The organization of the secretory machinery in chromaffin cells as a major factor in modeling exocytosis

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The organization of cytoplasm in excitable cells was a largely ignored factor when mathematical models were developed to understand intracellular calcium and secretory behavior. Here we employed a combination of fluorescent evanescent and transmitted light microscopy to explore the F-actin cytoskeletal organization in the vicinity of secretory sites in cultured bovine chromaffin cells. This technique and confocal fluorescent microscopy show chromaffin granules associated with the borders of cortical cytoskeletal cages forming an intricate tridimensional network. Furthermore, the overexpression of SNAP-25 in these cells also reveals the association of secretory machinery clusters with the borders of these cytoskeletal cages. The importance of these F-actin cage borders is stressed when granules appear to interact and remain associated during exocytosis visualized in acridin orange loaded vesicles. These results will prompt us to propose a model of cytoskeletal cages, where the secretory machinery is associated with its borders. Both the calcium level and the secretory response are enhanced in this geometrical arrangement when compared with a random distribution of the secretory machinery that is not restricted to the borders of the cage. [DOI: 10.2976/1.3338707]

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Chromaffin cells have been widely employed as an excellent neuroendocrine model to study the release of neurotransmitters by exocytosis. During this process dense granules containing biogenic amines were first transported and translocated to the plasma membrane, where finally both membranes fused to release the stored content in a calcium-dependent process (Bader *et al.*, 2002). Various types of proteins seem to play a central role in this process, cytoskeletal elements such as F-actin and myosins are clearly implicated in the initial steps of vesicle transport (Trifaro *et al.*, 2008), whereas voltage-dependent calcium

channels (Garcia *et al.*, 1997) and a fusion machinery apparatus (Bennett *et al.*, 1992; Rettig and Neher, 2002; Burgoyne and Morgan, 1998) will be responsible for the initiation of the intracellular signal triggering the process and the regulated membrane fusion event, respectively. Among the different elements that could be involved in membrane fusion, it is accepted that the ubiquitous soluble *N*-ethylmaleimide sensitive factor attachment protein (SNAP) receptor (SNARE) proteins may not only be essential to produce the specific docking or tethering of the vesicles at secretory sites (Toonen *et al.*, 2006) but probably constitute the minimal fusion ma-

chinery on its own (Weber *et al.*, 1998). Even though most of these elements are considered in mathematical models that try to explain exocytosis (Klingauf and Neher, 1997; Gil *et al.*, 2000; Segura *et al.*, 2000), it is also common for their disposition in the proximity of secretory sites to be randomized, ignoring any specific organization that helps to develop their physiological function. Therefore, in the present work we introduce a new combination of microscopy techniques that allows the simultaneous visualization of vesicles and SNAREs by total internal reflection of fluorescence (TIRF, (Oheim *et al.*, 1998) and the study of the F-actin cytoskeletal cortical network using transmitted light (Giner *et al.*, 2005; Giner *et al.*, 2007) in order to propose a specific geometrical arrangement for the elements that constitute the secretory machinery. The data presented here provide a new and original vision of the organization of the exocytotic elements in the border of cortical cytoskeletal structures of neuroendocrine cells. In addition, by using a stochastic mathematical model (Gil *et al.*, 2000), we also give some clues to the advantages offered by this specific arrangement to sustain fast secretory responses.

RESULTS AND DISCUSSION

A combination of fluorescence reflection and transmitted light images can be used to visualize the position of vesicles with respect to the cortical barrier

In recent works we have demonstrated that the dynamic changes suffered by the F-actin-myosin II cortical cytoskeleton could be studied using its transmitted light interference in confocal microscopes (Giner *et al.*, 2005; Giner *et al.*, 2007). Therefore, we conceived that the interaction of vesicles with this structure within the cellular cortex could be analyzed with increased detail by combining the visualization of fluorescent labeled granules provided by total internal reflection fluorescence microscopy (TIRFM) with transmitted light observation of F-actin cytoskeleton in live cultured chromaffin cells. This is easily achieved in the configuration of a TIRFM system shown in Fig. 1(A), where the vesicles are excited by an evanescent field generated by changing the laser incidence angle using a high numerical aperture objective, whereas transmitted light images were obtained by the selection of the green spectra of light from a tungsten lamp using appropriate filtering. In this microscopy configuration, the TIRFM images of vesicles obtained using an acidophilic dye emitting red fluorescence such as lysotracker red (Becherer *et al.*, 2003; Giner *et al.*, 2007) (Fig. 1(B)) could be separated from the green images of cytoskeletal structures provided by transmitted light (Fig. 1) using an image splitter before the images are acquired simultaneously using a high sensitivity ECCD camera. Finally, images were later combined using appropriate software (Fig. 1(A)), which resulted in high resolution dynamic images depicting the interaction of vesicles with the cortical network

Figure 1. TIRF/Transmission light microscopy allows the study of the cytoskeletal network in the vicinity of releasing sites. Left panel. Scheme of the microscopy configuration allowing simultaneous acquisition of TIRFM images (in this case using a through-the-lens system with a 543 nm laser beam and a high numerical aperture objective), and transmitted light images using the green spectra of light passing the tungsten halogen light for a BP546 filter. Red fluorescence of lysotracker red labeled vesicles was separated from green transmitted light images of the cytoskeletal structures using an image splitter. (A) Depicts an example showing both, vesicles, and structures at high magnification. (B) 3D reconstruction of the images obtained from focal planes separated by 50 nm and covering 1 μm using a motorized microscopy stage. (C) XY, XZ, and YZ planes obtained from a 3D reconstruction of confocal fluorescence images (20 images separated by 150 nm) depicting lysotracker green labeled vesicles and F-actin cortical structures decorated with rhodamine-phalloidin (red). Bars represent 1 μm .

of F-actin. From these images, we observed that vesicles appeared interacting with the borders of the cytoskeletal cages that were observed with transmitted light or locating just over these structures. A better appreciation of this disposition is observed in three-dimensional reconstructions of the cytoskeletal network obtained from images separated by 50 nm in the z plane and covering a 1 μm volume of cortical area [Fig. 1(B)]. Since TIRFM and transmitted light have different depths of field it is difficult to assess the real disposition of vesicles and structures in the z plane; therefore, we tested this spatial interaction using 3D-reconstruction studies based on confocal fluorescence microscopy. As can be observed in the example depicted in Fig. 1(C), F-actin cortical structures

labeled with rhodamine coupled to phalloidin are in contact with lysotracker green labeled vesicles as determined in *xy*, *xz*, and *yz* sections of these 3D reconstructions.

Expressed SNAREs associated with the borders of cortical cytoskeletal cages

In order to test the relevance of cytoskeletal cortical cages to locating exocytosis sites we conceived experiments to investigate the disposition of SNAREs using the microscopy configuration described above. DsRed-SNAP-25 was expressed in chromaffin cells as described recently (López *et al.*, 2009) and its fluorescence in the evanescent field imaged with simultaneous acquisition of transmitted light images. As can be observed in the two examples displayed in Figs. 2(A) and 2(B), the membrane microdomains formed by the expressed protein appear to locate adjacent to the empty spaces characteristic of cytoskeletal cages. These examples were representative of the location of 84 DsRed-SNAP-25 patches observed with cells from two different cell cultures and in no case were the SNARE microdomains observed in cytoskeleton empty spaces without contacting cage borders. This was further demonstrated using green fluorescent protein (GFP)-SNAP-25 expression and simultaneous labeling of F-actin cortical structures with rhodamine-phalloidin in chromaffin cells as visualized by confocal microscopy [Fig. 2(C)]. In this case, we have estimated that close to 90% of the 68 analyzed patches colocalized with F-actin, and most of them (70%) located in the borders. This is highly significant since randomly distributed patches of the same average size (around 500 nm in diameter) presented a 60% probability of colocalization with F-actin structures [see example of Figs. 2(D) and 2(E)]. Furthermore, randomly generated patches present a threefold higher probability of noncolocalization when compared with real patches [Fig. 2(E)].

Thus, it appears that expressed SNAP-25 microdomains appear to locate in contact with the F-actin structures and mostly facing the open spaces formed by cytoskeletal cortical structures. It has been established that SNAREs form membrane-associated clusters in chromaffin (Rickman *et al.*, 2004; Lopez *et al.*, 2007) and other secretory systems such as PC12 (Lang *et al.*, 2001) and MIN6 beta cells (Ohara-Imaizumi *et al.*, 2004); therefore, the observed microdomains of expressed SNAP-25 agree well with previous data. It is also not surprising that SNARE microdomains interact with cytoskeletal elements since it is a relatively general feature of plasma membrane proteins to interact with F-actin and other elements that structure the cellular architecture. Members of the SNARE syntaxin family have been proven to interact with α -fodrin, a major component of the chromaffin cell cortical cytoskeleton (Nakano *et al.*, 2001).

Fusions occur in the border of cytoskeletal cages

The data presented above demonstrate the association of SNAREs, which constitute the secretory machinery with the

Figure 2. SNARE proteins locate in the borders of cytoskeletal cortical cages. DsRed or GFP-SNAP-25 constructs were expressed in chromaffin cells as described in the Materials and methods section and the fluorescence investigated using the TIRF-transmitted light microscopy set-up described in the previous figure. (A and B) The images representative of experiments performed in cells from two different cultures show cytoskeletal structures in green and DsRed-SNAP-25 clusters in red. SNARE microdomains were found adjacent to the spaces devoid of cytoskeleton. (C) Expression of GFP-SNAP-25 patches (green) in cells, where F-actin structures are labeled with 5 μ M rhodamine-phalloidin (red) for 30 min. (D) Example of randomly generated patches to study the probability of colocalization with F-actin structures. (E) Probability of colocalization and noncolocalization of GFP-SNAP-25 patches as well as randomly generated patches with phalloidin labeled F-actin using a 50% intensity threshold in both channels. Bars represent 1 μ m.

borders of cytoskeletal cages. Therefore, it is suggested that even at the time of experiencing exocytosis, granules interact with cytoskeletal elements. In order to prove such interaction we studied the precise localization of exocytosis in relation to cytoskeletal structures by *in vivo* observation of the exocytotic events using TIRFM observation of acridin orange (AO) loaded granules (López *et al.*, 2009) and the cytoskeletal structures using transmitted light. As can be seen in the example of Fig. 3(A), brilliant AO-labeled vesicles appeared interacting with the borders of cytoskeletal cages, which avoided the empty spaces left in the interior of such struc-

Figure 3. Fusions occur in contact with the cage borders. Cultured cells were incubated with AO and exocytosis stimulated by depolarization with a high KCl 59 mM solution. Panel A shows a sequence of images separated by 6 s depicting the continuous interaction of three bright vesicles (indicated by arrows) with cytoskeletal cages, after depolarization two vesicles fused readily and the third one later on. At the time of fusion the three vesicles were contacting with the cytoskeletal structures. B. An example of similar experiments performed in cells incubated with 5 μ M fluorescein-phalloidin (green). Granules are visualized in the red spectra of AO due to the acidic interior of the vesicles, and during fusion a green flash resulted from vesicle neutralization (fused vesicle indicated by the arrow). Bars represent 10 μ m.

tures. The interaction continues till the moment of vesicle fusion occurs, when the KCl depolarizing solution is superfusing the cells; so far, in dozens of experiments we have not observed any vesicle fusions in areas devoid of cytoskeletal structure (284 fusions from 23 cells). Again, we have confirmed this observation using fluorescent labeling of F-actin with phalloidin coupled to fluorescein in our TIRFM experiments. As can be observed in the example depicted in Fig. 3(B) acidic granules are visualized in red with acridine orange labeling, and during fusion acridine orange neutralization results in a green flash, confirming the localization of the fused vesicle. Again in this example and others observed in 11 cells from two different cultures it was evident that vesicle fusions occur in close contact with fluorescein-phalloidin labeled structures. The localization of vesicle fusion with cage borders might be the consequence of the localization of SNAREs in these spots since it has been demonstrated that vesicle fusion takes place in the precise position of SNARE clusters (Lang *et al.*, 2001; Lopez *et al.*, 2007).

Modeling border influence on exocytosis

What will the influence on secretion of a border arrangement as described in our experiments be? In order to test these effects, we have developed a model based on Monte Carlo techniques (Gil *et al.*, 2000), which simulates chromaffin cell secretory response with this type of geometrical arrangement.

Figure 4. Schematic representation of the arrangements of secretory vesicles and clusters of calcium channels considered in the simulations. Configuration 1 corresponds to one in which the secretory machinery is located in the limits of a cortical F-actin cage. In configuration 2, the secretory vesicles large pixelated circles are randomly distributed inside the cytoskeletal cage. The clusters of calcium channels (groups of small circles) are colocalized, in both cases, with the secretory machinery.

We simulated calcium currents and computed secretory events in response to a short depolarizing pulse for two types of random configurations of secretory vesicles: configuration 1 corresponds to one in which the vesicles are located randomly within the limits of a prototype cortical F-actin cage while in geometrical configuration 2 the vesicles are randomly distributed on the surface of the submembrane domain of the cage. The cage is modeled as a cylinder of radius 0.3 μ m, a value which is estimated from experiments (Giner *et al.*, 2007). We also assume that the clusters of calcium channels are colocalized with the secretory machinery in cultured chromaffin cells, as suggested in previous experimental results (Lopez *et al.*, 2007) and that calcium diffusion is restricted to the cage. A schematic representation of both configurations is shown in Fig. 4. Using our simulation scheme we quantify average calcium levels and secretory events for several randomly generated sets of configuration types 1 and 2 (ten sets for each configuration), in response to a depolarizing pulse up to 20 mV (from -80 mV), which starts at $t = 2$ ms and which lasts 20 ms. The results shown correspond to the average values of the corresponding 10 random sets. We also simulate the effect of possible variations in the positioning of the calcium sensor of the vesicles with respect to the channels (Shahrezaei and Delaney, 2004) by averaging 16 vesicle configurations around each cluster of calcium channels. The computed average calcium concentrations for the submembrane domain for both configurations are very similar (figure not shown) and they correspond to the typical calcium profiles obtained using shell models. However, the possible differences in secretion are due not to the average calcium but to the local calcium. A measure of local differences in calcium concentrations is given in Fig. 5, which shows notched box plots summarizing the statistical description of the average temporal values of the ratio between the calcium concentration at the compartments having a vesicle, and the average calcium concentration at the submembrane domain. The

Figure 5. Comparison of the levels of local calcium for configurations 1 and 2. The plot shows notched box plots summarizing the statistical description of the average temporal values of the ratio between the calcium concentration at the compartments of the discretized domain having a vesicle and the average calcium concentration at the submembrane domain ($[Ca^{2+}]_{\text{vesicles}}/[Ca^{2+}]_{\text{average}}$) for configurations 1 and 2. The boxes have lines at the lower quartile, median, and upper quartile values.

boxes have lines at the lower quartile, median, and upper quartile values. The notches in the boxes represent a robust estimate of the uncertainty about the medians for box plot comparisons. As the notches in the boxes do not overlap, this is an indication that the medians of the two groups differ at the 5% significance level.

The ratio, which measures the level of local calcium at the secretory sensor with respect to the average, has a median value of 1.568 for configuration 1 and 1.311 for configuration 2. This means that the effect of colocalization of calcium channels with the secretory vesicles could be enhanced, on average, by up to 25% (taking 1 as the bottom value) when the secretory machinery is located in the borders of the F-actin cytoskeletal cage with respect to a random distribution of the vesicles inside the cage. Furthermore, Fig. 5 also shows that the variability in the levels of local calcium for the vesicles in configuration 1 is significantly larger than for vesicles in configuration 2 (the difference between the third and the first quartile values is 2.1 for configuration 1 and 1.65 for configuration 2). The impact of these effects on the secretory response is shown in Fig. 6, where the time courses of the average normalized accumulated secretory responses obtained using a noncooperative kinetic scheme for both configurations are plotted.

In consequence, the use of these secretory models based on Monte Carlo simulations suggest that the organization of the secretory machinery in contact with the borders of cytoskeletal cortical cages will result in a more efficient coupling of the calcium currents and secretory responses resulting in faster secretion; therefore, the interface between the cortical cytoskeleton and the cytoplasmic space could be a new and relevant subject of interest in the field of neurotransmission.

Figure 6. Comparison of normalized accumulated secretory responses for configurations 1 and 2. Temporal dynamics of the normalized accumulated secretory response for configurations 1 and 2 in response to a simulated depolarizing pulse from -80 mV to 20 mV.

MATERIALS AND METHODS

Isolation and culture of bovine chromaffin cells

Chromaffin cells were isolated from bovine adrenal glands following collagenase digestion and they were separated from the debris and erythrocytes by centrifugation on Percoll gradients as described elsewhere (Gil *et al.*, 1998). Cells were maintained in 35 mm Petri dishes as monolayer cultures with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, $10 \mu\text{M}$ cytosine arabinoside, $10 \mu\text{M}$ 5-fluoro-2'-deoxyuridine, 50 IU/ml penicillin, and $50 \mu\text{g/ml}$ streptomycin (500 000 cells/dish, Corning Inc., Corning, New York). The cells were used between the second and fourth day after plating and all experiments were performed at room temperature (21 – 22°C). Chemicals were obtained from Sigma (Sigma Co., Madrid, Spain).

Expression of DsRed and GFP-SNAP-25 constructs in chromaffin cells

Expression of DsRed and GFP-SNAP-25 was performed as described recently (López *et al.*, 2009). The cDNA corresponding to the SNAP-25a isoform (Bark and Wilson, 1994) was cloned into the XhoI and BamHI sites of pDsRed-C3 or pEGFP-C3 (Clontech, Palo Alto, California) to express this protein fused in-frame at the C-terminus. The constructs were transferred to the pHSVpUC vector (Geller *et al.*, 1993) and primary cultures of chromaffin cells were infected with the Herpes Simplex virus (HSV-1) amplicon containing these constructs. The packaging of the different helper viruses (HSV-1 IE2 deletion mutant 5dl 1.2) was carried out as described previously (Lim *et al.*, 1996). The viral infection efficiency was determined by fluorescent microscopy using serial dilutions of the purified virus. The dilution producing 20–40% infection efficiency (usually 20–40 μl virus per 35 mm plate containing 1 ml of medium) was chosen for the sub-

sequent experiments. DsRed or GFP fluorescence was observed one day after infection and it persisted for at least two days.

TIRFM studies and combination with visualization of the cortical cytoskeleton by transmitted light

A through-the-lens TIRFM system was configured using the Olympus IX-71 inverted microscope using a 60× PlanApo 1.45 N.A. Olympus TIRFM objective. Epifluorescence and laser illumination (488 nm argon ion 40 mW or 543 nm He/Ne 10 mW; Melles Griot, Carlsbad, California) were selected using an Olympus TIRFM IX2-RFAEVA combiner system, and modifying the angle of laser incidence. Fluorescence emission was split using an Optosplit II system (Cairn Research Ltd., Faversham, UK) equipped with GFP and rhodamine filter sets. The separated images were simultaneously acquired side by side at 20 ms per frame using an electron multiplier CCD cooled camera (C9100-02 model, Hamamatsu Photonics, Japan) and stored in an IBM compatible PC. TIRFM calibration was performed using 100 nm fluorescent beads (Molecular Probes, Invitrogen detecting technologies, Carlsbad, California). The fluorescence intensities were determined at different vertical planes with step lengths of 100 nm using the motorized system mounted on the microscope and the image was obtained for both epifluorescence and TIRFM. The depth of penetration for the evanescent field was estimated as ~ 200 nm ($1/e$ depth of 180 ± 16 nm) mainly permitting the visualization of the static beads adhered to the coverslip. In contrast, beads in suspension undergoing random movement were infrequently seen in TIRFM and the vast majority were visualized by epifluorescence. Vesicle labeling was performed using either 1 μ M lysotracker red or green (Becherer *et al.*, 2003) (Molecular Probes, Invitrogen detecting technologies, Carlsbad, California), for experiments studying the motion of granules in relation to the cytoskeleton. In experiments designed to study vesicle fusion in response to 59 mM KCl depolarizing solutions, vesicles were stained for 15 min with 2 μ M acridine orange, granule position was assessed by the red acridine orange fluorescence of mature acidic vesicles, whereas its fusion was followed by the green flashes produced after matrix neutralization during exocytosis (López *et al.*, 2009). In some experiments F-actin was visualized by incubating the cells with fluorescein coupled to phalloidin at a 5 μ M concentration for 30 min after acridine orange labeling.

Transmitted light has been proved a useful technique to visualize the organization of the F-actin cytoskeleton in chromaffin cells (Giner *et al.*, 2005, Giner *et al.*, 2007). To combine cytoskeletal images with TIRFM, the halogen lamp light used for light transmission was turned into green (BP546 filter) for simultaneous visualization of lysotracker red labeled vesicles (see Fig. 1). In this way, the images acquired after separation by the image splitter corresponded to epifluorescence emission of the lysophilic dye loading the vesicles excited by the evanescent field and the transmitted

light image of the cytoskeletal structures (theoretical depth of field of 400–500 nm, using the TIRFM objective, Giner *et al.*, 2005).

Confocal Fluorescence Microscopy

Fluorescence from cells incubated with 1 lysotracker green and 5 μ M rhodamine-phalloidin for 30 min was visualized using an Olympus Fluoview FV300 confocal laser system mounted in an IX-71 inverted microscope incorporating a 100× UPlanSApo oil-immersion objective. This system allows for z axis reconstruction with theoretical z slices about 0.5 μ m thick and sequential mode studies in double labeling experiments.

Analysis of TIRFM, transmitted light, and confocal fluorescence microscopy images

Images were processed using the IMAGEJ program with Plugins for: particle centroid tracking, ROI measurements, image average, multiple channel image comparison, and colocalization analysis (Neco *et al.*, 2004).

Graphics were obtained with IGORPRO, GRAPHPAD PRISM (GraphPad software, San Diego, California) and ADOBE PHOTOSHOP 7.0. The Student's t -test for paired samples or the two-way ANOVA test were used to establish statistical significance among the experimental data (samples were considered significantly different when $p < 0.05$). Non-Gaussian data distributions were analyzed using the nonparametric Mann–Whitney U test. The data were expressed as mean $\pm$ SEM from experiments performed in a number (n) of individual cells, vesicles, or fusion events from at least three different cultures.

Calcium and secretory Monte Carlo simulations

Monte Carlo models are a powerful tool for the quantitative modeling of calcium dynamics and, in particular, for simulating the secretory response from readily releasable pools of secretory vesicles in neuroendocrine cells and presynaptic terminals. Monte Carlo techniques provide a microscopic particle simulation of the system in which the fundamental variables are the number of ions and buffers; in contrast, methods based on the solution of differential equations are macroscopic and deal with concentrations. The average values of the output of the Monte Carlo simulations converge to macroscopic results as the number of particles increase. The simulation scheme used in the present study is an extension of the algorithm developed by some of the authors (Gil *et al.*, 2000; Segura *et al.*, 2000). The Monte Carlo code for the simulation includes 3D calcium entry through L and P/Q -type calcium channels, calcium and mobile buffer diffusion, endogenous buffering of calcium, and kinetics for vesicle fusion (see model parameters in Table I). All these mechanisms are considered as stochastic processes taking place in a cylindrical domain, representing a prototype cage of the F-actin cytoskeleton. A 3D orthogonal grid divides the

Table I. Parameters used in simulations.

Geometrical parameters	
Cytoskeletal cage radius	$r=0.3 \mu\text{m}^a$
Height	$h=1 \mu\text{m}$
Spatial resolution	$\Delta x=30 \text{ nm}$
Basal concentration	$0.1 \mu\text{M}^b$
Ca^{2+} coefficient of diffusion	$220 \mu\text{m}^2/\text{s}^c$
Vesicle-fusion kinetic scheme	
Number of binding sites	3^d
Forward binding rate	$k^+=8 \cdot 10^6 \text{ M}^{-1} \text{ s}^{-1}^d$
Dissociation constant	$K_D=13 \mu\text{M}^d$
Endogenous buffering	
Concentration	$[\text{B}]=500 \mu\text{M}$
Forward binding rate	$k^+=5 \cdot 10^8 \text{ M}^{-1} \text{ s}^{-1}^b$
Dissociation constant	$K_D=10 \mu\text{M}^b$

^aGiner *et al.* 2007.^bKlingauf and Neher 1997.^cAllbritton *et al.* 1992.^dHeinemann *et al.* 1994.

cylindrical section in small cubes of $\Delta x=30 \text{ nm}$ per side. On the upper side of the cylinder we distribute calcium channels and vesicles. The structure of the algorithm is summarized in [Supplementary Fig. S1](#) for supplementary information.

To simulate currents through calcium channels, we use a stochastic scheme, where every channel of the total population may transit from its present state to an open, closed, or inactive state in response to voltage and calcium concentrations. Then, the total current is the sum of unitary currents due to open channels and the unitary currents are specific to each channel type and depend on its unitary conductance. In our simulations, we consider that each channel cluster is formed by two *P/Q*- and one *L*-type calcium channels, according to experimental estimations of channel populations involved in secretion in chromaffin cells (Lukyanetz and Neher, 1999). The scheme for the three-state model for each calcium channel and plots of the voltage-to-current curves for the *L*- and *P/Q*-type Ca channels are given in the [supplementary information](#).

For the secretory sensor, we adopt a noncooperative kinetic scheme to model each vesicle (Table I). In this scheme, three calcium ions have to bind to the calcium sensor to be in a prefusion state, then vesicle fusion occurs following a constant rate, which represents the delay between fusion and release and which is about 1 ms for chromaffin cells (Klingauf and Neher, 1997).

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REFERENCES

- Allbritton, NL, Meyer, T, and Stryer, L (1992). "Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate." *Science* **258**, 1812–1815.
- Bader, MF, Holz, RW, Kumakura, K, and Vitale, N (2002). "Exocytosis: the chromaffin cell as a model system." *Ann. N. Y. Acad. Sci.* **971**, 178–183.
- Bark, IC, and Wilson, MC (1994). "Human cDNA clones encoding two different isoforms of the nerve terminal protein SNAP-25." *Gene* **139**, 291–292.
- Becherer, U, Moser, T, Stuhmer, W, and Oheim, M (2003). "Calcium regulates exocytosis at the level of single vesicles." *Nat. Neurosci.* **6**, 846–853.
- Bennett, MK, Calakos, N, Kreiner, T, and Scheller, RH (1992). "Synaptic vesicle membrane proteins interact to form a multimeric complex." *J. Cell Biol.* **116**, 761–775.
- Burgoyne, RD, and Morgan, A (1998). "Analysis of regulated exocytosis in adrenal chromaffin cells: insights into NSF/SNAP/SNARE function." *BioEssays* **20**, 328–335.
- Garcia, AG, Albillos, A, Cano-Abad, MF, Garcia-Palomero, E, Hernandez-Guijo, M, Herrero, CJ, Lomax, RB, and Gandia, L (1997). "Calcium channels for exocytosis in chromaffin cells." *Adv. Pharmacol.* **42**, 91–94.
- Geller, AI, Doring, MJ, Haycock, JW, Freese, A, and Neve, R (1993). "Long-term increases in neurotransmitter release from neuronal cells expressing a constitutively active adenylate cyclase from a herpes simplex virus type 1 vector." *Proc. Natl. Acad. Sci. U.S.A.* **90**, 7603–7607.
- Gil, A, Segura, J, Pertusa, JA, and Soria, B (2000). "Monte carlo simulation of 3-D buffered Ca^{2+} diffusion in neuroendocrine cells." *Biophys. J.* **78**, 13–33.
- Gil, A, Viniegra, S, and Gutierrez, LM (1998). "Dual effects of botulinum neurotoxin A on the secretory stages of chromaffin cells." *Eur. J. Neurosci.* **10**, 3369–3378.
- Giner, D, Lopez, I, Villanueva, J, Torres, V, Viniegra, S, and Gutierrez, LM (2007). "Vesicle movements are governed by the size and dynamics of F-actin cytoskeletal structures in bovine chromaffin cells." *Neuroscience* **146**, 659–669.
- Giner, D, Neco, P, Frances, MM, Lopez, I, Viniegra, S, and Gutierrez, LM (2005). "Real-time dynamics of the F-actin cytoskeleton during secretion from chromaffin cells." *J. Cell Sci.* **118**, 2871–2880.
- Heinemann, C, Chow, RH, Neher, E, and Zucker, RS (1994). "Kinetics of the secretory response in bovine chromaffin cells following flash photolysis of caged Ca^{2+} ." *Biophys. J.* **67**, 2546–2557.
- Klingauf, J, and Neher, E (1997). "Modeling buffered Ca^{2+} diffusion near the membrane: implications for secretion in neuroendocrine cells." *Biophys. J.* **72**, 674–690.
- Lang, T, Bruns, D, Wenzel, D, Riedel, D, Holroyd, P, Thiele, C, and Jahn, R (2001). "SNAREs are concentrated in cholesterol-dependent clusters that define docking and fusion sites for exocytosis." *EMBO J.* **20**, 2202–2213.
- Lim, F, Hartley, D, Starr, P, Lang, P, Song, S, Yu, L, Wang, Y, and Geller, AI (1996). "Generation of high-titer defective HSV-1 vectors using an IE 2 deletion mutant and quantitative study of expression in cultured cortical cells." *BioTechniques* **20**, 460–469.
- Lopez, I, Giner, D, Ruiz-Nuno, A, Fuentelba, J, Viniegra, S, Garcia, AG, Davletov, B, and Gutierrez, LM (2007). "Tight coupling of the t-SNARE and calcium channel microdomains in adrenomedullary slices and not in cultured chromaffin cells." *Cell Calcium* **41**, 547–558.
- López, I, Ortiz, JA, Villanueva, J, Torres, V, Torregrosa-Hetland, CJ, del Mar, FM, Viniegra, S, and Gutierrez, LM (2009). "Vesicle motion and fusion are altered in chromaffin cells with increased SNARE cluster dynamics." *Traffic* **10**, 172–185.
- Lukyanetz, EA, and Neher, E (1999). "Different types of calcium channels

- and secretion from bovine chromaffin cells." *Eur. J. Neurosci.* **11**, 2865–2873.
- Nakano, M, Nogami, S, Sato, S, Terano, A, and Shirataki, H (2001). "Interaction of syntaxin with alpha-fodrin, a major component of the submembranous cytoskeleton." *Biochem. Biophys. Res. Commun.* **288**, 468–475.
- Neco, P, Giner, D, Viniegra, S, Borges, R, Villarroel, A, and Gutierrez, LM (2004). "New roles of myosin II during vesicle transport and fusion in chromaffin cells." *J. Biol. Chem.* **279**, 27450–27457.
- Ohara-Imaizumi, M, Nishiwaki, C, Nakamichi, Y, Kikuta, T, Nagai, S, and Nagamatsu, S (2004). "Correlation of syntaxin-1 and SNAP-25 clusters with docking and fusion of insulin granules analysed by total internal reflection fluorescence microscopy." *Diabetologia* **47**, 2200–2207.
- Oheim, M, Loerke, D, Stuhmer, W, and Chow, RH (1998). "The last few milliseconds in the life of a secretory granule. Docking, dynamics and fusion visualized by total internal reflection fluorescence microscopy (TIRFM)." *Eur. Biophys. J.* **27**, 83–98.
- Rettig, J, and Neher, E (2002). "Emerging roles of presynaptic proteins in Ca^{2+} -triggered exocytosis." *Science* **298**, 781–785.
- Rickman, C, Meunier, FA, Binz, T, and Davletov, B (2004). "High affinity interaction of syntaxin and SNAP-25 on the plasma membrane is abolished by botulinum toxin E." *J. Biol. Chem.* **279**, 644–651.
- See supplementary material at <http://dx.doi.org/10.2976/1.3338707>. Fig. S1: sequence of the simulation in the Monte Carlo algorithm. Fig. S2: models and current-to-voltage curves employed for L and P/Q calcium channels used in the Monte Carlo simulations.
- Segura, J, Gil, A, and Soria, B (2000). "Modeling study of exocytosis in neuroendocrine cells: influence of the geometrical parameters." *Biophys. J.* **79**, 1771–1786.
- Shahrezaei, V, and Delaney, KR (2004). "Consequences of molecular-level Ca^{2+} channel and synaptic vesicle colocalization for the Ca^{2+} microdomain and neurotransmitter exocytosis: a Monte Carlo study." *Biophys. J.* **87**, 2352–2364.
- Toonen, RF, Kochubey, O, De, WH, Gulyas-Kovacs, A, Konijnenburg, B, Sorensen, JB, Klingauf, J, and Verhage, M (2006). "Dissecting docking and tethering of secretory vesicles at the target membrane." *EMBO J.* **25**, 3725–3737.
- Trifaro, JM, Gasman, S, and Gutierrez, LM (2008). "Cytoskeletal control of vesicle transport and exocytosis in chromaffin cells." *Acta Physiol. (Oxf.)* **192**, 165–172.1748-1708
- Weber, T, Zemelman, BV, McNew, JA, Westermann, B, Gmachl, M, Parlati, F, Sollner, TH, and Rothman, JE (1998). "SNAREpins: minimal machinery for membrane fusion." *Cell* **92**, 759–772.