Exocytosis in Bovine Chromaffin Cells: Studies with Patch-Clamp Capacitance and FM1-43 Fluorescence

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ABSTRACT In response to physiological stimuli, neuroendocrine cells secrete neurotransmitters through a Ca^{2+}-dependent fusion of secretory granules with the plasma membrane. We studied insertion of granules in bovine chromaffin cells using capacitance as a measure of plasma membrane area and fluorescence of a membrane marker FM1-43 as a measure of exocytosis. Intracellular dialysis with [Ca^{2+}] (1.5–100 μM) evoked massive exocytosis that was sufficient to double plasma membrane area but did not swell cells. In principle, in the absence of endocytosis, the addition of granule membrane would be anticipated to produce similar increases in the capacitance and FM1-43 fluorescence responses. However, when endocytosis was minimal, the changes in capacitance were markedly larger than the corresponding changes in FM1-43 fluorescence. Moreover, the apparent differences between capacitance and FM1-43 fluorescence changes increased with larger exocytic responses, as more granules fused with the plasma membrane. In experiments in which exocytosis was suppressed, increasing membrane tension by osmotically induced cell swelling increased FM1-43 fluorescence, suggesting that FM1-43 fluorescence is sensitive to changes in the membrane tension. Thus, increasing membrane area through exocytosis does not swell chromaffin cells but may decrease membrane tension.

INTRODUCTION

Ca^{2+}-dependent exocytosis in neurons and neuroendocrine cells occurs through fusion of intracellular membrane compartments with the plasma membrane. After fusion and release of secretory cargo, secretory membrane can either be retrieved on a time scale that ranges between a few milliseconds (kiss-and-run) (Ceccarelli et al., 1973) and 45 min (Patzak and Winkler, 1986) or can remain fused producing swelling of the plasma membrane (Heuser and Reese, 1981; Smith and Betz, 1996). Ultrastructural studies demonstrate that the membrane of neuronal cells displays both types of behavior (Ceccarelli et al., 1973; Heuser and Reese, 1981; Koenig and Ikeda, 1989; Shupliakov et al., 1997). In bovine chromaffin cells, intracellular dialysis with high [Ca^{2+}] through a patch pipette stimulated massive exocytosis and concomitant cell swelling (Smith and Betz, 1996). In contrast, when exocytosis was stimulated by exposing chromaffin cells to nicotine (Vitale et al., 1995) or elevated KCl (Fox, 1996), cells did not swell. Interestingly, cell swelling per se represents a potent stimulus for a broad range of cellular functions including gene expression, protein phosphorylation, ion transport (for review, see Lang et al., 1998), as well as the balance between exocytosis and endocytosis (Dai and Sheetz, 1995; Dai et al., 1997). Moreover, numerous studies using bovine chromaffin cells as a model to study exocytosis showed that intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) dynamics vary substantially with different stimuli (Engisch et al., 1997; Knight and Kesteven, 1983; Neher and Augustine, 1992; O'Sullivan and Burgoyne, 1989; O'Sullivan et al., 1989). Consequently, it is not known whether Ca^{2+}-dependent exocytosis regulates cell swelling in neuroendocrine cells.

Secretion from neuroendocrine cells has been studied in detail using patch-clamp techniques to measure membrane capacitance (Neher, 1998) and fluorescence of a membrane marker FM1-43 (Cochilla et al., 1999). The principle of the capacitance method is based on the fact that plasma membrane capacitance is directly proportional to membrane area ($\sim$10 fF/μm^2). Thus, whenever exocytosis (increase in membrane area) occurs, changes in the capacitance can be detected using this approach. The fluorescent membrane marker FM1-43 provides complementary information. FM1-43 binds to membranes but does not cross lipid bilayers. Furthermore, it is not fluorescent when free in a solution, but upon membrane binding its quantum yield increases $\sim$350 times (Betz et al., 1992). This relationship implies that fluorescence intensity is directly proportional to the amount of membrane exposed to FM1-43, and the overall change in FM1-43 fluorescence provides in real time a measure of the sum of all exocytic events. Consequently, in the absence of endocytosis the changes in capacitance and FM1-43 fluorescence should be the same. Under these conditions, the relationship between capacitance and FM1-43 fluorescence responses is approximately one-to-one in bovine chromaffin cells (Smith and Betz, 1996) and rat pituitary somatotrophs (Kilic et al., 2001a). However, recent studies indicate that these measurements can be dissociated in rat pituitary lactrotrophs (Angleson et al., 1999) and the disparity between capacitance and FM1-43 fluorescence is thought to be due to FM1-43 staining of the matrix of dense core (Angleson et al., 1999; Cochilla et al., 2000). Furthermore, the properties of FM1-43 may differ in cell

Submitted September 11, 2001, and accepted for publication April 10, 2002.

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0006-3495/02/08/849/09 $2.00

TABLE 1 Mixtures of Ca^{2+} buffers and Ca^{2+} used to stimulate exocytosis

Buffer (5 mM)	$[CaCl_2]$ (mM)	Free $[Ca^{2+}]_i$ (μM)
EGTA	4.5	1.5
HEDTA	3.75	10
NTA	2.3	100

versus granule membranes because the fluorescence of FM1-43 is strongly influenced by its environment (Betz et al., 1996).

Based on these observations, the aim of the present study was to quantitate the magnitude of cell swelling after intracellular dialysis with different $[Ca^{2+}]_i$ and to evaluate the relationship between changes in the capacitance and FM1-43 fluorescence under these conditions. In bovine chromaffin cells, we found that exocytic insertion of granules in the plasma membrane does not swell cells. Moreover, when endocytosis was minimal, capacitance and FM1-43 fluorescence responses were markedly different, and the disparity between capacitance and FM1-43 fluorescence responses increased as more granules fused with the plasma membrane. These studies also indicate that FM1-43 fluorescence is directly proportional to the extent of cell swelling, and consequently the disparity between capacitance and FM1-43 fluorescence responses may be due in part to changes in membrane tension.

MATERIALS AND METHODS

Cell preparation and solutions

Bovine chromaffin cells were isolated from adrenal glands and enzymatically treated as described (Fenwick et al., 1982). Cells were then plated on petri dishes and stored in an incubator at 37°C in 5% CO_2 and 95% air atmosphere. Cells were used 2 to 3 days after isolation.

For most experiments the standard external solution contained: 140 mM NaCl, 2 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, and 3 μM of fluorescent dye FM1-43 (Molecular Probes, Eugene, OR). To stimulate exocytosis, cells were dialyzed with pipette solutions that contained mixtures of Ca^{2+} buffers and $CaCl_2$ (Table 1) and 130 mM Cs-D-glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 2 mM ATP-Mg_2, and 0.3 mM GTP. Free $[Ca^{2+}]_i$ was calculated as described (Kilic et al., 2001a). To suppress exocytosis, cells were dialyzed with nonstimulating pipette solution that contained: 145 mM Cs-D-glutamate, 8 mM NaCl, 1 mM $MgCl_2$, 2 mM ATP-Mg, 0.3 mM GTP, and 0.1 mM EGTA (free $[Ca^{2+}]_i \sim 0.1$ μM). Osmolarity of the external solution was 300 mosM and of pipette solutions was 295 to 300 mosM

To swell the cells and increase plasma membrane tension without stimulating exocytosis, cells were dialyzed with a hypertonic solution composed of nonstimulating solution and 50 mM sucrose. The osmolarity of hypertonic solution was ~350 mosM. In another set of experiments, after dialyzing the cells with nonstimulating pipette solution, membrane tension was increased by exposing cells to hypotonic solution. Hypotonic solution (180 mosM) was similar to standard external solution except that concentration of NaCl was reduced to 70 mM. To assess the potential role of ionic strength on FM1-43 fluorescence, cells were exposed to a low ionic strength solution that was composed of hypotonic solution and sucrose to match the osmolarity of standard external solution (300 mosM). The pH of all solutions was 7.2

Capacitance and conductance measurements

Changes in plasma membrane area were assessed by measuring the capacitance in whole-cell configuration of the patch-clamp technique. The cells were voltage clamped at holding potential of −80 mV, and eight hyperpolarizing pulses of 4 ms in duration were applied (pulse amplitude −20 mV). Current responses were filtered with 8-pole Bessel filter (30-kHz cutoff) and acquired with a sampling time of 5 μs using Pulse Control software (Horrigan and Bookman, 1994) in conjunction with an interface ITC16 (Instrutech, Greatneck, NY) and IgorPro3 (WaveMetrics, Lake Oswego OR). The currents were averaged and inverted, and then fitted to an equation $I(t) = I_{ss} + (I_0 - I_{ss})\exp(-t/\tau)$. From the fitted parameters, membrane capacitance (C_m), membrane conductance (G_m), and access resistance (R_a) were determined (Lindau and Neher, 1988). This procedure was repeated every 3 s.

Fluorescence imaging and analysis

Exocytosis and cell swelling were monitored using fluorescence of a membrane marker FM1-43 (Smith and Betz, 1996). Before patch-clamp experiments, cells were transferred to coverslips and exposed to the standard external solution that contained FM1-43. Fluorescence of FM1-43 was excited with 480-nm light through Nikon 60× water immersion objective CFN PlanAPO (NA = 1.2) and then collected at 535 nm. Chromaffin cells were spherical in shape and fluorescent images were sampled at the cell equator every 5 to 15 s. Images were acquired with a PXL1400 cooled CCD camera (Photometrics, AZ) controlled by software (Inovision, NC) on a Silicon Graphics (Sunnyvale, CA) Indigo2 computer.

Analysis was performed on raw images using NIH Image (Bethesda, MD). Assuming spherical geometry, cell swelling was monitored optically by measuring the cell surface area ($4\pi r^2$), which was determined from the area of cell equatorial cross-section (πr^2). Total cellular FM1-43 fluorescence was measured from individual cells excluding regions with adherent debris. Background fluorescence was measured in the same way from regions containing no cells and was subtracted from total cellular fluorescence. In patch-clamp experiments, total cellular FM1-43 fluorescence was normalized to the values obtained before or immediately after achieving whole-cell configuration.

To compare fluorescence and capacitance changes from different cells, the following procedure was performed. Because capacitance data were acquired at higher rate than fluorescence images, both parameters were interpolated in time using a linear function approximation. In this manner, the time-dependence of both quantities was eliminated, and each fluorescence value had its corresponding capacitance value. Then, changes in fluorescence (ΔF) were taken from different cells and grouped according to their capacitance changes (ΔC). The ΔF values from the groups $\Delta C = 5$, 15, 25, . . . , 125 representing fluorescence responses from different cells were averaged (see Fig. 4).

Data are expressed as mean ± SE. All experiments were performed at 24°C.

RESULTS

Exocytosis does not swell cells

To stimulate exocytosis in bovine chromaffin cells, cells were dialyzed with different $[Ca^{2+}]_i$ (0.1–100 μM) through a patch pipette. Increases in plasma membrane area were assessed by measuring membrane capacitance. Fig. 1 *A* shows a representative recording from a cell dialyzed with 100 μM $[Ca^{2+}]_i$. After achieving access to the cell interior (arrow), during Ca^{2+} dialysis capacitance gradually increased and for long experiments (>5 min) reached a pla-

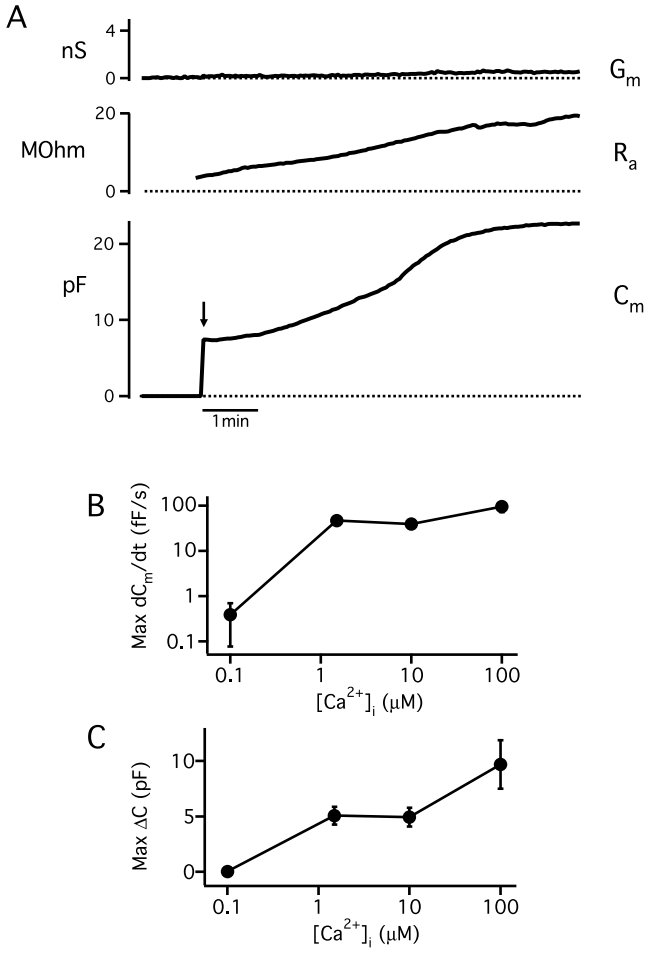

FIGURE 1 Ca^{2+}-dependent exocytosis in chromaffin cells revealed using patch-clamp method. (*A*) Membrane conductance (G_m), access resistance (R_a), and membrane capacitance (C_m) were measured in the whole-cell configuration. Arrow indicates break-in with the pipette solution that contained 100 μM $[Ca^{2+}]_i$. During dialysis cell capacitance increased from 7.4 to 22.7 pF. Consistent feature of the recordings was also an increase in R_a (5–30 MΩ). Holding potential was −80 mV. (*B*) Maximal rate of capacitance rise (dC_m/dt) during dialysis is plotted versus $[Ca^{2+}]_i$ on logarithmic scale. (*C*) Maximal change in the capacitance (ΔC) is shown versus $[Ca^{2+}]_i$. The ΔC is a difference between the capacitance at the end of dialysis and initial capacitance. The number of cells was 5 to 18.

teau. The corresponding conductance change was <1 nS. Both the maximal rate of capacitance increase (Fig. 1 *B*) and the maximal change in capacitance (5–10 pF, Fig. 1 *C*) were Ca^{2+}-dependent. Because the initial capacitance of chromaffin cells was 7.5 ± 0.2 pF (37 cells), these results suggest that Ca^{2+}-dependent exocytosis approximately doubles the plasma membrane area of chromaffin cells.

To test whether this increase in membrane area swells cells, the cell surface area ($4\pi r^2$) was monitored optically assuming spherical geometry. Before dialysis, cells were stained with fluorescent membrane marker FM1-43, and the cell surface area as well as total cellular fluorescence were measured from fluorescent images (Smith and Betz, 1996). The left panel of Fig. 2 shows a fluorescent image of a cell before break-in to establish the whole-cell recording configuration. FM1-43 stained the surface membrane and adherent debris (bright spots). The debris showed no consistent change in fluorescence intensity during Ca^{2+} dialysis and was excluded from analysis. The right panel in Fig. 2 shows the cell ~6 min after break-in with a pipette solution that contained 1.5 μM $[Ca^{2+}]_i$. Although, the capacitance increased by ~100%, the cell surface area increased only by 7%. The same results were obtained with other $[Ca^{2+}]_i$ (Fig. 3 *A*). Large increases in plasma membrane area as measured by capacitance changes did not swell cells. Moreover, maximal changes in cell surface area were not correlated with maximal changes in the capacitance (Fig. 3 *B*, correlation coefficients = 0.1). These results suggest that insertion of granule membrane into the plasma membrane of chromaffin cells does not swell cells.

Capacitance and FM1-43 fluorescence responses are different

In Fig. 3 *A*, during Ca^{2+} dialysis the changes in FM1-43 fluorescence were smaller than or similar to the changes in capacitance. Capacitance changes represent a measure of the net change of plasma membrane area (exocytosis-endocytosis), and FM1-43 fluorescence measures the sum of all fusion events (exocytosis) that occur in the presence of dye. Therefore, in the absence of endocytosis, the relative change in the two quantities should be similar, and consequently if endocytosis occurred FM1-43 fluorescence should exceed capacitance (Kilic et al., 2001a; Smith and Betz, 1996). To further explore the relationship between fluorescence and capacitance, changes in FM1-43 fluorescence and capacitance were compared from different cells. In Fig. 4 *A*, average fluorescence changes are shown versus capacitance changes at different $[Ca^{2+}]_i$. The straight line indicates the expected result if FM1-43 fluorescence correlated directly with capacitance. Notably, the difference between fluorescence and capacitance changes was more pronounced at 1.5 μM than at higher $[Ca^{2+}]_i$. For example, at 1.5 μM $[Ca^{2+}]_i$ cells that doubled their initial capacitance increased in fluorescence only by approximately one-half as much.

To compare fluorescence changes at different capacitance responses, averaged ΔF from Fig. 4 *A* were divided by ΔC. These normalized ΔF were plotted versus ΔC at different $[Ca^{2+}]_i$ (Fig. 4 *B*). Interestingly, at 1.5 μM $[Ca^{2+}]_i$ the ratio seems not be constant but to decrease with larger capacitance changes. At higher $[Ca^{2+}]_i$, the ratio appears to be constant with increasing capacitance changes as more granules fuse with the plasma membrane. These result suggest that at low $[Ca^{2+}]_i$, the discrepancy between the changes in fluorescence and capacitance increases with a larger number of fused granules.

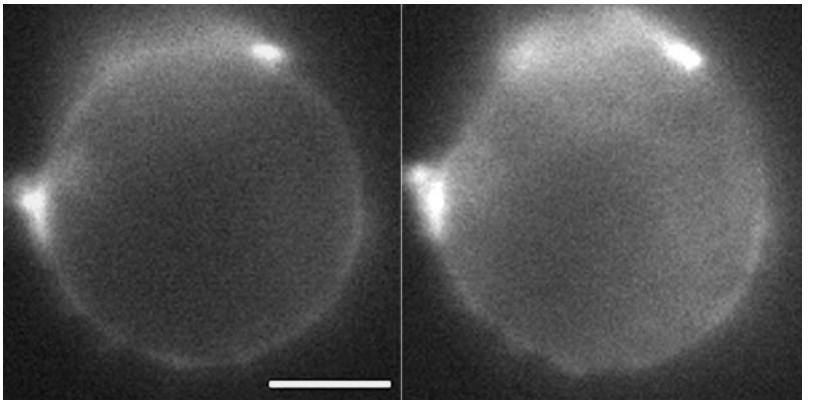

FIGURE 2 Exocytosis in chromaffin cells does not swell cells. A cell was exposed to standard external solution that contained 3 μM FM1-43. Left panel shows fluorescent image of the cell just before break-in to dialyze 1.5 μM $[Ca^{2+}]_i$. The bright debris at the cell surface was excluded from analysis. Approximately 6 min after dialysis the capacitance increased from 6.8 to 13.2 pF, and total cellular fluorescence increased by $\sim$ 50% (*right panel*). Note that the cell surface area did not increase in proportion. Scale bar = 5 μm.

FM1-43 fluorescence is sensitive to membrane tension and ionic strength

Exocytic insertion of new membranes into the plasma membrane has been reported to decrease membrane tension in many cells (Dai et al., 1997; Togo et al., 1999, 2000). To evaluate the potential effect of membrane tension on FM1-43 fluorescence, cells were swelled either by dialysis with hypertonic solution or exposure to hypotonic solution. In voltage clamped chromaffin cells, these manipulations are known not to stimulate exocytosis (Moser et al., 1995) and to increase membrane tension (Dai et al., 1997, 1998; Raucher and Sheetz, 2000). In these experiments with a pipette $[Ca^{2+}]_i \sim 0.1$ μM, the capacitance did not increase (Max $\Delta C = -0.07 \pm 0.04$ pF, 14 cells), but the conductance transiently increased (2.1 ± 0.6 nS, 14 cells) within a few minutes after stimulation (not shown) consistent with an activation of volume-sensitive Cl^- conductance in chromaffin cells (Doroshenko and Neher, 1992; Doroshenko et al., 1991). Interestingly, dialysis with hypertonic solution increased the cell surface area and FM1-43 fluorescence (Fig. 5 *A*), suggesting that elevated membrane tension increases FM1-43 fluorescence.

Staining of the plasma membrane of T-lymphocytes with FM1-43 is dependent on ionic strength of extracellular media (Zweifach, 2000). To test if ionic strength has an effect on FM1-43 fluorescence in chromaffin cells, cells were exposed to a solution that had lower ionic strength but the same osmolarity. This manipulation markedly increased

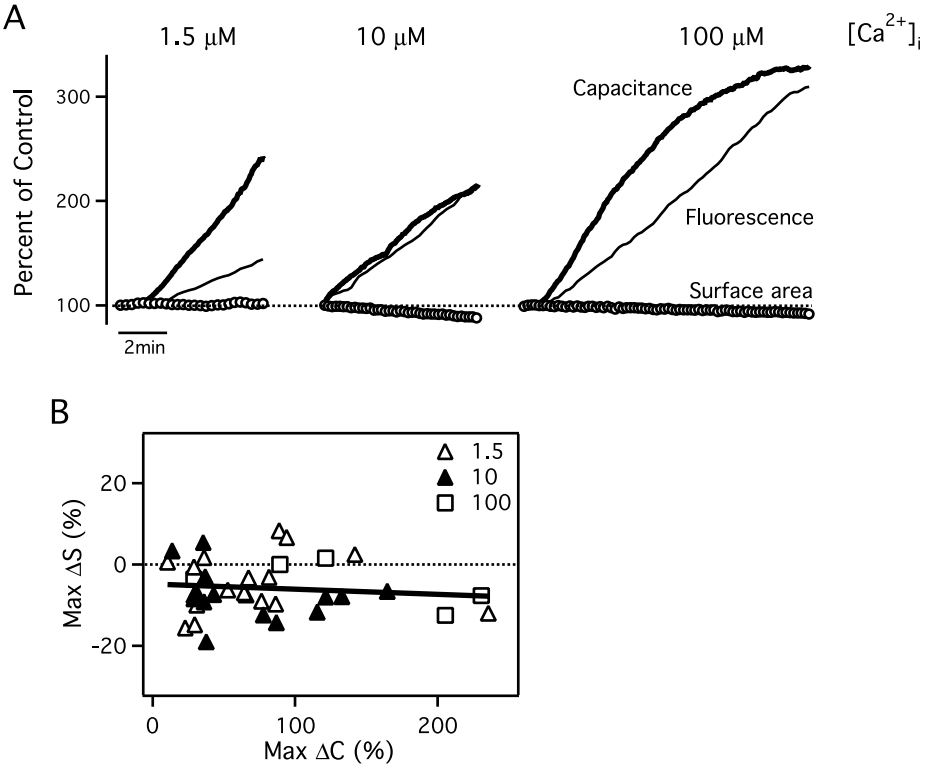

FIGURE 3 Swelling is absent in cells dialyzed with different $[Ca^{2+}]_i$. (*A*) Capacitance and FM1-43 fluorescence were simultaneously measured from chromaffin cells dialyzed with three different $[Ca^{2+}]_i$ (indicated at *top*). Both parameters substantially increased during Ca^{2+} dialysis, but cell surface area changed little if any. (*B*) Maximal change in the cell surface area is shown versus maximal capacitance change. Maximal ΔS and ΔC are not correlated. Number of cells for 1.5, 10, and 100 μM $[Ca^{2+}]_i$ were 18, 14, and 5, respectively.

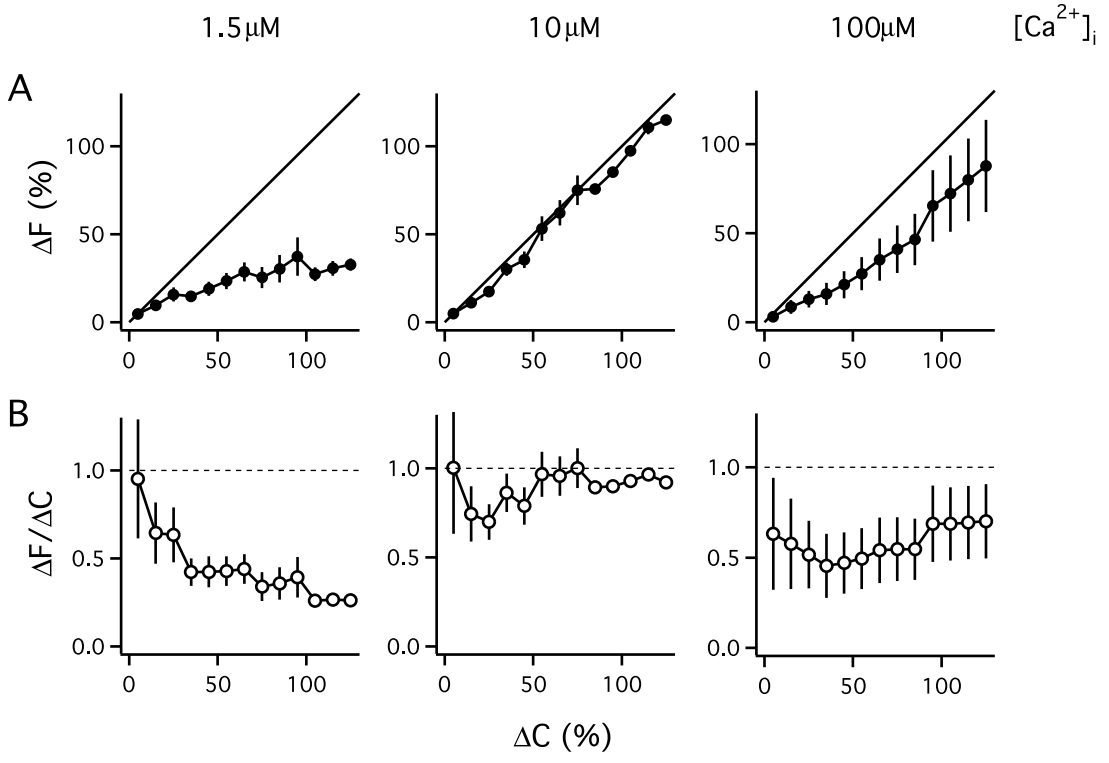

FIGURE 4 Discrepancy between FM1-43 fluorescence and capacitance at different $[Ca^{2+}]_i$. (*A*) During Ca^{2+} dialysis, changes in FM1-43 fluorescence were averaged from different cells as described in Materials and Methods and plotted versus capacitance changes every 10%. Unity lines are also shown. Different $[Ca^{2+}]_i$ are indicated at top. (*B*) The changes in FM1-43 fluorescence were compared at different capacitance response by dividing averaged ΔF from Fig. 4 *A* with ΔC. The normalized fluorescence responses ($\Delta F/\Delta C$) are shown versus ΔC at different $[Ca^{2+}]_i$ (indicated at *top*). Note that the ratio progressively decreases as ΔC increases at 1.5 μM $[Ca^{2+}]_i$, and it is approximately constant at higher $[Ca^{2+}]_i$. Number of cells is as indicated in Fig. 3.

FM1-43 fluorescence (10.1 ± 1.9%, 4 cells, Fig. 5 *B*), suggesting that the staining of membranes with FM1-43 in chromaffin cells is influenced by the ionic strength of extracellular media.

Additional experiments were performed to assess the potential role of membrane tension on FM1-43 fluorescence. Cells were dialyzed with a nonstimulating pipette solution and then swelled by exposure to hypotonic solution. A representative recording is shown in Fig. 5 *C*. Hypotonic solution increased the cell surface area and FM1-43 fluorescence without changing the capacitance. Even when FM1-43 fluorescence was corrected for lower ionic strength of hypotonic media (dashed line) using values from Fig. 5 *B*, it remained elevated during hypotonic exposure. These results suggest that increasing membrane tension by exposing cells to hypotonic media increases FM1-43 fluorescence.

To further examine the relationship between membrane tension and FM1-43 fluorescence, maximal changes in the cell surface area are plotted versus maximal changes in FM1-43 fluorescence obtained from cells that were not stimulated to secrete (Fig. 6). These quantities were correlated (correlation coefficient 0.8). Assuming that changes in optically measured cell surface area are representative measures of membrane tension in the absence of exocytosis (Dai et al., 1997, 1998; Raucher and Sheetz, 2000), these results imply that in chromaffin cells FM1-43 fluorescence is proportional to changes in the membrane tension. Consequently, lower-than-expected changes in FM1-43 fluorescence observed during massive exocytosis at 1.5 μM $[Ca^{2+}]_i$ may be due to the decreased membrane tension.

DISCUSSION

During physiological stimulation, chromaffin cells secrete catecholamine through Ca^{2+}-dependent fusion of secretory granules with the plasma membrane. The present studies in bovine chromaffin cells demonstrate that 1) insertion of granule membrane with the plasma membrane does not swell cells and 2) staining of membranes with a fluorescent marker FM1-43 increases with increasing membrane tension. Moreover, during strong exocytic activity in the absence of endocytosis, increases in the plasma membrane area are substantially larger than corresponding increases in FM1-43 fluorescence. Consequently, this effect may be in part due to decreased

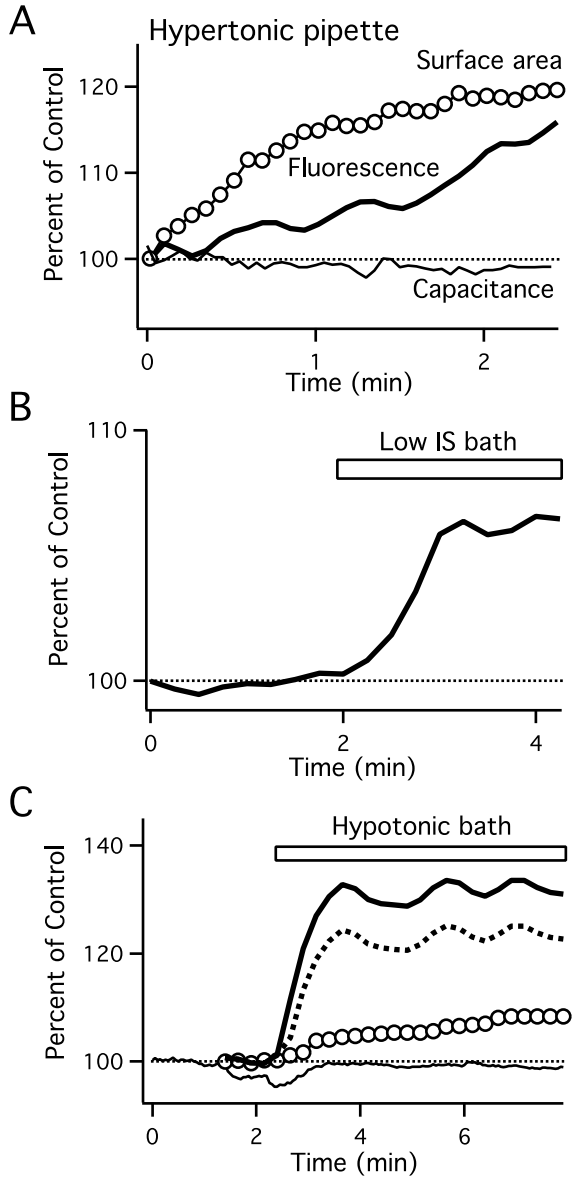

FIGURE 5 Membrane tension and ionic strength influence FM1-43 fluorescence. (*A*) FM1-43 fluorescence, cell surface area, and the capacitance were measured from a cell dialyzed with the hypertonic solution (350 mosM). Note that an increase in the cell surface area elevated FM1-43 fluorescence but not capacitance. (*B*) Intact cell was exposed to a solution that had lower ionic strength (low IS) than standard external solution, but the same osmolarity (300 mosM). Note that FM1-43 fluorescence increased after decreasing ionic strength. (*C*) Cell was dialyzed with a nonstimulating pipette solution. After increasing membrane tension by exposure to hypotonic solution (180 mosM), FM1-43 fluorescence and cell surface area increased, but the capacitance remained unchanged. The dashed line represents fluorescence after correcting for the effect of decreased ionic strength of hypotonic solution. A small decrease in capacitance shortly before and after hypotonic exposure is a perfusion artifact.

membrane tension induced by exocytic granule insertion that may occur in chromaffin cells in a similar manner reported in other cells (Dai et al., 1997; Togo et al., 1999, 2000).

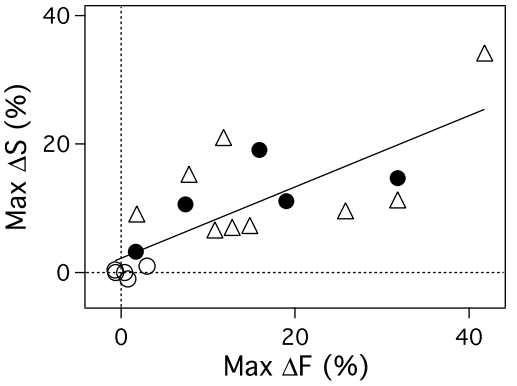

FIGURE 6 Changes in cell swelling and FM1-43 fluorescence are correlated. The cell surface area, FM1-43 fluorescence, and capacitance were measured from cells that were not stimulated to secrete. In all cells the capacitance remained flat (not shown). Cells were either dialyzed with nonstimulating solution (○, 6 cells), or swelled by exposure to hypotonic solution (△, 9 cells), or dialyzed with hypertonic solution (●, 5 cells). Maximal change in cell surface area is shown versus maximal change in FM1-43 fluorescence. Note the strong correlation between these parameters.

Cell swelling and exocytosis

Intracellular dialysis with a stimulating level of $[Ca^{2+}]$ on average doubled plasma membrane area without causing an apparent increase in cell surface area (Figs. 2 and 3). Thus, under iso-osmotic conditions, increasing plasma membrane area through exocytosis does not swell cells. Similar to this study, no swelling was observed in intact chromaffin cells stimulated with elevated KCl or nicotine (Fox, 1996; Vitale et al., 1995). In contrast, when chromaffin cells were dialyzed with 50 μM $[Ca^{2+}]_i$ using similar experimental conditions, capacitance increased in parallel with cell surface area, indicating a direct linear relationship between exocytosis and cell swelling (Smith and Betz, 1996). Although the reasons for contradictory findings are not known, one point merits emphasis and may in part explain the observed difference. A slight mismatch in the osmolarities of the pipette and external solutions in the study by Smith and Betz (1996) could lead to swelling of chromaffin cells in a manner unrelated to the capacitance changes. Consistent with this explanation, when mast cells were dialyzed with micromolar $[Ca^{2+}]$, a slight positive hydrostatic pressure swelled cells, and this swelling was not accompanied by an increase in capacitance (Penner and Neher, 1988). Thus, cells can swell without increasing capacitance, as observed in bovine chromaffin cells (Moser et al., 1995; Solsona et al., 1998) (Fig. 5 this study), liver cells (Graf et al., 1995), and CHO cells (Solsona et al., 1998). Consequently, swelling of chromaffin cells during massive exocytosis (Smith and Betz, 1996) may be related in part to an increased sensitivity of cells to the osmotically unbalanced solutions caused by a Ca^{2+}-dependent alterations of the cytoskeleton (Penner and Neher, 1988) rather than exocytosis.

FM1-43 fluorescence and capacitance

When cells were dialyzed with low $[Ca^{2+}]_i$ (1.5 μM) to minimize endocytosis, capacitance increased more than fluorescence. Further experiments support the concept that this discrepancy is related in part to changes in plasma membrane tension during exocytosis. In theory, capacitance changes could exceed fluorescence changes. For example, the behavior of FM dye molecules may be different in the surface and granule membranes, which are known to differ in biochemical composition (de Oliveira Filgueiras et al., 1981; Dreyfus et al., 1977). This is an important consideration because the fluorescence of FM1-43 is strongly influenced by its environment (Betz et al., 1996). For example, the emission properties of the dye are different in membranes of synaptic vesicles and myelin at the frog neuromuscular junction (Betz et al., 1992). Moreover, calorimetric and nuclear magnetic resonance studies of FM1-43 in lipid vesicles indicate that the partitioning of the dye into the lipid bilayer is affected by the charge of the lipids (Schote and Seelig, 1998). In addition, nonlipid components may also contribute to FM1-43 fluorescence. It has recently been reported that FM1-43 can stain the matrix of dense core granules in pituitary lactotrophs (Angleson et al., 1999). Thus, if chromaffin granule membranes take up fewer dye molecules than an equivalent amount surface membrane, then the change in fluorescence signal upon exocytosis would be lower than the change in capacitance. This could be related to a lower dye partition coefficient, restricted access of the dye, or lower quantum yield of dye molecules in granule membranes. There is no direct evidence about these possibilities. However, they each should produce a constant disparity between capacitance and fluorescence, regardless of size of capacitance change. Experiments showed that the $\Delta F/\Delta C$ ratio was not constant with 1.5 μM $[Ca^{2+}]_i$, but fell progressively with larger capacitance changes (Fig. 4 *B*) as if the fluorescence signal progressively waned with massive exocytic responses.

The increase in the fluorescence-capacitance disparity with increasing exocytosis supports another explanation related to effects of surface membrane tension (Dai et al., 1997; Togo et al., 1999, 2000), which falls as more granules fuse. To test this possibility, membrane tension was increased without stimulating exocytosis (Moser et al., 1995). Under these conditions, FM1-43 fluorescence markedly increased, suggesting that FM1-43 fluorescence increases with increasing membrane tension (Figs. 5 and 6). If the converse holds during exocytosis, then increase in the fluorescence due to addition of granule membrane to the surface will be reduced as surface tension falls. Furthermore, as more granules undergo exocytosis, plasma membrane tension will be progressively reduced, which will increase the disparity between fluorescence and capacitance changes, as we observed experimentally (Fig. 4 *B*).

A different result was obtained when cells were dialyzed with higher $[Ca^{2+}]_i$ (10 or 100 μM). The fluorescence-capacitance disparity was smaller or nearly disappeared (Fig. 4). One possible explanation concerns endocytosis. If this process was stimulated with higher $[Ca^{2+}]_i$, then an increase in the fluorescence relative to the capacitance would be measured, and this would offset the relative decrease in fluorescence due to changes in membrane tension. However, to produce similar changes in the capacitance would require more exocytosis to occur at higher $[Ca^{2+}]_i$. In fact, electrophysiological (Augustine and Neher, 1992; Heinemann et al., 1994), biochemical (Bittner and Holz, 1992; von Grafenstein and Knight, 1993), and electrochemical (Finnegan and Wightman, 1995; Jankowski et al., 1992) studies in bovine chromaffin cells demonstrated that there is more exocytosis at 10 or 100 μM $[Ca^{2+}]_i$ than at 1.5 μM. Moreover, if during Ca^{2+} dialysis the relationship between FM1-43 staining of plasma membrane and granule membranes is independent of $[Ca^{2+}]_i$ in the pipette, then our data (Fig. 4) suggest that there was substantially more endocytic activity at 10 or 100 μM $[Ca^{2+}]_i$ than at 1.5 μM. Endocytosis in chromaffin cells is dependent on $[Ca^{2+}]_i$. Although the time dependence of endocytic activity in chromaffin cells varies substantially, it has been shown that higher $[Ca^{2+}]_i$ stimulates membrane retrieval (Ales et al., 1999; Artalejo et al., 1995; Burgoyne, 1995; Engisch and Nowycky, 1998; Heinemann et al., 1994; Neher and Zucker, 1993; Smith and Neher, 1997). Consequently, apparent disappearance of the disparity between the fluorescence and capacitance changes observed at high $[Ca^{2+}]_i$ may be due to stimulation of endocytosis.

Although the reasons for the disparity between capacitance and FM1-43 fluorescence changes at different $[Ca^{2+}]_i$ is not known, decreases in membrane tension and FM1-43 fluorescence induced by exocytosis, and stimulation of endocytosis could explain these results. Because the present study did not measure membrane tension during exocytosis, alternative mechanisms for the disparity between capacitance and FM1-43 fluorescence changes may be possible as well. For example, perhaps the cell membrane are ruffled at rest (Solsona et al., 1998) and the osmotic imbalance leads to an unruffling, which then relieves steric hindrance or electrical repulsion of the amphipathic dye molecule, allowing a greater two-dimensional concentration within the membrane. If this mechanism could explain an increase in FM1-43 fluorescence after cell swelling, then under isoosmotic conditions an insertion of granule in the plasma membrane regions where a dye access is restricted would result in smaller FM1-43 fluorescence response relative to the capacitance response. However, it is not clear whether disappearance of the disparity between ΔF and ΔC at high $[Ca^{2+}]_i$ is due to an unrestricted access of FM1-43 to fusing granules and/or a simple stimulation of endocytosis as described above. However, it is clear that increasing membrane tension by osmotically induced swelling (Dai et al.,

1997; Raucher and Sheetz, 2000) increases FM1-43 fluorescence (Figs. 5 and 6). Finally, in addition to the changes in membrane tension during exocytosis the disparity between FM1-43 fluorescence and capacitance changes at least at 100 μM $[Ca^{2+}]_i$, may be due to a lack of FM1-43 staining of small synaptic-like vesicles. These vesicles do not contain neurotransmitters, and they fuse with the plasma membrane at high $[Ca^{2+}]_i$ (Xu et al., 1998). Although the identity of these vesicles in chromaffin cells is not known, they should in principle stain with FM1-43 in a manner similar to synaptic vesicles in hipocampal neurons (Klingauf et al., 1998) or constitutive vesicles in liver cells (Kilic et al., 2001b). Therefore, it is not likely that insertion of a separate vesicle pool into the plasma membrane could explain the disparity between ΔF and ΔC at 100 μM $[Ca^{2+}]_i$.

Assuming that changes in membrane tension affect FM1-43 fluorescence, an additional point merits emphasis. In the study by Smith and Betz (1996) FM1-43 fluorescence continued to increase after the cell stopped swelling, limiting further changes in membrane tension. If the swelling observed by Smith and Betz (1996) was osmotic as discussed above, then at least two processes contributed to the increase in FM1-43 fluorescence, including 1) Ca^{2+}-driven exocytosis and 2) swelling-induced increases in membrane tension. Therefore, Ca^{2+}-driven exocytosis may explain why FM1-43 fluorescence continued to increase after the cell stopped swelling. Thus, some caution is warranted in attributing FM1-43 fluorescence to exocytosis alone because other factors could alter fluorescence as well.

CONCLUSION

In summary, these studies demonstrate that in bovine chromaffin cells, Ca^{2+}-dependent insertion of granule membrane into the plasma membrane does not swell cells. In addition, the fluorescence intensity of a membrane marker FM1-43 is sensitive to changes in the membrane tension. Thus, in addition to binding and staining granule matrix (Angleson et al., 1999; Cochilla et al., 2000), the properties of FM1-43 are influenced by changes in the membrane tension that may occur during exocytosis (Dai et al., 1997; Togo et al., 1999, 2000). Because FM1-43 is widely used to study exocytosis, attention to characterization of the relationship between capacitance and FM1-43 fluorescence measurements under a variety of conditions in the system being studied is emphasized.

We are grateful to William Betz for using research facilities at the Department of Physiology and Biophysics at University of Colorado Health Sciences Center. We also thank Steven Fadul for technical assistance and Joseph Angleson and Gregory Fitz for helpful suggestions. Support for this work was provided by fellowship from Human Frontier Science Program (to G.K.).

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