo et al., 1993; Oberhauser et al., 1995; Robinson et al., 1995). One advantage of this method of following secretion is that it only monitors the release of secretory products into the extracellular milieu and as such is not complicated by many of the factors affecting capacitance measurements. It is also very sensitive and can detect the release from single vesicles that are too small to be detected as individual events by capacitance measurements using the whole-cell configuration (Ureña et al., 1994; Chen et al., 1994; Bruns and Jahn, 1995). A time integral of the amperometric trace is a measure of the total amount of secretory products that were released by a cell after its stimulation and as such allows a direct comparison of the events measured with amperometry with those measured by capacitance (von Rüden and Neher, 1993; Oberhauser et al., 1995).

In this study, fusion of secretory granules with the plasma membrane and the subsequent release of their contents were simultaneously monitored by using membrane capacitance and amperometric measurements. The two measurements were then compared with one another. Secretory responses were triggered by raising intracellular concentrations of either GTPγS or Ca^{2+}, using photolabile caged compounds (Walker et al., 1989; Kaplan and Ellis-Davies, 1988). In response to a GTPγS stimulus, we found that an integral of the amperometric current recording followed a sigmoidal time course similar to that of the capacitance measurement. The superimposition of the integrated amperometric signal on the capacitance trace demonstrates that it can provide an alternative method of assessing the extent and kinetics of the secretory response of a cell. Very different results were obtained when photolysis of caged Ca^{2+} (DM-nitrophen) was used to stimulate secretion in either mast cells or chromaffin cells. In mast cells, after the uncaging of Ca^{2+}, there was an immediate graded increase in the membrane capacitance (Kirillova et al., 1993). However, during this smooth phase, no step increases in capacitance were observed, and more importantly, no release of oxidizable secretory products was detected. In chromaffin cells we also observed immediate increases in capacitance, triggered by uncaging Ca^{2+}, that were not accompanied by the release of oxidizable secretory products. The cause of these "smooth" phase changes in capacitance is not yet clear, but they are clearly not the result of secretory granules that contain oxidizable substances.

MATERIALS AND METHODS

Preparation of mast cells and chromaffin cells

Mast cells from normal mice (C57BL/6J strain; Jackson Laboratories, Bar Harbor, ME) were obtained by peritoneal lavage after a procedure described elsewhere (Alvarez de Toledo and Fernandez, 1990). Chromaffin cells were prepared from bovine adrenal medullae by enzymatic digestion and cultured for 1–5 days before use (Burgoyne et al., 1988).

Solutions

The standard extracellular solution used for mast cells was (in mM): 130 NaCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES (pH 7.2). In experiments using caged GTPγS, the internal solution was (in mM): 140 K-glutamate, 7 $MgCl_2$, 0.2 ATP-Mg, 0.3 (1-(3-S-(4,5-dimetoxy-2-mytrophenyl)ethyl)thio ester) (DMNPE) caged GTPγS (Molecular Probes), 10 HEPES (pH 7.2), 10 EGTA, and 1 $CaCl_2$. Solutions containing the photolabile Ca^{2+} chelator DM-nitrophen (Calbiochem) had the following composition (in mM): 140 K-glutamate, 1.5 GTP, 10 HEPES (pH 7.2), 0.2 ATP-Mg, 3 $CaCl_2$, and 10 DM-nitrophen. In experiments with chromaffin cells the external solution was 140 NaCl, 30 HEPES, 2 $MgCl_2$, 2 $CaCl_2$, 2 mg/ml glucose, and 1 μM tetrodotoxin at pH 7.2. The internal solution consisted of (in mM): 130 Cs-glutamate, 0.3 GTP, 10 HEPES (pH 7.2), 0.2 ATP-Mg, 3 $CaCl_2$, and 10 DM-nitrophen. In experiments using caged GTPγS, the internal solution was (in mM): 140 Cs-glutamate, 7 $MgCl_2$, 0.2 ATP-Mg, 0.3 DMNPE caged GTPγS, 10 HEPES (pH 7.2), 10 EGTA, and 1 $CaCl_2$.

Photolysis system

Photolysis of caged compounds was achieved by coupling a 200-W Xe(Hg) arc lamp (Oriel) to the epifluorescence pathway of a microscope (IM-35; Zeiss) with a liquid light guide (7-mm-diameter; Oriel). The exposure time of the cell to UV light (<395 nm; dichroic mirror) was controlled by an electronic shutter (Uniblitz) placed between the lamp and the liquid guide. The cell was viewed with an 100× oil-immersion Neofluar objective (NA 1.25; Zeiss).

Measurement of membrane capacitance

Exocytosis was monitored by measuring the cell membrane capacitance, using the whole-cell mode of the patch-clamp technique (Hamill et al., 1981), in conjunction with a digital phase detector (Joshi and Fernandez, 1988) implemented on a system comprising an AT-MIO16x interface (National Instruments) and an Axopatch 200a patch-clamp amplifier (Axon, Rapid City, CA). All of the data acquisition and analysis programs were written in Labview (v3.0.1; National Instruments). A sinusoidal voltage (833 Hz, 54 mV peak to peak) on top of a holding potential of 0 mV for mast cells or −60 mV for chromaffin cells was applied to the patch-clamped cell, and the current was measured at two different phase angles, θ and $\theta - \pi/2$, relative to the stimulus. The phase was periodically adjusted by using the phase tracking technique (Fidler and Fernandez, 1989), so that the output at $\theta - \pi/2$ reflected changes in the real part of the cell admittance (Re [ΔY]), whereas the output at θ reflected changes in the imaginary part of the admittance, (Im [ΔY]). Capacitance and AC conductance data, along with voltage and DC conductance, were captured with a temporal resolution of 9.6 ms per point.

Amperometric detection of release

Amperometric detection of secretory products was monitored with a carbon fiber electrode with a tip diameter of ~14 μm, fabricated as

described elsewhere (Kawagoe et al., 1993). The holding voltage applied to the electrode was 700 mV, and the redox current was monitored with an EPC-7 patch-clamp amplifier (List Electronics, Darmstadt, Germany). The carbon fiber was placed 1–2 μm over the cell. The use of a carbon fiber electrode with a large detecting surface, placed in close proximity to the cell, was important for minimizing the probability of missing exocytotic events.

RESULTS

Simultaneous membrane capacitance and amperometric measurements of the secretory response induced by photolysis of caged GTPγS in mast cells and chromaffin cells

Fig. 1 *A* shows the secretory response elicited in a mast cell by photolysis of caged GTPγS in the presence of 10 mM EGTA to buffer any increase in $[Ca^{2+}]_i$. Secretion was followed by capacitance (*bold trace*) and amperometry (*lower trace*). Exposure of the cell to a single pulse of UV light uncaged sufficient GTPγS to trigger a complete degranulation (i.e., the fusion of all the cell's secretory granules with the plasma membrane). The capacitance trace is sigmoidally shaped. This shows that granule fusion with the plasma membrane was initially slow, then increased as a greater number of granules fused with the plasma membrane, only to slow again, as the supply of granules became exhausted. Each step increase in the capacitance trace represents the fusion of a single secretory granule (Fernandez et al., 1984), and each amperometric spike represents the release of serotonin contained in the secretory granule (Alvarez de Toledo et al., 1993).

A careful examination of the amperometry signal (Fig. 1) shows that as the secretory response progresses, the frequency of the amperometric spikes increases, and that the highest frequency of spikes corresponds to the maximum rate of increase in the capacitance trace. This can be seen more clearly in the integrated amperometric signal (*dotted line*), which has been superimposed on the capacitance trace. The integrated amperometric signal represents the total amount of oxidizable secretory products that were detected by the carbon fiber from the cell during the secretory response. The superimposition of the integrated amperometric signal on the capacitance trace shows that there was a good correlation between the two methods used to monitor secretion. Similar results were obtained from adrenal chromaffin cells (Fig. 1 *B*). In chromaffin cells it is only the fusion of the larger granules that can be recorded as individual step changes in membrane capacitance. In these cells amperometry is a more sensitive means of monitoring the secretion from individual vesicles.

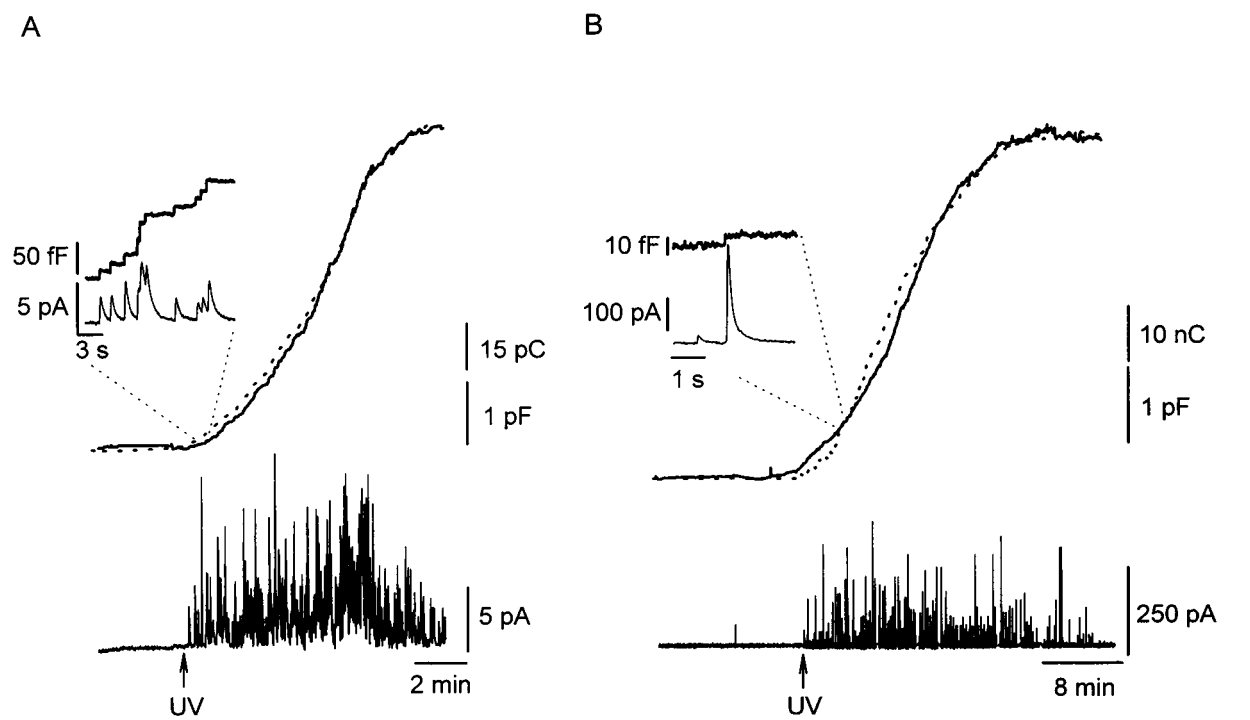

FIGURE 1 (*A*) The secretory response induced by photolysis of caged GTPγS is delayed in mast cells. Secretion was stimulated by photolysis of 300 μM DMNPE-GTPγS by exposure to a 5-s UV light flash 4 min after entering the whole-cell configuration. The time interval between the stimulus and the first fusion event was 35 s. The time integral of the amperometric trace is shown as a dotted line superimposed on the cell membrane capacitance trace. The amperometric recording is shown at the bottom of the figure. An expansion of these traces (*inset*) shows that each step increase in membrane capacitance (C_m) is associated with a spike in the amperometric current recording. (*B*) DMNPE-GTPγS (300 μM) was uncaged in an adrenal chromaffin cell 15 min after entering the whole cell configuration by exposure to UV light for 10 s. An integral of the amperometric trace (dotted line) could be superimposed on the capacitance trace (top). The amperometric recording is shown at the bottom of the figure.

Photolysis of caged Ca^{2+} induced secretion in mast cells

Fig. 2 *A* shows the secretory response induced by rapidly and transiently increasing the $[Ca^{2+}]_i$ by photolysis of DM-nitrophen (30% loaded with Ca^{2+}). Vesicle fusion was measured by monitoring the cell membrane capacitance. Photolysis was achieved by illuminating the cell and the very tip of the patch pipette with UV light (<395 nm) for 500 ms (at times indicated by the arrows in Fig. 2 *A*). Each time DM-nitrophen was photolyzed, there was an immediate increase in membrane capacitance that stopped after several seconds, reflecting a return of the $[Ca^{2+}]_i$ to resting levels (Oberhauser et al., 1995). The total increase in membrane capacitance represents the fusion of about 30% of the secretory granules in the cell. Surprisingly, the increase in membrane capacitance triggered by the first elevation in $[Ca^{2+}]_i$ had two clear phases: a very rapid phase (smooth phase) in which no steps could be resolved, followed by a slower increase, which was composed of clearly distinguishable steps (see insets in Fig. 2 *A*). The smooth phase increase in membrane capacitance was only observed the first time Ca^{2+} was uncaged and was not seen in the same cell for either the second or subsequent stimulations.

In the experiment described in Fig. 2 *A*, Ca^{2+} was uncaged in the presence of 1.5 mM GTP. In a similar experiment, Ca^{2+}-loaded DM-nitrophen was photolyzed in the absence of guanine nucleotides (Fig. 2 *B*). This elicited only a smooth phase increase in capacitance that was not followed by the stepwise increases normally associated with mast cell degranulation (Fig. 2 *B*; see also Robinson et al., 1994). This smooth phase increase in the capacitance trace is not due to a photoproduct of DM-nitrophen, as no change in the membrane capacitance of the cell was recorded when Mg^{2+} was uncaged from DM-nitrophen (Fig. 2 *C*; see also Robinson et al., 1994). This result also shows that the smooth phase increase in membrane capacitance was not a nonspecific effect of divalent cations.

The secretory response to the first transient increase in $[Ca^{2+}]_i$ starts with a long delay in mast cells

It is possible that the smooth phase increase in membrane capacitance seen after photolysis of caged Ca^{2+} may be a result of the fusion of secretory vesicles that are too small (<200 nm) to be detected as stepwise increases. The amperometric technique has proved to be more sensitive for monitoring exocytosis of small vesicles (Chow et al., 1992; Ureña et al., 1994; Chen et al., 1994; Robinson et al., 1995; Bruns and Jahn, 1995). Simultaneous capacitance and amperometric recordings were used to determine whether release of oxidizable secretory products occurred during this smooth phase increase in membrane capacitance (Fig. 3 *A*). In response to the first rapid increase in $[Ca^{2+}]_i$, the membrane capacitance increased first smoothly and then in a stepwise fashion (Fig. 3 *A*, *top bold trace*). However, the

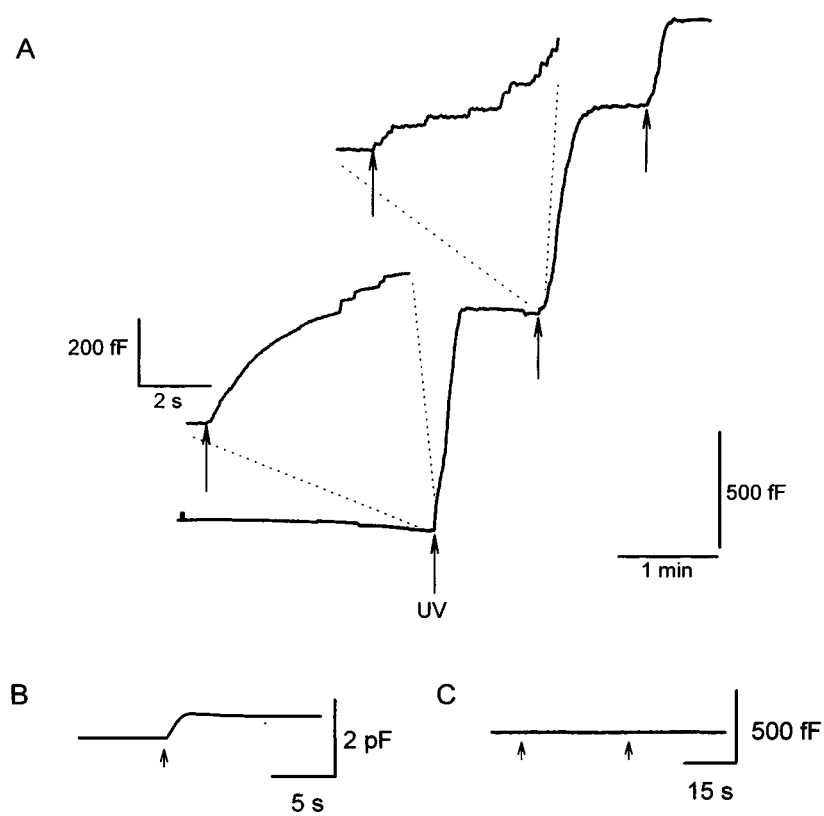

FIGURE 2 (*A*) The release of caged Ca^{2+} triggers transient increases in membrane capacitance in mast cells. At the times indicated by the arrows, the cell was illuminated with UV light for 500 ms. The initial capacitance of the cell was 3.4 pF and increased to 5.4 pF after the third flash. The insets show expanded time scales of the initial phases of the first and second stimulations of the cell. It can be seen that each phase of membrane capacitance increase comprises individual fusion events (steps), and that in the case of the first stimulation, the step increases in capacitance were preceded by a smooth phase increase. (*B*) Stepwise fusion events were not seen after the photolysis of caged Ca^{2+} in the absence of guanine nucleotides. Only a smooth phase change in capacitance was observed. (*C*) The smooth phase change in capacitance was not caused by photoproducts of DM-nitrophen. After the uncaging of DM-nitrophen 30% loaded with Mg^{2+} (indicated by the arrows), no change in membrane capacitance could be observed. This also shows that the smooth phase change in capacitance is not a nonspecific effect of cations.

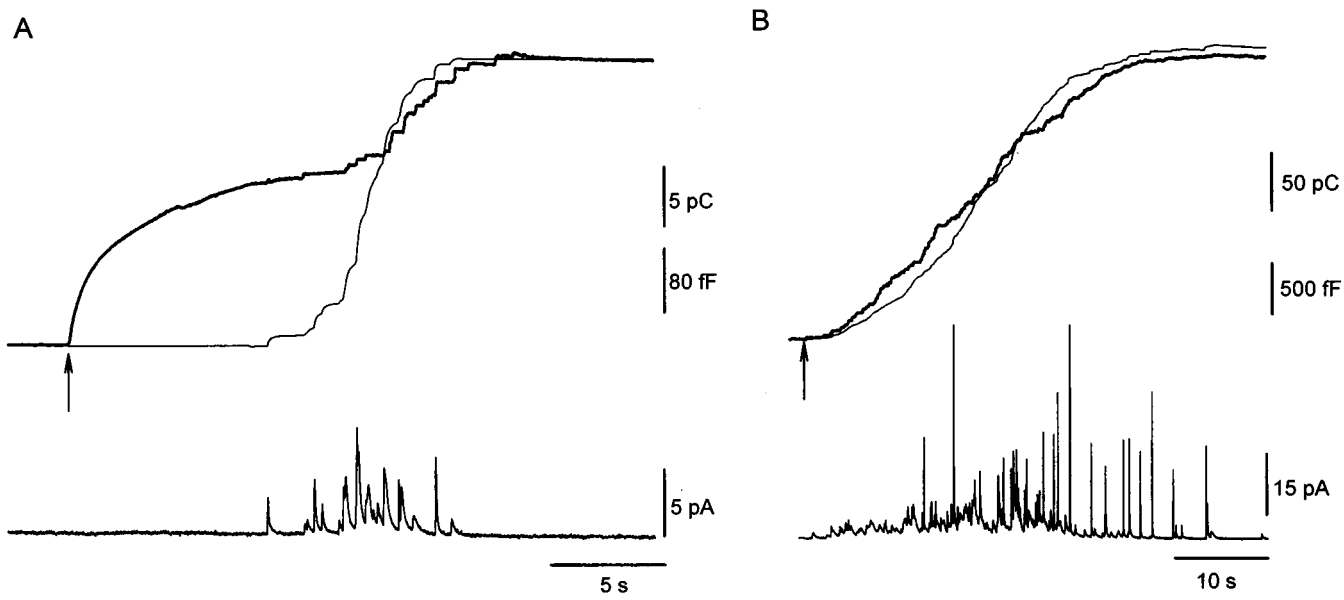

FIGURE 3 Secretory responses to the first (*A*) and second (*B*) transient increases in $[Ca^{2+}]_i$ triggered by photolysis of DM-nitrophen in a patch-clamped mast cell. The cell membrane capacitance (top traces, thick lines) was measured simultaneously with the amperometric current (bottom traces). The time integral of the amperometric current is shown superimposed on the membrane capacitance trace (thin lines). Exposure to UV light lasted for 440 ms. It can be seen that there was a long delay (5.3 s) between the first increase in membrane capacitance and the first discernible step increase. This step increase coincided with the recording of the first amperometric spike (*A*). The second time the cell was stimulated, the integrated amperometric signal was more closely correlated with the capacitance recording (*B*). This is because there was no large smooth phase increase seen in the capacitance trace.

first amperometric spike was only seen after a long delay (5.3 s in this cell) and was seen to coincide with the first detectable step increase in the capacitance trace. We calculated the integral of the amperometric response to more readily compare this with the capacitance recording. The integrated amperometric signal is shown as the thin line superimposed on the capacitance trace. Again this emphasizes the lack of release of oxidizable substances during the smooth phase of the capacitance increase. It is not clear at present what this smooth phase increase in membrane capacitance represents, but it has been proposed that this represents the fusion of small synaptic-like vesicles with the plasma membrane (Kirillova et al., 1993).

The secretory response elicited by a second exposure to UV light was much more robust (Fig. 3 *B*). The delay between the uncaging of Ca^{2+} and the first observed fusion of a granule with the plasma membrane was much shorter (0.3 s) than that seen after the first stimulation of the cell (5.3 s). The number of fusion events was increased after this second stimulus, 161 ± 13 (n = 7), compared to only 60 ± 10 (n = 7) events induced by the first flash. There was a higher degree of correlation between the capacitance trace and the integral of the amperometric signal after the second stimulus, because there was no smooth phase increase in membrane capacitance. Table 1 shows that the average delay to the first fusion event was 4.0 ± 0.7 s (n = 7) for

TABLE 1 Summary of the secretory responses induced by photolysis of Ca^{2+}-DM-nitrophen in patch-clamped cells and chromaffin cells

	1st flash					2nd flash				
	Delay (ms)	Fusion-ready granules	Max. rate (I_{amp}; spikes/s)	Max. rate (C_m; vesicles/s)	Total no. of exocytotic events	Delay (ms)	Fusion-ready granules	Max. rate (I_{amp}; spikes/s)	Max. rate (C_m; vesicles/s)	Total no. of exocytotic events
Mast cells (n = 7)	4018 ± 701	0	5 ± 1	36 ± 6	60 ± 10	280 ± 120	3–8	10 ± 1	14 ± 3	161 ± 13
Chromaffin cells (n = 19)	520 ± 117	1.8 ± 0.8	9 ± 1	506 ± 63	132 ± 21	84 ± 20	39 ± 7	79 ± 11	145 ± 23	108 ± 49

The maximum rate of secretion was calculated from the steepest slope in capacitance trace. These rates were converted into vesicles per second, assuming that the average mast cell vesicle contributes 25 fF and each chromaffin vesicle 2fF. In a similar fashion, the average charge under an amperometric spike (1.25 pC in mast cells; 0.57 pC in chromaffin cells) was used to calculate the rate of vesicle fusion from the steepest part of the integrated amperometric trace. The number of fusion-ready granules was determined by counting the number of amperometric spikes that occurred 500 ms after stimulation of the cells.

the first flash. This delay was reduced to 0.28 ± 0.12 s (n = 7) for a second stimulus delivered 3 min after the first. The maximum rate of granule fusion was increased after a second stimulation of the cell.

The secretory response induced by transient increases in $[Ca^{2+}]_i$ in chromaffin cells

We have shown that in mast cells there is a long delay between the uncaging of Ca^{2+} and the evoked secretory response. We wanted to know whether this was a phenomenon peculiar to mast cells, or if such delays could be observed in other cells. To examine this question we chose to study a neuroendocrine cell, the adrenal chromaffin cell, because these cells have already proved to be amenable to both membrane capacitance and amperometric measurements of secretion (Neher and Marty, 1982; Wightman et al., 1991; von Rüden and Neher, 1993; Robinson et al., 1995). Fig. 4 shows a typical recording of the secretory responses triggered by several rapid transient increases in $[Ca^{2+}]_i$ triggered by the photolysis of caged Ca^{2+}. Secretion was simultaneously monitored by measuring membrane capacitance (*top trace*, C_m) and by using a carbon fiber electrode to detect the release of catecholamines (*bottom trace*, I_{amp}). The trace in the center shows the integral of the amperometric signal, which is a measure of the total catecholamine release (detected by the carbon fiber) triggered by each of the four stimuli applied. At the time indicated by the arrows, the cell was briefly exposed (140 ms) to UV light to trigger the release of Ca^{2+} by photolysis of DM-nitrophen. A comparison of the capacitance trace (*top*) with the time integral of the amperometric recording (*middle*) reveals that the two methods, in this particular instance, report distinct differences in the secretory response of the cell. The first stimulation of the cell resulted in a very rapid increase in the cell membrane area that was accompanied by a concomitant increase in the number of amperometric spikes recorded. The very rapid increase in cell membrane capacitance, stimulated by uncaging Ca^{2+}, was followed by a decrease that presumably reflects a reuptake of plasma membrane by the cell due to an endocytic process (Neher and Zucker, 1993; von Rüden and Neher, 1993; Parsons et al., 1994; Thomas et al., 1994; Burgoyne, 1995). During this phase of membrane retrieval, however, there was still a significant amount of secretion from the cell, which was evident from the amperometric recording.

A second uncaging stimulus also elicited an increase in capacitance that was followed by a declining phase. The amperometric recording shows that the secretory phase of the cell was also more prolonged for the second stimulation of the cell. Photolysis of the caged compound for a third and fourth time evoked an increase in membrane capacitance that was more closely correlated to the time integral of the amperometric current trace.

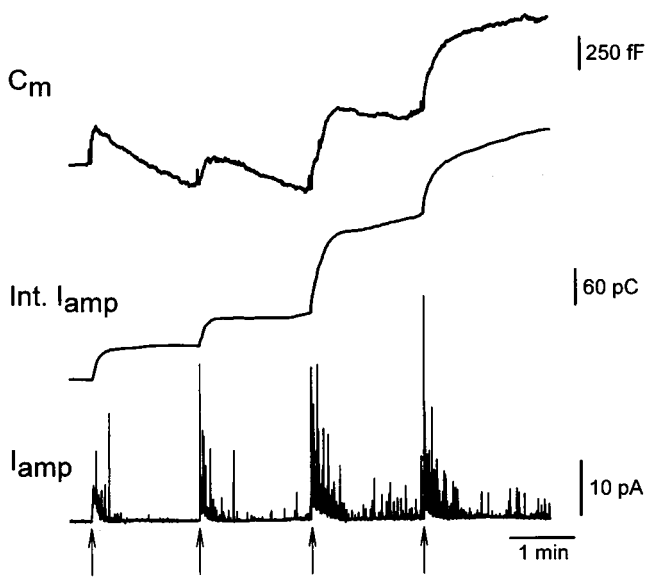

FIGURE 4 Secretory responses induced by step increases in $[Ca^{2+}]_i$ in chromaffin cells. Secretion was followed by the measurement of membrane capacitance (upper trace) and by the amperometric detection of released catecholamines (bottom trace). The time integral of the amperometric current trace is also shown (center). At the times indicated by the arrows, the cell was illuminated by UV light for 140 ms to release Ca^{2+} by photolysis of DM-nitrophen (10 mM plus 3 mM $CaCl_2$). The first flash was given 4 min after a whole-cell recording was established. Clear differences can be seen between the membrane capacitance trace and the time integral of the amperometric signal, in particular for the first two stimulations of the cell.

The secretory response to the first transient increase in $[Ca^{2+}]_i$ is also delayed in chromaffin cells

Fig. 5 shows simultaneous membrane capacitance recordings and amperometric signals from an adrenal chromaffin cell that was stimulated to secrete in response to a rapid transient increase in $[Ca^{2+}]_i$ caused by the photolysis of caged Ca^{2+}. The top trace in each instance shows the membrane capacitance record, and the lower trace shows the amperometric signal. Fig. 5 *A* shows the first stimulation, and Fig. 5 *B* shows the second stimulation of the cell (in both cases only the initial part of the response obtained in the first few seconds is shown). Fig. 5 *A* shows that there was a clear delay from the increase in capacitance to the first amperometric spike (550 ms). During this time the membrane capacitance increased to 577 fF. Most of the granules in these cells are too small to be detected as step changes in capacitance; however, assuming 2 fF per granule (Horrigan and Bookman, 1994), this change in membrane capacitance would represent the fusion of about 290 granules. However, this increase in membrane capacitance is not due to the fusion of catecholamine-containing granules, because the amperometric fiber did not report any events during this phase. Fig. 5 *B* shows that the delay between the increase in capacitance and the first observable amperometric spikes seen for the second stimulation of the cell was very much reduced (also see Table 1). This suggests that in

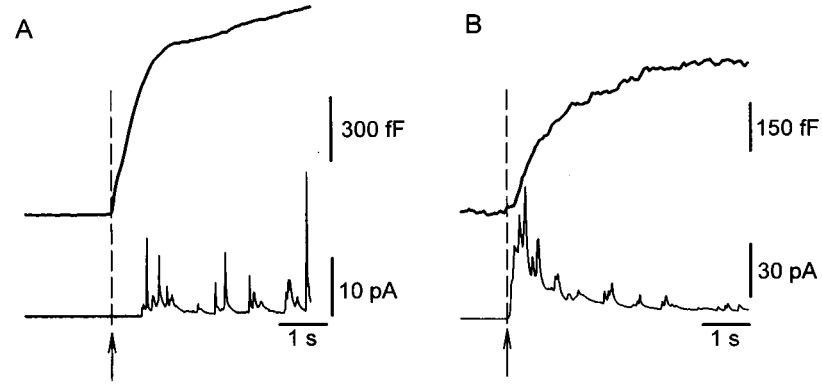

FIGURE 5 Secretory responses induced by rapid transient increases in $[Ca^{2+}]_i$ in chromaffin cells. (*A* and *B*) The first and second secretory responses induced by consecutive UV light photolysis (arrows) of Ca^{2+}–DM-nitrophen in the same cell. The membrane capacitance is shown in the top trace, and the simultaneously recorded amperometric signal is shown in the lower trace. The interval between stimulations of the cell was 3 min. A distinct lag (550 ms) between the increase in membrane capacitance after the uncaging of Ca^{2+}–DM-nitrophen and the first amperometric spike can be seen after the first stimulation of the cell (*A*). This delay was very much reduced after the second stimulus (*B*).

chromaffin cells, just as in mast cells (see Fig. 2 *A*), there is an increase in membrane capacitance that is not associated with the fusion of granules that contain oxidizable compounds.

Estimation of the rates of vesicle fusion in mast cells and chromaffin cells

Table 1 shows a summary of the secretory responses induced by a rapid, transient increase in $[Ca^{2+}]_i$ in mast cells and chromaffin cells. The delay time to the fusion of the first granule for a second stimulation of a given cell was considerably reduced in both mast cells and in chromaffin cells. The number of fusion-ready vesicles was determined by counting the number of amperometric spikes present in the first 500 ms after exposing the cell to UV light. The response to the first flash shows that there are no fusion-ready granules in mast cells ($n = 7$), and between 0 and 3 granules in chromaffin cells (1.8 ± 0.8, $n = 19$). The number of fusion-ready granules increased to 3–8 ($n = 7$) in mast cells and 39 ± 17 ($n = 19$) in chromaffin cells for the second stimulus.

The average charge under individual amperometric spikes was 1.25 ± 0.10 pC ($n = 38$) for mast cells and 0.57 ± 0.05 pC ($n = 30$) for chromaffin cells. These values were used to estimate the rate of granule fusion from the integrated amperometric signal. The maximum rate of secretion was calculated from the steepest slope of the integrated amperometric signal and corresponded to a rate of 5–6 granules/s in mast cells or 9–10 granules/s in chromaffin cells (Table 1) for the first flash. In contrast, much higher rates of secretion were obtained from the capacitance traces (rates of secretion were converted to granules/s, assuming a magnitude of 25 fF for an average mast cell granule and 2 fF for a chromaffin granule). In both cases, this discrepancy was due to the large smooth phase changes that were recorded from the cells that were not accompanied by the fusion of secretory vesicles that contained oxidizable substances. For a second stimulation of the same cell, it can be seen that the rate of secretion increased and that the values of the maximum rates of secretion that were obtained from the amperometric recording and the capacitance recording are more closely correlated. This is particularly true in the case of mast cells (Table 1).

DISCUSSION

When photolysis of caged GTPγS was used to stimulate secretion, in either mast or chromaffin cells, it was possible to superimpose an integral of the amperometric signal on the capacitance recording (Fig. 1, *A* and *B*). The integrated amperometric signal is a measure of the total amount of secretory products that were detected by the carbon fiber electrode during the time course of secretion. The superimposition of the integrated amperometric signal on the capacitance trace demonstrates that it can provide an alternative method of assessing the extent and kinetics of the secretory response of a cell. The close agreement between these two traces also showed that if the carbon fiber missed some exocytotic events, the missed events occurred at random during the degranulation and did not bias the time course of the integral of the amperometric signal.

When photolysis of caged Ca^{2+} (DM-nitrophen) was used to stimulate secretion in either mast cells or chromaffin cells, a very rapid increase in membrane capacitance that could not be resolved as single step increases was seen. This smooth phase capacitance change was not accompanied by the release of oxidizable secretory products. This gradual increase in membrane capacitance has previously been reported in mast cells and was attributed to the fusion of small dense core vesicles (Kirillova et al., 1993). There is no published morphological or biochemical evidence to support the existence of such vesicles in mast cells. However, adrenal chromaffin cells do contain synaptic-like microvesicles (Unsicker and Chamley, 1977; Thomas-Reetz and De Camilli, 1994), and fusion of these vesicles could account for some of the change in capacitance that was not accompanied by amperometric spikes. The contents of these microvesicles have not been determined yet; thus whether they are oxidizable and hence capable of detection by the carbon fiber is not known. Another possible explanation of the smooth phase is that endocytic vesicles were triggered to fuse with the plasma membrane, in a manner similar to that previously reported in chromaffin cells (von Graffenstein

and Knight, 1992). When guanine nucleotides are omitted from the pipette solution, the uncaging of Ca^{2+} from DM-nitrophen resulted only in the smooth phase change in capacitance and no step changes in capacitance (indicative of the fusion of secretory granules) (Fig. 3 *C*; Robinson et al., 1994). No change in capacitance was seen when Mg^{2+} was used to load DM-nitrophen, suggesting that this smooth phase is not merely due to a nonspecific effect of cations (Fig. 3 *D*; Robinson et al., 1994). This result also shows that the smooth phase increase in membrane capacitance was not due to photoproducts of the caged compounds. It has previously been reported that mast cells swell when they are perfused with buffers that contain high concentrations of Ca^{2+} (Penner and Neher, 1988), and this may provide yet another alternative explanation for this smooth phase change in membrane capacitance.

The maximum rates of fusion of granules with the plasma membrane (estimated from the integral of the amperometric trace) are much slower than those estimated from capacitance measurements for the first uncaging event (Table 1). This is because a rapid elevation of $[Ca^{2+}]_i$ triggers fast, smooth increases in membrane capacitance that are not associated with the exocytotic fusion of granules that contain oxidizable products. In contrast, the step increases in membrane capacitance that we have observed in mast cells are accompanied by an amperometric spike, demonstrating that these events correspond to the exocytosis of single secretory granules (Fig. 1 *A*). Exactly what causes these smooth phase membrane capacitance increases remains an open question. However, it is clear that extrapolating kinetic information from the cell membrane capacitance recording about the rate of secretion in cells is complicated, particularly when the fusion of single secretory vesicles (observed as step increases in capacitance) cannot be detected. We propose that the numbers of fusion-ready vesicles and the rate of their fusion can be more accurately determined from the integrated amperometric recording.

In any mammalian cell there will be a number of intracellular organelles that can fuse with the plasma membrane, given a large enough increase in the cytosolic calcium concentration. These organelles can be several types of secretory vesicles, endosomes, and small vesicles that underlie constitutive traffic (Mayorga et al., 1994; Bauerfeind et al., 1994). Because it is now known that calcium stimuli are highly localized (e.g., Llinas et al., 1992; Issa and Hudspeth, 1994; Robinson et al., 1995), it is unlikely that under physiological conditions indiscriminate fusion of these organelles will occur. However, caged compounds are believed to create a global calcium stimulus throughout the cytosol, which may trigger fusion events that are not otherwise observed. In this case, estimates of the number of fusion-ready vesicles and rates of vesicle fusion events will be complicated by the inability of the capacitance technique to discriminate among the fusions of different populations of small vesicles. Our results clearly demonstrate this point. A smooth increase in capacitance of unknown origin is observed in response to the photolysis of DM-nitrophen. However, the lack of amine secretion during this smooth phase and the stepwise fusion events observed later showed that there are at least two types of events during these responses. Furthermore, as discussed in the Introduction, changes in the cell shape, surface morphology, or the gating charges of transmembrane proteins that are sensitive to the binding of calcium might alter the cell's input admittance, creating changes in the calculated membrane capacitance that are unrelated to vesicle fusion (e.g., Fernandez et al., 1982; Horrigan and Bookman, 1994). In those cases in which step increases in capacitance are observed and result in the measurable release of secretory products, there can be little doubt that these are true exocytotic fusion events. However, the results of this work suggest that capacitance measurements alone, unless corroborated by independent measurements such as electron microscopy (Neher and Marty, 1982; Fernandez et al., 1984; Lollike et al., 1995), amperometry (von Rüden and Neher, 1993; Alvarez de Toledo et al., 1993; Robinson et al., 1995), or other techniques, are more difficult to interpret.

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