The Sea Anemone Toxin Bc2 Induces Continuous or Transient Exocytosis, in the Presence of Sustained Levels of High Cytosolic Ca\(^{2+}\) in Chromaffin Cells*

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We have isolated and characterized a new excitatory toxin from the venom of the sea anemone Bunodosoma caissarum, named Bc2. We investigated the mechanism of action of the toxin on Ca\(^{2+}\)-regulated exocytosis in single bovine adrenal chromaffin cells, monitoring simultaneously fura-2 fluorescence measurements and electrophysiological recordings using a carbon fiber micro-electrode. Bc2 induced quantal release of catecholamines in a calcium-dependent manner. This release was associated with a sustained rise in cytosolic Ca\(^{2+}\) and displayed two different patterns of response: a continuous discharge of prolonged duration that changed to a transient burst as the toxin concentration (or incubation time) increased. Continuous secretion was dependent on the activity of native voltage-dependent Ca\(^{2+}\) channels and showed a pattern similar to that of α-latrotoxin; however, its kinetics adjusted better to that of continuous cell depolarization with high K\(^{+}\) concentration. In contrast, transient secretion was independent of Ca\(^{2+}\) entry through native voltage-dependent Ca\(^{2+}\) channels and showed inhibition of late vesicle fusion that was accompanied by “freezing” of F-actin disassembly. These new features make Bc2 a promising new tool for studying the machinery of neurotransmitter release.

Neurotoxins have been fundamental tools to analyze the basic mechanisms involved in the control of exocytotic release of neurotransmitters at peripheral and central synapses. Exocytosis occurs selectively at active zones, a specialized region of the cell membrane, and is initiated by depolarization, Ca\(^{2+}\) influx into the cell by opening of voltage-dependent Ca\(^{2+}\) channels, and increase of the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)])\(_i\), 1 that triggers vesicle fusion to the membrane (1). The relevant role of [Ca\(^{2+}\)], changes in controlling neurotransmitter release has been established in part through the judicious use of selective-cation ionophores and pore-forming toxins to induce exocytosis (2–4). A new class of toxins with interesting properties to study exocytotic mechanisms are those extracted from sea anemones.

Sea anemone cytolysins are pore-forming proteins (16–20 kDa) (5–8) that have a wide variety of cellular effects, including hemolysis (5, 9), cytotoxicity (10, 11), and cardiotoxicity (12). Equinatoxins and stichotoxins, for instance, are well studied sea anemone cytolysins and they form cation-selective channels in lipid bilayers and cells (6, 13–16). The pore they form consists of 3 or 4 monomers inserted into the lipid membrane with an estimated diameter of approximately 1 nm, that allows the leakage of 400–900 Da molecules (6, 7, 9, 17). The effects of these cytolysins can be strongly inhibited by sphingomyelin, a membrane phospholipid that likely serves to anchor the toxin within the plasma membrane (5). They are also able to increase the [Ca\(^{2+}\)], (10, 18) and [Na\(^{+}\)], concentrations (19). The increase of [Ca\(^{2+}\)], is probably a result of Ca\(^{2+}\) entry through the membrane pores formed by cytolysins.

More recently, we have seen that Bc2 (20 kDa), the main cytolysin present in the brazilian sea anemone Bunodosoma caissarum, induces extensive glutamate release from rat cortical synaptosomes (20). This effect was independent of extracellular Ca\(^{2+}\) and Na\(^{+}\) and was inhibited by the lipid sphingomyelin, a classical inhibitor of the sea anemone cytolysins activity. In addition, removal of Bc2 allowed the synaptosomes to restore their responsiveness to a subsequent KCl stimulation, indicating that this toxin does not cause unspecific disruption of cell membranes. Some of these effects resemble those observed with α-latrotoxin (LTX), a large neuroactive protein that produces massive release of a wide variety of neurotransmitters (21). LTX shows pore forming activity and has been used as a powerful tool to study the Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent exocytosis in cell lines (2, 22, 23), neurons (3, 4), and chromaffin cells (24–26).

We report here the first detailed study on the effects of Bc2 on [Ca\(^{2+}\)], and exocytosis. To measure simultaneously these two parameters in a single cell with high time resolution we chose the bovine adrenal chromaffin cell, a model widely used to study basic mechanisms of exocytosis as a function of Ca\(^{2+}\) dynamics at the single-cell level (27). For comparative purposes we have also employed LTX and direct depolarization of the cell with high concentration of K\(^{+}\). Although we found some similarities between Bc2, LTX, and K\(^{+}\), we also depicted drastic differences in their effects on [Ca\(^{2+}\)], changes and the release of catecholamines. This makes Bc2 an interesting neurotoxin to...
explore different aspects of basic neurotransmitter release mechanisms.

**EXPERIMENTAL PROCEDURES**

**Preparation and Culture of Bovine Chromaffin Cells—**Bovine adrenal medulla chromaffin cells were isolated following standard methods (28) with some modifications (29). Cells were suspended in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum, 50 IU/ml penicillin, and 50 µg/ml streptomycin. Cells were kept in an incubator at 37 °C, in a 5% CO₂ and 95% air atmosphere, and used 1–5 days thereafter.

**Electrochemical Detection of Catecholamine—**Microelectrodes for the detection of catecholamine release were prepared by cannulating individual carbon fibers (12 µm radius) into polyethylene tubing (Portex, Kent, United Kingdom), insulated as described (30), and sealed into glass micropipettes with epoxy (CIBA-GEIGY). Microelectrodes (20–60 Gohm) were back-filled with 3 M KC1 solution and connected to a home-made amplifier. A constant voltage of 780 mV versus Ag/AgCl reference was applied to the electrode. The tip of the carbon-fiber electrode was gently pressed against the cell surface. The amperometric current was filtered at 2 kHz and sampled at 200 Hz. An ADInstrument MacLab Chart application were used to record, display, and analyze simultaneously calcium and electrochemical data. Current integrals and curve fittings were also calculated using the program Igor Pro 3.14 and individual spike characteristics were analyzed using Igor Pro macros supplied by Dr. Ricardo Borges (Departamento de Farmacología, Facultad de Medicina, Universidad de La Laguna, La Laguna, Tenerife, Spain).

Coverslips containing the cells were placed on an experimental chamber mounted on the stage of a Nikon Diaphot inverted microscope. The chamber was continuously perfused at room temperature (22 ± 2 °C) with Krebs-HEPES containing (mM): NaCl, 144; KCl, 5.9; MgCl₂, 1.2; CaCl₂, 2 mM; HEPES, 10; glucose, 11; pH 7.3, titrated with NaOH. Control experiments were changed using a multibarrelled concentration clamp device (31).

**Intracellular Ca²⁺ Measurements—**Cells attached to glass coverslips were loaded with the acetoxyethyl ester form of the fluorescent dye fura-2 (fura-2 AM) (2.5 µM for 40 min at 37 °C, in the dark). Then, the cells were washed with Krebs-HEPES solution and kept for 10 min at 37 °C in an incubator before being placed in a perfusion chamber. Solutions were applied to the cell under investigation using the fast superfusion device employed in the amperometric studies. Only one experimental protocol was run on each single coverslip. Single cell fluorescence measurements were performed by exciting the fura-2 loaded cells with alternating 360- and 390-nm filtered light. The apparent [Ca²⁺] was calculated from the ratio of the fluorescence signal (32).

\[ [Ca^{2+}] = K_a(R - R_1)/R - R \]  
\[ \text{(Eq. 1)} \]

where \( K_a \) is an “effective binding constant,” \( R \) is the fluorescence ratio at zero Ca²⁺, and \( R_1 \) is the limiting ratio at high Ca²⁺. These calibration constants were experimentally determined as described previously (33). \( R \) is the observed or experimental ratio.

**Fluorescence and Confocal Microscopy—**Chromaffin cells were plated on poly-D-lysine-coated coverslips contained in a 6-well plastic plate at a density of 5 × 10⁴ per 35-mm dish. Chromaffin cells were stained for F-actin and for dopamine β-hydroxylase (DBH), an antigen present in epinephrine- and norepinephrine-containing cells (34, 35). Cultured cells were washed in phosphate-buffered saline (PBS); incubated for different time periods in PBS solution in the presence or absence of high K⁺ (70 mM) or Bc2 at different time intervals, to evoke the stimulus required. Following these incubations, chromaffin cells were fixed in 3.7% formaldehyde for 10 min. Then, the cells were washed several times with PBS solution and incubated with blocking solution consisting of 1% bovine serum albumin in PBS for 20 min. The coverslips were then washed in PBS solution and incubated with mouse anti-DBH monoclonal antibody (1:200; Chemicon) for 45 min and washed with PBS several times; bound primary antibodies were revealed by incubation for 45 min with BODIPY FL goat anti-mouse IgG conjugate (diluted 1:100).

For the staining of F-actin, cells were permeabilized with Triton X-100 at 0.2%, for 2 min. Thereafter, the cells were washed with PBS for several times and then stained with 0.6 µM rhodamine-phalloidin for 20 min. Finally, coverslips were rinsed with PBS and mounted in glycerol-PBS (1:1, v/v). All the incubations were carried out at room temperature. Preparations were examined with a Bio-Rad MRC-1024 confocal microscope and a Nikon planapo 60X/1.4 oil-immersion objective.

**RESULTS**

**K⁺-induced Amperometric Secretion and Intracellular Ca²⁺ Signals—**Carbon fiber amperometry records were combined with fura-2 fluorescence measurements to allow simultaneous on-line monitoring of catecholamine quantal release and intracellular Ca²⁺ signals. A short depolarization (5 s) of 70 mM K⁺ caused a fast and transient increase of [Ca²⁺], from a resting level of approximately 75 ± 12 nM (n = 20) to 921 ± 82 nM (n = 18). The transient is attributed to activation of voltage-dependent Ca²⁺ channels; thereafter there is a sustained Ca²⁺ influx from the extracellular medium, followed by the release from intracellular Ca²⁺ stores and simultaneous extrusion of [Ca²⁺] (36) (Fig. 1A). The evoked electrochemical signal correlated well with the Ca²⁺ signal and exhibited a single secretory component. Many superimposed amperometric secretory spikes appeared over an elevation of the basal amperometric current that can be identified as quantal release of many single vesicles of oxidizable neurotransmitter that fused with the plasma membrane at high rate and that persisted until the Ca²⁺ signal decayed. This initial massive release followed by a rapid fall in the amperometric current probably reflects fusion and release of readily releasable vesicles, that only require an elevation of [Ca²⁺], for exocytosis (37, 38). Despite the similar initial increases in [Ca²⁺], continuous depolarization with 70 mM K⁺ presented a slower decay in the fura-2 fluorescence, attributed to inactivation of Ca²⁺ channels (39, 40) that blocks the entry of Ca²⁺. The secretory pattern induced by continuous depolarization persisted for several minutes (Fig. 1B) in contrast to that induced by a short depolarizing pulse (5 s). The secretory pattern consisted in a first rapid phase similar to that observed with short depolarizations followed by a second slow-rate secretion, where individual spikes could be distinguish easily; they represent vesicles that need to undergo priming and maturation before they are ready for fusion (38).

\[ \text{[Ca²⁺]}, \text{and Exocytotic Signals Induced by LTc and Bc2} \]

Exposure of chromaffin cells to 5 mM LTc resulted in a gradual increase in [Ca²⁺], that took place several minutes (2.5 ± 0.3 min; n = 7) after exposing the cells to the toxin; this latency can be attributed to the time required for the organization of the toxin to form pore structures. LTc increased the basal levels of [Ca²⁺], to a sustained plateau of approximately 660 ± 220 nM (n = 7) (Fig. 2A). Exposure of the cells for 2 min to LTc was not sufficient to induce a response. In contrast to the stimulation with K⁺, changes in [Ca²⁺], induced by LTc were not transient, but rather steady. Following the increase in [Ca²⁺], continuous discharges of amperometric spike events could be seen at a
constant rate of release during the whole course of the experiment. This secretory behavior is different to that observed with sustained depolarization with 70 mM K\(^{+}\), where two distinct secretory phases can be appreciated.

On the other hand, 5 so fsubnanomolar (0.3 nM) concentrations of the sea anemone toxin Bc2 were enough to cause a rapid (latency: 21.8 ± 2.7 s; \( n = 11 \)) increase in [Ca\(^{2+}\)]\(_{c}\), reaching a plateau at approximately 0.4 μM; the amperometric current persisted for at least 3 min.

FIG. 1. [Ca\(^{2+}\)]\(_{c}\) transient and amperometric events triggered by depolarization. A, application of high [K\(^{+}\)] solution (70 mM) for 5 s gave a transient increase in [Ca\(^{2+}\)]\(_{c}\), that was accompanied by an amperometric current burst that reflects exocytosis of catecholamine-containing vesicles. B, continuous perfusion with high [K\(^{+}\)] solution gave an initial [Ca\(^{2+}\)]\(_{c}\), transient that gradually declined to a plateau at approximately 0.4 μM; the amperometric current persisted for at least 3 min.

transient over pathing of amperometric spikes suggests a fast fusion and neurotransmitter release of a finite number of chromaffin vesicles. Interestingly, at high concentrations of Bc2 we obtained a dissociation of the exocytotic and [Ca\(^{2+}\)]\(_{c}\) signals; so, despite having increased levels of [Ca\(^{2+}\)]\(_{c}\), no measurable exocytosis occurred.

The Membrane Fusion Stages of Exocytosis Were Not Affected by Bc2—Detailed analysis of individual amperometric spikes is a good approach to study if the toxin could affect the last step of exocytosis, consisting in the fusion of the vesicle membrane with the plasma membrane, formation of an exocytotic fusion pore and the release of catecholamines from the vesicle matrix (41). Fig. 3 plots the frequency histograms of quantal charge (time integral of individual amperometric spikes), peak amplitude, and half-width (duration of the amperometric signal at 50% of its peak amplitude) (Fig. 3A), in cells stimulated with 70 mM K\(^{+}\) (Fig. 3B) or 0.3 nM Bc2 (Fig. 3C). The charge distribution for K\(^{+}\) and Bc2-induced exocytosis was rather similar; the mean charge value for K\(^{+}\) was 2.51 ± 0.13 pC (\( n = 448 \)) and for Bc2, 2.39 ± 0.13 pC (\( n = 459 \)). Half-width histograms fitted to
responses of cells stimulated with 70 mM K⁺, the parameters measured. Individual spikes were analyzed from the responses of cells stimulated with 70 mM K⁺ (B) and 0.3 nM Bc2 (C). The quantal charge was estimated from the time integral of individual amperometric events and the data binned at 0.2 pC intervals. The peak amplitude data were binned at 5 ms and the half-width was measured as the difference between rising and falling half-height times for each spike and histograms were built using 10 pA bin size.

Lorentzian distributions centered at \( t_{1/2} \) of 37.2 ± 0.7 ms for 70 mM K⁺ and 35.2 ± 0.5 ms for Bc2-treated cells; this difference was not statistically significant. The amplitude distribution was similar in K⁺ (42.8 ± 2.4 pA) and Bc2 (38.3 ± 2.1 pA)-treated cells. Altogether, these results suggest that Bc2 does not alter the quantal size and pattern of vesicle release. Therefore, it is unlikely that the toxin is affecting the last step of the exocytotic process.

Exocytotic Kinetic Components Induced by Bc2—In order to study the kinetic components of the secretory response when cells were exposed to different concentrations of Bc2, we measured the accumulated charge of the amperometric spikes. Fig. 4A depicts the average cumulative integral from experiments performed in 70 mM K⁺ (n = 6) and cells incubated with 0.3 nM (n = 7) and 0.6 nM (n = 9) Bc2. Continuous secretion induced by the low concentration of the toxin showed a marked increase of the amplitude of the exocytotic response (1504 ± 313 pC), with respect to cells exposed to prolonged depolarization (303 ± 82.9 pC). This difference is probably due to the difference in [Ca²⁺], since as shown in Figs. 1B and 2B, the total Ca²⁺ influx was lower in 70 mM K⁺ than in Bc2-treated cells. However, despite the different levels of [Ca²⁺], reached with the different stimuli, the amperometric transient response obtained with the high concentration of Bc2 (454 ± 147 pC) was 3.3-fold lower than that obtained with the low concentration of Bc2 and only 1.5-fold higher than that induced by K⁺. Fig. 4B shows the normalized data. Superfusion of the cells with 0.6 nM Bc2 saturated the cumulative integral after 70 s. Interestingly, the continuous secretory response to low Bc2 and 70 mM K⁺ were well fitted to a double exponential; however, the transient exocytotic Bc2 response could be adequately fitted to a single exponential (see detail in the inset of Fig. 4B). The time constant of the exponential of the transient response was slightly faster (\( \tau = 10.5 \) s) than the time constant of the faster component of the K⁺ response (\( \tau = 15.1 \) s) and much faster than the time constant obtained with low toxin concentration (\( \tau = 87.1 \) s).

These results suggest that low Bc2 concentrations (0.3 nM) induce massive exocytosis, probably due to the massive entry of Ca²⁺ into the cell. The size and rate of release for the fast and slow component of the secretion is probably dependent on [Ca²⁺]. The biexponential secretion behavior induced by Bc2 and high K⁺ indicate that these two stimuli seem to induce secretion through a similar mechanism of action. However, high Bc2 concentrations (0.6 nM) act in a very different manner; they induce an initial massive transient secretory release that fit to a single exponential.

When we express the integral current in terms of fused vesicles (knowing that the averaged spike integral has a value of 2.51 ± 0.13 pC/vesicle under our recording conditions and the estimated secretion with a 12-μm diameter carbon-fiber electrode is about 20% of the total cell surface area), we obtain about 904 vesicles/cell which are rapidly released when treated with the high concentration (0.6 nM) of Bc2; the estimation of vesicles released in the early detected component (\( \tau = 15.1 \) s) by 70 mM K⁺ is 228, a number in agreement with the estimated vesicle number obtained through capacitance measurements.
The Transient Secretion Induced by Bc2 Is Accompanied by “Freezing” of F-actin Disassembly—In order to correlate the cortical actin network dynamics and the exocytotic responses induced by Bc2 we used rhodamine-phalloidin and anti-DHB-IgG staining. In resting cells, staining of F-actin with rhodamine-phalloidin revealed a weak and diffuse cytoplasmic staining and a continuous cortical ring (Fig. 5A). Stimulation of cells with 70 mM K\(^+\) for 2 min induced the disruption of the cortical fluorescent ring (Fig. 5B). Because phalloidin is a probe for F-actin, the disappearance of rhodamine fluorescence indicates disassembly of actin filaments at subplasmalemmal areas (35), allowing translocation of vesicles to the plasmalemma in preparation for exocytosis (45). This effect is confirmed using the staining with anti-DHB-IgG to detect the presence of chromaffin vesicle membranes on the cell surface, in cells treated for 2 min with 70 mM K\(^+\) (Fig. 5B’). Treatment of cells for 5 s with Bc2, followed by 2 min in Krebs-HEPES, to induce the “transient” secretory pattern did not cause an important disruption of the subplasmalemmal F-actin network (Fig. 5D) and only a few spots of DBH were observed at the plasma membrane level (Fig. 5D’). This was in some way unexpected because elevated [Ca\(^{2+}\)] has been suggested to modulate the supply of release-competent vesicles in chromaffin cells (46) by inducing breakdown of the barrier of cortical actin network (45). However, despite the high and sustained level of measured [Ca\(^{2+}\)], in the presence of the toxin, the cortical F-actin disruption and hence the delivery of vesicles to exocytotic sites is inhibited. On the other hand, these results could explain why the measured secretion by amperometry was suddenly arrested after 70 s of intensive release. The cortical F-actin freezing would act as a barrier preventing the access or morphological docking of new chromaffin vesicles from the depot pool to the plasmalemma. This consideration implies that only vesicles that would be relatively close to the plasma membrane could be released by the toxin at high concentrations.

Ca\(^{2+}\) Dependence of the Action of Bc2—In order to study the dependence of external Ca\(^{2+}\) for the toxin effects on the quantal release of catecholamines and the levels [Ca\(^{2+}\)]\(_{\text{c}}\), fura-2-loaded cells were bathed in a Ca\(^{-}\)-free solution (i.e. no added Ca\(^{2+}\) + 0.5 mM EGTA) (Fig. 6). Applications of LTx (5 nM) during 5 min (more than enough to induce a response) produced no quantal release nor increase in [Ca\(^{2+}\)]\(_{\text{c}}\); however, the subsequent addition of Krebs-HEPES containing 2 mM Ca\(^{2+}\) produced a response of abrupt onset in [Ca\(^{2+}\)]\(_{\text{c}}\), and secretion, that persisted while Ca\(^{2+}\) was present in the extracellular medium (Fig. 6A), confirming that LTx-induced release is triggered by the accompanying rise in cytosolic Ca\(^{2+}\) (26). Similarly, when cells were stimulated with Bc2 (0.3 nM) during 30 s in the absence of Ca\(^{2+}\) no response was observed (Fig. 6B). However, addition of Bc2 in the presence of 2 mM [Ca\(^{2+}\)]\(_{\text{c}}\) induced an abrupt increase of Ca\(^{2+}\) and quantal release proving an association of toxin-induced release with a rise of [Ca\(^{2+}\)]\(_{\text{c}}\). The lack of effect of LTx and Bc2 on [Ca\(^{2+}\)]\(_{\text{c}}\) in the absence of [Ca\(^{2+}\)]\(_{\text{c}}\), and presence of EGTA suggests that the toxin-induced [Ca\(^{2+}\)]\(_{\text{c}}\) increase are due solely to Ca\(^{2+}\) influx and not to release of calcium from intracellular stores.

Ca\(^{2+}\) Entry Through Voltage-dependent Ca\(^{2+}\) Channels and Bc2-induced Exocytosis—Exocytosis could in principle be triggered by Ca\(^{2+}\) entering through toxin-induced channels, or native voltage-gated Ca\(^{2+}\) channels. In synaptosomes it has been reported that Ca\(^{2+}\) entry induced by LTx is mediated by both of these pathways (47). In chromaffin cells, Ca\(^{2+}\) entry is through LTx-induced channels (25, 26). To determine the Ca\(^{2+}\) entry pathway for Bc2, we blocked endogenous voltage-dependent Ca\(^{2+}\) channels with a mixture of specific Ca\(^{2+}\) channel blockers (nifedipine, L-type channels blocker, and the toxins \(\omega\)-conotoxin MVIIIC, \(\omega\)-conotoxin GVIA, and \(\omega\)-agatoxin IVA,
FIG. 6. Effect of extracellular Ca\(^{2+}\) on cytosolic Ca\(^{2+}\) and secretion evoked by LTx and Bc2. A, the action of LTx (5 nM) on cytosolic Ca\(^{2+}\) and catecholamine release was totally abolished in the absence of external Ca\(^{2+}\). Reintroduction of Ca\(^{2+}\) induced a brisk response of both parameters measured. For control purposes, initial depolarization with 70 mM K\(^+\) was measured. B, the addition of Be2 (0.3 nM) for 30 s in the absence of external Ca\(^{2+}\) produced no Ca\(^{2+}\) signal and no quantal release demonstrating the inability of the toxin to induce secretion independently of Ca\(^{2+}\) entry. However, the subsequent addition of Be2 in the presence of 2 mM extracellular Ca\(^{2+}\) solution produced an abrupt onset of Ca\(^{2+}\) signal and secretion. In some experiments a slow and mild elevation of the amperometric trace could be observed during the application of Be2 that we have identified as an oxidative response to the toxin or to salt residues during its purification.

FIG. 7. Relationship between Ca\(^{2+}\) entry induced by Be2 and the functional state of native voltage-dependent Ca\(^{2+}\) channels. A, continuous response: a 5-s pulse of 0.3 nM Be2 failed to produce a response when voltage-dependent Ca\(^{2+}\) channels were blocked by a mixture of specific Ca\(^{2+}\) channel blockers (nifedipine, 3 μM; ω-agatoxin IVA, 1 μM; ω-agatoxin GVIA, 1 μM). Washout of Ca\(^{2+}\) channel blockers allowed the recovery of the response by the toxin. B, transient response: however, the [Ca\(^{2+}\)] increase and the transient amperometric signals induced by 0.6 nM Be2 were not affected when the voltage-dependent Ca\(^{2+}\) channels were blocked. In all experiments blockade of voltage-dependent Ca\(^{2+}\) channels was assessed by observing total blockade of the Ca\(^{2+}\) and amperometric signals induced by 70 mM K\(^{+}\).

blockers of N and P/Q channels). Under these conditions, in which the majority of Ca\(^{2+}\) currents are blocked, the addition of 0.3 nM Be2 did not evoke [Ca\(^{2+}\)], increments nor secretory responses (Fig. 7A); washout of the Ca\(^{2+}\) channel blockers resulted in the appearance of both the increase in [Ca\(^{2+}\)], and the secretory response upon addition of Be2, likely due to the reversible effects of nifedipine. That Ca\(^{2+}\) channels were fully blocked during the toxins + nifedipine application was clear, since the application of a depolarizing pulse with 70 mM K\(^{+}\) abolished completely Ca\(^{2+}\) entry and the excytotic signal. However, Be2 at high concentration (0.6 nM) still caused an increase in [Ca\(^{2+}\)], and a transient release of catecholamine (Fig. 7B). These results suggest that the continuous response (low concentration or short exposition time) induced by Be2 requires the activity of native voltage-dependent Ca\(^{2+}\) channels, while the transient response (higher concentrations or longer exposure times) to Be2 is probably secondary to Ca\(^{2+}\) entry through Be2-formed channels that are permeable to Ca\(^{2+}\).

DISCUSSION

There is general consensus that a rise in the [Ca\(^{2+}\)] near the plasma membrane due to Ca\(^{2+}\) influx through Ca\(^{2+}\) channels is the main mechanism to induce exocytosis (24, 48, 49). However, it is also known that selective-cation ionophores and pore-forming toxins can induce exocytosis (2–4). In the present study we have investigated the effects of a novel toxin derived from the brazilian sea anemone B. caissarum Be2 related on the [Ca\(^{2+}\)], and exocytosis of catecholamines from adrenal chromaffin cells.

In chromaffin cells, LTx and Be2 share some common actions; both toxins were able to elicit prolonged and sustained inward calcium fluxes, in contrast to K\(^{+}\) that induced a transient rise in [Ca\(^{2+}\)], (even during a prolonged depolarization). In contrast, all stimuli evoked a prolonged secretory pattern. Neurortransmitter release requires extracellular Ca\(^{2+}\) and is associated with a local rise in [Ca\(^{2+}\)], in the micromolar range. However, while Ca\(^{2+}\) entry induced by LTx to enhance quantal release is based on Ca\(^{2+}\) entry through toxin-formed channels (26), Be2 (at least at the low concentration) mediates Ca\(^{2+}\) entry via voltage-dependent Ca\(^{2+}\) channels (Fig. 7A). It is noteworthy that at higher Be2 concentrations (0.6 nM), release begins vigorously but is transient (Fig. 2C). A similar pattern of transient release has been reported with LTx at high concentrations in rat chromaffin cells (25). In contrast to LTx, the increase in [Ca\(^{2+}\)], caused by Be2 is not gradual but fast (latency: 17.8 ± 2.7 s). The possibility that the fast increase in [Ca\(^{2+}\)], is due to pore formation cannot be discounted (at high doses of Be2), but seems unlikely when considering that 5–20 min are required for organization of the toxin to form the pore structures (50). Most likely the interaction of Be2 with sphingomyelin changes the phase-transition properties of phospholipids (51) of the chromaffin cell plasma membrane, resulting in

[Image 88x441 to 258x729]

[Image 311x436 to 551x729]
a rapid influx of Ca\textsuperscript{2+} from the extracellular milieu. In fact, an excess of sphingomyelin added during the application of Bc2 resulted in the absence of both Ca\textsuperscript{2+} and exocytosis signals (data not shown).

Depolarization-induced secretion in neurosecretory cells often displays two kinetic components: a fast phase component, highly synchronous with the opening of Ca\textsuperscript{2+} channels, followed by a slower and more sustained component (52–55). The exact mechanisms underlying this biphasic secretory behavior remains a matter of debate. The fast and slow phases could represent exocytosis of a immediately releasable pool of docked vesicles and mobilization of vesicles from a reserve pool to the releasable pool, respectively. Also, the specific localization of vesicles with respect to Ca\textsuperscript{2+} channels has been suggested on the basis of a biphasic secretory behavior (44), indicating that Ca\textsuperscript{2+} gradients have a profound influence on the kinetics of depolarization-induced neurosecretion. This supports our results. We can observe two phases of exocytosis in the experiments with prolonged depolarization or Bc2 toxin at low concentration; however, LTx failed to reveal a biphasic secretory response. This toxin acts by forming ion channels at random in the plasma membrane, thereby allowing a massive arbitrary entry of Ca\textsuperscript{2+} to the cell surface. In contrast, exocytosis occurs selectively at active zones where the local transient increase in [Ca\textsuperscript{2+}]\textsubscript{i} generated by activation of calcium-ion channels triggers primarily the fusion of readily releasable vesicles. The intracellular Ca\textsuperscript{2+} gradient distribution and the compartmentalization induced by LTx must be different at the functional unit Ca\textsuperscript{2+} channel/mitochondria/endoplasmic reticulum, that has been reported to modulate exocytosis by controlling the availability of Ca\textsuperscript{2+} for secretion (56). This would lead to the massive and monophasic catecholamine secretion that we observed (Fig. 2A).

Adrenal chromaffin cells offer the advantage that exocytosis can be explored with a high temporal resolution at the single cell level, using either amperometry or membrane capacitance measurements (27). When [Ca\textsuperscript{2+}]\textsubscript{i} is raised by Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+} channels activated by a long depolarization, an early detected component (τ = 15.1 s) is observed during which 228 vesicles are rapidly released. Subsequently, exocytosis continues at a slower rate (sustained component). As mentioned above, the interpretation of this sequence of events is that the fast initial release represents the fusion of release-competent vesicles that are more or less close to the Ca\textsuperscript{2+} channels in the active zones of the plasma membrane and the sustained component represents the recruitment of new vesicles that will become competent for release, followed by exocytosis (37, 38, 57). Using high resolution capacitance measurements techniques and clostridial neurotoxins, the sizes of the pool of vesicles and the molecular machinery that underlie the fast kinetic component (previously termed exocytotic burst) have been well studied. However, due to the mixture of endocytosis (that contaminate the capacitance measurement) and the lack of pharmacological tools that affect the early stages of the exocytotic process, the sustained component is interpreted as a mixture of vesicles in different maturation stages that form the so-called depot pool (4000 vesicles). Amperometry in intact cells provides a good alternative to study the sustained component of quantal release and the mechanism of action of new toxins that could help to understand better the early exocytotic process. We found that Bc2 (0.6 nM) is able to release rapidly about 900 vesicles in a time of approximately 70 s; this effect was irreversible but it is not related to cell damage since lactate dehydrogenase measurements performed under the same experimental conditions as described here remained unaltered in comparison to control (data not shown) and also previously observed (20). We have fitted the exocytotic rate curve to a single exponential with a time constant of 10.5 s, faster than the time constant calculated for the fast exocytotic component induced by prolonged depolarization with 70 mM K\textsuperscript{+} (τ = 15.1) where 228 vesicles are rapidly released. There is a good correlation between the size of the fast component detected in the present work and the reported value of about 200 vesicles governing the fusion step studied by amperometry in previous work (58) and capacitance measurements (42–44). However, it is surprising that the toxin can release 904 vesicles at such a high rate. One exciting possibility is that Bc2 can release rapidly all docked granules. It has been postulated that only a fraction of the docked vesicles appear readily releasable (59, 60). Morphometric analysis on melanotrophs and chromaffin cells concluded that a pool of docked granules can be identified, and this pool is larger than the functionally defined pool of readily releasable vesicles. Calculations to measure the number of vesicles touching the plasmalemma have been performed in many morphometric studies (59–62). Despite the lack of agreement, the calculated number of docked vesicles is estimated to be between 450 (61) and 1010 (60) vesicles. This number is in line with the 904 vesicles released at the high concentration of Bc2.

Elevated cytosolic Ca\textsuperscript{2+} modulates the supply of release-competent vesicles in chromaffin cells. On the other hand, it has been suggested that Ca\textsuperscript{2+} and protein kinase C enhance the supply of release-ready vesicles to the plasma membrane and by inducing breakdown of the barrier of cortical actin network (45). Recently, a model has been presented in which the GTP-binding protein Rho regulates secretion and cortical F-actin in a manner dependent on/or synergistic with Ca\textsuperscript{2+} in mast cells (63). We have observed that Bc2 is able to produce an intriguing dissociation between the Ca\textsuperscript{2+} signal and the F-actin disassembly, supporting the recent model. This result matches with the brisk arrest of exocytosis, preventing vesicles retained by the cytoskeleton replenishing the plasmalemma of new vesicles to secrete. This implicates a role of Bc2 controlling early stages of the secretory process.

Bc2 seems to require the membrane phospholipid sphingomyelin and/or cholesterol (5, 9), to exert its action. Sphingomyelin hydrolysis produces diacylglycerol and ceramide. The generated ceramide can function as a second messenger, modulating the activities of different kinases as well as phosphatases mediating its biological responses (64–66). For instance, in rat pinealocytes, ceramide inhibits L-type Ca\textsuperscript{2+} channel (67). On the other hand, in adrenal chromaffin cells, an annexin termed calpain I (68), is a Ca\textsuperscript{2+}-dependent protein that is unable to restore the secretory activity in cells where protein kinase C is inhibited by sphingosine, a mediator of the sphingomyelin pathway. Also, proteins involved in the regulation of synaptic transmission (Munc 13) have been defined as presynaptic targets of diacylglycerol, acting in parallel with protein kinase C to regulate secretion (69). Finally, ceramide and inositol sphingolipid synthesis have been involved in the trafficking of secretory vesicles in yeast mutants that appear to bypass the known synaptobrevin/VAMP (v-SNARE) requirement in secretion (70); this protein is thought to be essential for vesicle docking and exocytosis. These considerations evidence Bc2, a toxin secreted by the brazilian sea anemone B. caissarum, as a powerful tool to study the mechanism of neuroexocytosis.

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