
CALCIUM SIGNALING AND DIFFERENT SECRETORY VESICLES IN CHROMAFFIN CELLS

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Introduction

Exocytosis is one of the main cellular processes which enables the cell to influence its environment. This mechanism underlies the intercellular communication in the nervous system via neurotransmitter release in synapses and thus is a basis for integrative function of the brain. The involvement of numerous intracellular molecules in secretory pathway has been already shown. However, a major intracellular stimulus for exocytotic proceeding in excitable cells is a rise of intracellular Ca^{2+} concentration (Ca_i). It has been previously shown that Ca^{2+} -dependence of secretion has a two-step character, which can be explained by several models, viz. a "pools" model in chromaffin cells (1, 2) or a "prepared stage" model of secretion in nerve terminals (3). Recently the involvement of a protein kinase C mechanism into biphasic phenomenon has also been proposed (4). The "pools" model assumes the existence of three vesicular pools and vesicle "migration" from large reserve to a release-ready and then to secreted pools. The existence of large reserve and releaseready pools determines a two-step secretion with the third-

power Ca^{2+} -dependence of the final secretory response. The "prepared stage" model describes two steps, namely a threshold phase being a preparative stage for secretion and a secretory phase itself. The preparative stage serves as Ca^{2+} -dependent "priming" step of secretion. The "PKC model" explains the two phases of secretion by PKC-dependent and -independent processes depending on Ca_i . In this paper we propose a 'vesicular' mechanism which describes experimental data and implies significance of this phenomenon in the function of secretory cells.

Materials and methods

Cell culture. Chromaffin cells were prepared by enzymatic dissociation of bovine adrenal glands and maintained in tissue culture for 2-5 days. Then they were plated on poly-L-lysine-coated glass coverslips and maintained in Medium 199 supplemented with 10% fetal calf serum, 1% bovine serum albumin and 2 mM glutamine for cell culture; see (5) for more details. We did not separate adrenaline and noradrenaline-secreting cells.

Electrophysiological measurements. Cells were voltage clamped using the whole-cell patch-clamp technique (6). Whole-cell Ca_2 currents were recorded at high time resolution with a computer-controlled patch-clamp amplifier EPC-9 and "Pulse" software (HEKA Electronic, Lambrecht, Germany). Standard bath solution contained in mM: NaCl, 140; KCl, 2.8; CaCl_2 , 2; MgCl_2 , 2; Hepes, 10; TEACl, 10; tetrodotoxin, 0.01; pH 7.2; bath solutions with high Ca_2 concentration contained: NaCl, 40; CaCl_2 , 60; MgCl_2 , 2; Hepes, 10; TEACl, 40; tetrodotoxin, 0.01; pH 7.2. Protocol of experiments was as described previously (5). The intracellular solution contained in mM: CsCl, 64; Cs_2SO_4 , 28; ATP, 2; MgCl_2 , 2; EGTA, 0.5; Hepes, 10; N-methyl-D-glucamine, 10; GTP, 0.3; pH 7.4. The osmolarity of the solutions was adjusted to 320 mOsm with glucose. All compounds were obtained from Sigma. All experiments were performed at room temperature (21-24 °C).

Capacitance measurements. To measure the secretion, we used a high-resolution measurement of membrane capacitance (C_m) (7). The measurements were realized in whole-cell experiments by applying a sine wave stimulus of a DC holding potential 270 mV with a software "Lock-in". A 1-kHz, 10 mV peak-to-peak sine wave was generated by an ITC-16 multichannel interface (Instrutech, Inc., NY) controlled by Mac computer. The sine generation as well as phase sensitive detection of C_m were fulfilled with the software 'Lock-in amplifier' controlled by "Pulse" software and EPC-9 amplifier (HEKA Electronics). Differences in C_m (ΔC_m) before and after a depolarizing pulse were estimated off-line (5, 8). Data were analyzed statistically using Origin 5.0, Microcal Inc. (U.S.A.). Data are given as mean $\pm$ SE unless indicated otherwise.

Fluorescence measurements. Patch-clamp and capacitance records were combined with Fura-2 fluorescence measurements to allow simultaneous on-line monitoring of Ca_2 currents, exocytosis and Fura-2 fluorescence signals to measure

Cai. 100 mM Fura-2 was loaded into the cells via patch pipets. Ca^{2+} concentration calculated from the ratios of light emission produced by 360 and 390 nm wavelengths according to Grynkiewicz et al. (9) was displayed on-line using a system by Luigs and Neumann (Ratingen, Germany); see for more details (10).

Materials. Thermanox and Aklar were obtained from Nunc Inc. (U.S.A.); Araldite was from Fluka AG, Germany. All other chemicals were purchased from Sigma.

Results

Efficiency of Ca^{2+} entry to induce the exocytosis depends on resting Cai

In our experiments, we used a high bath Ca^{2+} concentration (60 mM) to induce secretion by single depolarizations of 50 or 200 ms durations. At such concentration a maximum of current-voltage curve was located at 140 mV. Chromaffin cells were depolarized with the trains of depolarizing pulses to induce a wide range of secretory responses evoked by Ca^{2+} current (ICa). The membrane potential was held at 270 mV and the trains of depolarizations were started at a reference potential (140 mV) corresponding to the maximum of current-voltage relationship (I-V) for ICa followed by a series of depolarizations to different membrane potentials which activated ICa (210 to 160 mV). The reference potential was randomly repeated several times in the series the same protocol as described previously (5). The amount of Ca^{2+} ions entering *into* the cell during membrane depolarization was estimated as a Ca^{2+} charge (Q), the integral of ICa and Cai was directly measured by fura-2. Fig. 1 demonstrates typical records of Cai transients, membrane capacitance, C_m as well as ICa traces evoked by reference potential (V_{Ref} 5 140 mV) in the same chromaffin cell. Series conductance remained constant for all presented recordings.

As seen in Fig. 1, membrane depolarizations induced a rapid increase of C_m with a subsequent decline, which we attributed to endocytosis. Such 'reversible' C_m responses were observed in most of our experiments. As we described in our recent investigations, in the case of stable prepulse resting level of Cai, similar Q values induced similar changes in the membrane capacitance, ΔC_m (Fig. 2, b from (5)). On the contrary, an increase of resting Cai affected the secretory responses of the cell. Fig. 1 presents evidence that the value of DCm changes strongly depends on the resting level of Cai. Thus, despite even smaller values of the ICa peak due to its run-down (Fig. 1, Ca current traces 4-6), at higher resting Cai (right part of the Ca^{2+} trace), the Q-induced changes in C_m (ΔC_m) were significantly larger (right part of the C_m trace). Fig. 2, a presents an example of the dependence of ΔC_m changes evoked by reference potential (140 mV) on resting Ca_i measured in one of the cells. In these experiments, Q values induced by the reference potential were in the range of 30-70 pC.

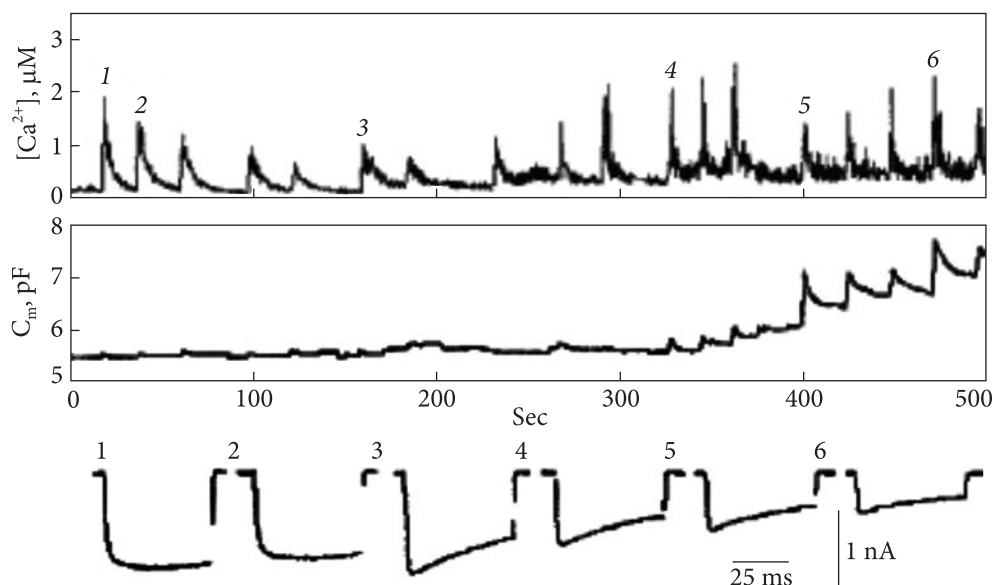


Fig. 1. Depolarization-induced exocytosis in bovine chromaffin cells. Changes in the intracellular Ca^{2+} concentration, Ca_i , membrane capacitance, C_m and Ca^{2+} current (I_{Ca}) traces induced by 50 ms depolarizations from a holding potential of 270 mV to different test potentials. Numbers pointed near Ca^{2+} current traces and Ca_i transients represent responses to reference potential (+40) which corresponds to a maximum of the current-voltage curve

The experimental points can be well approximated by the third-power calcium dependence curve (Fig. 2, *a*, solid line) as was supposed previously (1). Such a dependence was characteristic for all the tested cells ($n = 47$). Since the data recorded from different cells were scattered in absolute values, we analyzed one of the tested cells in detail. However, Fig. 2, *a* shows that the dependence of secretory response of the cell on Ca_i is composed of two parts. The first one represents slight dependence of ΔC_m on Ca_i (similar to previously described ‘preparative’ step (3)). This step was observed when Ca_i changes were in the range below a critical level of Ca_i^* (Ca^*), which in our experiments was about $200 \div 300$ nM for different tested cells. When Ca_i reached Ca^* , ΔC_m evoked by the same Q was dramatically increased. Similar critical point (at $\text{Ca}_i \sim 300$ nM) was also observed by the cited authors (3). Fig. 2, *b* demonstrates differences in mean values of ΔC_m induced by V_{Ref} measured at two different levels of resting Ca_i in the same cell. Thus, at low $\text{Ca}_i \leq \text{Ca}^*$, the mean value of ΔC_m induced by Q_{ref} accounted for 72.78 ± 18.97 fF ($n = 6$), and at high $\text{Ca}^* < \text{Ca}_i \leq 600$ nM it was equal to 276.54 ± 134.8 fF, $n = 5$ in the same cell. We calculated these values as averaged within the corresponding range of Ca_i . Since in the range of high Ca_i the ΔC_m growth is considerable, significant deviations of ΔC_m values and low statistical significance ($P < 0.2$) were due to essential difference in ΔC_m laying in the poles of the range, see Fig. 2, *a*.

Two components of Ca^{2+} -dependent secretion

Taking into account the observation described above, we estimated the influence of resting level of Ca_i on depolarization-induced secretion. ΔCm changes induced by Q were calculated separately for two levels of resting Ca_i , below and above the critical level $\text{Ca}^* = 250 \text{ nM}$. Fig. 2, *c* shows an example of ΔCm dependence on Q recorded at low resting $\text{Ca}_i \leq \text{Ca}^*$ in the same cell. As seen, the experimental points can be well approximated by a linear graph with a slope of $B1 = 4.43$. Fig. 2, *d* demonstrates the dependence obtained for the same cell but at high level of resting, Ca_i

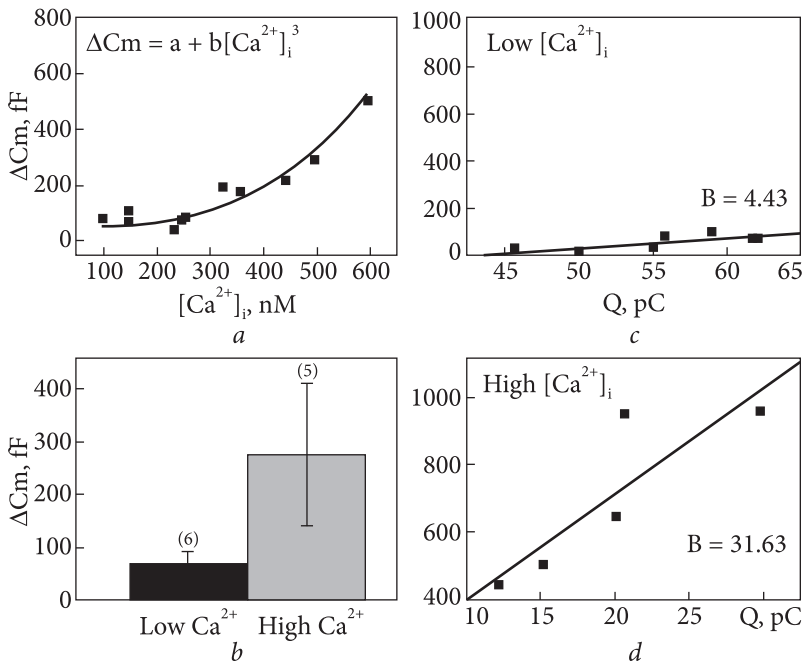


Fig. 2. Dependence of exocytosis on resting Ca_i level (*a*). Calcium concentration was measured using Fura-2 loaded into the cell by diffusion from the patch pipet solution (for more details see (5)). Experimental points represent ΔCm plotted against calcium concentration Ca_i recorded just before every depolarization in the same cell. ΔCm changes were induced by ICa with a Ca^{2+} charge lying in the range of 30–70 pQ. The equation $\Delta\text{Cm} = a + b[\text{Ca}^{2+}]_i^3$, where a is a proportionality constant (solid line), was fitted to the plot of ΔCm versus Ca_i ; *b* — Representation of comparison of changes in ΔCm against the resting level of Ca_i for the same cell at low ($\text{Ca}_i \approx 200 \text{ nM}$, black bar) and high ($\text{Ca}_i > 200 \text{ nM}$, gray bar) resting Ca_i preceding depolarizing pulses. Error bars represent SD and numbers of tested values are presented near the bars. (*c-d*) Two components of exocytosis recorded at different resting Ca_i in bovine chromaffin cells; *c* — Linear increase in the value of ΔCm depending on Q value observed at low resting $\text{Ca}_i \leq 200 \text{ nM}$ was well approximated by linear function with a slope $B = 4.43$; *d* — Similar linear function with the slope $B = 31.63$ approximated data points obtained at Ca_i higher than 200 nM in the same cell

> Ca^* . In this case, the experimental points can also be approximated by a linear function, but the slope is obviously steeper ($B2^* = 31.63$).

Vesicular model of two-step Ca^{2+} -dependent secretion

Suppose two steps of secretion are triggered by different levels of resting Ca_i , then the simplest explanation for this could be the participation of two types of vesicles in the fusion process. If assume that at low level of Ca_i only small vesicles (SV) fused with the cell membrane, then this fusion would induce an insignificant increase in the cell membrane area proportionally to the vesicular surface area (S_1) and the number (n_1) of fused vesicles. It is generally accepted that the cell membrane capacitance, reflecting the cell membrane area, is usually calculated from the equation for a parallel plate capacitor. The changes in C_m (ΔC_m) produced by vesicle fusion are

$$\Delta C_m(n_1) = n_1 \frac{\varepsilon \varepsilon_0 \cdot S_1}{d}, \quad (1)$$

where $\varepsilon_0 = 8.9 \cdot 10^{-12}$ F/m is the dielectric permeability in the vacuum, $\varepsilon = 3$, relative dielectric permeability, and $d = 3$ nm is the membrane thickness. If one takes into account that $\varepsilon \varepsilon_0 \pi / d$ is a constant which equals to about 0.03 F/m² (30 fF/mm²), Eq. [1] can be easily transformed to a simpler expression

$$\Delta C_m(n_1) = 30 n_1 D_1^2, \quad (2)$$

where D_1 is the diameter of SV taken in micrometers. The linear graph constructed from Eq. [2] is presented in Fig. 3, *a* (curve D_1) and looks like that describing the experimental points obtained at low Ca_i , Fig. 2, *c*.

(Ca^*) large vesicles (LVs) start fusing with the membrane, then the function describing the fusion also will be linear but with a steeper slope (Fig. 3, *a*, line D_2) similar to the linear function describing experimental data (Fig. 2, *d*). For this case, the equation is as follows:

$$\Delta C_m(n_2) = 30 n_2 D_2^2, \quad (3)$$

where n_2 is the number of fused LVs and D_2 is their diameter.

Obviously, due to the linearity of $\Delta C_m(Q)$ obtained from experimental data (Fig. 2, *c* and *d*), it is reasonable to consider that the number of fused vesicles depends linearly on Q . Taking into account the fact that an increase of Ca_i due to Ca^{2+} entering the cell during depolarization is proportional to Q (Fig. 2, *a* from (5)) and also considering Eqs. [2] and [3], the overall process of ΔC_m dependence on Ca_i induced by similar Q will not be linear and will be described by conditional equation presented in Fig. 3, *b* (curves D_1 and D_2) where $Ca^* = 200$ nM, k_1 and k_2 are the proportionality coefficients. Thus, sup-

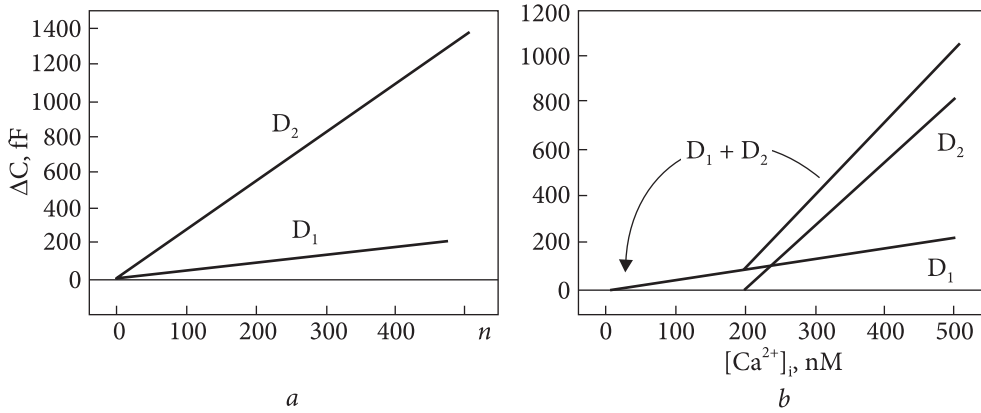


Fig. 3. Calculated dependence of exocytosis on the fusion of two types of vesicles: *a* — Simulation of changes in cell secretory response depending on the number (*n*) of fusing vesicles of two different diameters. Curve *D*₁ represents an increase of DC_m due to fusion of small vesicles and curve *D*₂ describes fusion of large vesicles with the cell membrane; *b* — Summary solid line (*D*₁ + *D*₂) describing the fusion of small (*D*₁) and large (*D*₂) vesicles in conditions when large vesicles start fusing after some delay determined by level of resting *Ca*_i. Corresponding separate graphs for *D*₁ and *D*₂ are presented by dotted straight lines. Start point of large vesicles fusion is determined by *Ca*_i level; 200 nM

pose the rate of fusion is constant, then the slope of linear functions *will reflect* the sizes of vesicles.

$$\Delta C_m(Ca_1) = \begin{cases} 30k_1D_1^2Ca_1, & \text{for } Ca_1 \leq Ca^* \\ 30(k_1D_1^2 + k_1D_2^2)Ca_1, & \text{for } Ca_1 > Ca^*, \end{cases} \quad (4)$$

Assuming that at higher *Ca*_i the SVs continue to fuse with the membrane until their pool is emptied, the *B**2 slope will reflect the sum of two processes, the fusion of SVs and LVs (*B**2 = *B*₁ + *B*₂). From our experimental electrophysiological data (in accordance with the slopes), it can be as follows: (*B*₁ + *B*₂)/*B*₁ = 7.14; consequently, the ratio of vesicular diameters is

$$\frac{D_2}{D_1} = 2.48 \cdot \sqrt{\frac{k_1}{k_2}}. \quad (5)$$

This means that the diameters of secretory vesicles participating in depolarization-induced exocytosis differ by about 2.5 times.

Discussion

We used electrophysiological measurements of calcium currents and membrane capacitance in combination with microfluorimetric measuring intracellular *Ca*²⁺ concentration to investigate the fine mechanisms of *Ca*²⁺-dependent secretion. Our experiments have shown two components of *Ca*²⁺-dependent exocytosis that

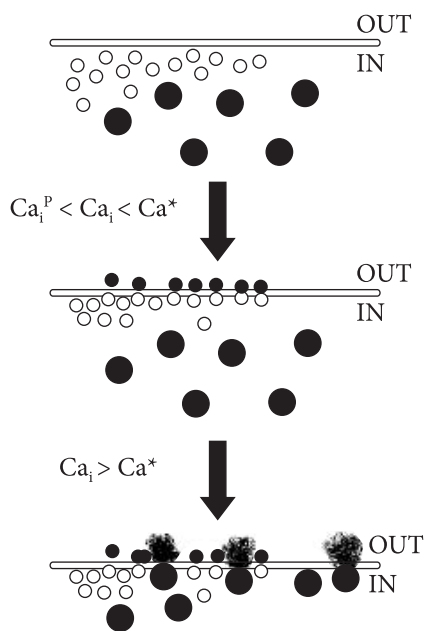


Fig. 4. Schematic representation of vesicular model. Small and large secretory vesicles are shown near the membrane. Abbreviations: Ca_i , cytoplasmic Ca^{2+} concentration; Ca_i^* , critical point; Ca_i^p , physiological normal Ca^{2+} concentration

coincide with the data reported previously on chromaffin and other secretory cells (1-4, 11). We have established a linear dependence of both stages of exocytosis on Ca^{2+} influx during depolarization. Similar linear dependencies were also observed earlier, when the authors used EGTA buffers to fix Ca_i at two different levels (12). Here the data allow us to explain why a discrepancy between data describing $DCm(Ca_i)$ dependence (linear or nonlinear was reported) does exist in the literature (1-3, 12). Linear dependence will be observed in the case if resting Ca_i level is

stable during the experiment. If it is changed only in the range below a critical level (about 200-300 nM), then a linear function with a low grade slope can be observed. If stable Ca_i is above the critical level, the function will be represented also by a linear graph with a sharper slope. Linear dependence will also be observed when resting Ca_i level passes both ranges, but if DCm is normalized to the reference values of the series. On the other hand, if resting level of Ca_i passes both ranges during experiment, then $DCm(Ca_i)$ will correspond to the sum of two linear graphs and will look like an exponential-like dependence (Figs 2, *a* and 3, *b*).

Finally, we have presented a simple explanation of a two-step relationship between Ca_i and exocytosis, which is schematically presented in Fig. 4. The proposed 'vesicular' model consists in assumption that every phase of two-step secretion linearly depends on Ca_i and is provided by fusion of two different sized vesicles. According to presented data, the level of basal Ca_i determines which of the vesicular types starts to fuse. At slight increase of Ca_i which does not exceed the critical resting level ($Ca_i < 200 \div 300$ nM), fusion of small vesicles starts. Vesicles that have been filled with neurotransmitter translocate to the active zone by diffusion or by a cytoskeleton-based transport process where they dock and fuse with the plasma membrane. When Ca_i reaches the critical point, the larger vesicles start to fuse with plasmatic membrane. This process can provide very important physiological process — to trigger a release of different neurotransmitters which can be differently distributed among diverse secretory vesicles. For example, classical neurotransmitters located in synaptic-like microvesicles and peptide transmitters located in large dense core vesicles. Taking into account the fact that

chromaffin cells contain several types of secretory vesicles which are quite heterogeneous in their diameters (13-15), it is important to find types participating in exocytosis evoked by depolarization-induced increase of Ca_i .

Acknowledgements. We should like to thank sincerely Prof. P. Kostyuk and Prof. E. Neher. This work was supported by grants from the U.S. Civilian Research and Development Foundation for the Independent States of the Former Soviet Union (UB2-2049). This work was previously published (Lukyanetz, 2001).

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