Cytosolic calcium facilitates release of secretory products after exocytotic vesicle fusion

Rafael Fernández-Chacón, Guillermo Alvarez de Toledo*

Departamento de Fisiología Médica y Biofísica, Facultad de Medicina, Universidad de Sevilla, Avda. Sánchez Pizjuán 4, 41009 Sevilla, Spain

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Abstract We monitored single vesicle exocytosis by simultaneous measurements of cell membrane capacitance as an indicator of fusion and amperometric detection of serotonin release. We show here that vesicle–plasma membrane fusion in rat mast cell granules is followed by a variable, exponentially distributed, delay before bulk release. This delay reflects the time required for the expansion of the exocytotic fusion pore, lasting, on average, 231 ms in resting cytosolic calcium, $[Ca^{2+}]_i$ (50 nM). In the presence of $[Ca^{2+}]_i$ in the low micromollar range, the lag between fusion and release was reduced to 123 ms. The characteristics of the amperometric signals were unchanged by $[Ca^{2+}]_i$. These results show a novel site of regulation in the exocytotic process, the fusion pore, which may represent a different mechanism facilitating transmitter release.

Key words: Exocytosis; Amperometry; Cell capacitance; Transmitter release

1. Introduction

Exocytotic release begins with the formation of a narrow connection between the lumen of a secretory vesicle and the extracellular space, the fusion pore [1]. On its formation, the pore has a conductance similar to a gap junction channel, around 230 pS [2]. To release granule contents the fusion pore must enlarge its conductance up to several nS [3]. Recent application of amperometric techniques has shown that catecholamine release from single chromaffin granules occurs in two stages: first, a small release lasting several milliseconds, followed by a spike like release attributable to the solubilization of the granule core [4]. In this preparation the first phase of release, called 'foot', was thought to occur by leak of catecholamines through the fusion pore, appearing the amperometric spike once the pore has completely opened. This hypothesis was proved to be true in beige mouse mast cell granules by monitoring simultaneously the release of serotonin with amperometry and the time course of fusion pore expansion by cell membrane capacitance measurements [5]. These experiments showed that during fusion pore expansion release is limited by the small size of the pore, apparently delaying bulk release with respect to secretory vesicle fusion [5]. However, the factors controlling the process of fusion pore expansion are unknown. We have examined the dynamics of fusion pores in beige mouse mast cell granules and tested the effect of $[Ca^{2+}]_i$ on the lag period between fusion and release in rat peritoneal mast cells by measuring the cell surface area with the whole cell patch clamp technique [6]. Release from the same vesicles was monitored by simultaneous detection of serotonin molecules by an amperometric electrode [4,5,7,8]. Our results show that the lag period between fusion and release of serotonin is shortened by increased cytosolic calcium in the low micromollar range, suggesting that fusion pore expansion be under cellular control even at these late stages of the exocytotic process.

*Corresponding author. Fax: (34) (5) 455 1769.
E-mail: GATOLEDO@CICA.ES

Abbreviations: EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfo nic acid), GTPγS, guanosine-5'-*O*-(3-thiotriphosphate).

2. Materials and methods

2.1. Cells and solutions

Beige mouse and normal rat peritoneal mast cells were obtained by peritoneal lavage as previously described [9]. Cells were plated on all-glass chambers and incubated in a 5% CO_2, 95% air, atmosphere until use. All experiments were done at room temperature (22–25°C) in a bath solution containing, in mM: 140 NaCl, 10 HEPES, 3 KOH, 2 $MgCl_2$, 1 $CaCl_2$ and 10 glucose. The pH was 7.2 and the osmolality 300–310 mmol·kg^{-1}. The patch pipette solution contained, in mM: 140 K-glutamate, 7 $MgCl_2$, 10 HEPES, 3 KOH, 0.2 Mg-ATP and 10 Ca-EGTA buffers. Two different combinations of Ca-EGTA buffers were used: a low (50 nM) calcium concentration was obtained by combining 9 mM K_2-EGTAwith 1 mM Ca-EGTA; high calcium concentration (1.1 μM), which greatly accelerate the rate of secretion in mast cell, was obtained by combining 2.5 of K_2-EGTA with 7.5 Ca-EGTA [10]. The final Ca^{2+} concentration in the internal solution was measured with the ETH 1001 (Glasbasseri, Geneva, Switzerland) calcium membrane. To induce degranulation GTPγS (0.5 to 25 μM) was added to the pipette solution.

2.2. Measurements of the cell membrane capacitance

Mast cells were subjected to whole cell patch clamp to measure the cell membrane capacitance. To resolve small capacitance changes, the initial values of cell membrane capacitance and series conductance in every cell were cancelled with the C-slow and G-series potentiometers of an EPC-7 patch clamp amplifier. After compensation, a calibration was obtained by changing the compensation circuitry in the patch clamp amplifier by 100 fF. The V-command was a 50-mv sine wave (peak-to-peak, 800 Hz). Capacitance and conductance values were estimated from the real and imaginary components of the complex admittance, which were obtained on line by a software-based phase detector. The phase angle was periodically adjusted by using the phase tracking technique [11]. One data point was obtained every 9.5 ms. The time course of pore resistance in beige mouse mast cells was calculated off line from the real and imaginary components of the admittance [1]. The same measurements could not be done in rat mast cells because of the small granule capacitance.

2.3. Electrochemical detection

Eight micrometer diameter carbon fibres were used to monitor release of serotonin. The electrodes were prepared as previously described [8]. We held the carbon fibre electrode at a constant voltage of +650 mV. The amperometric current was registered with an EPC-7 patch-clamp amplifier. At this voltage, serotonin is the main secretory product detected in peritoneal mast cells by amperometry [5,8]. Capacitance and amperometric recordings were combined into the same computer program, which was written in C-lab (INDEC, Sunnyvale, CA, USA).

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3. Results

Fig. 1A (top) shows the step like changes in cell surface area in a beige mouse mast cell reflecting the fusion of single secretory vesicles with the plasma membrane. Every step change in cell surface area is accompanied with release of vesicle contents (Fig. 1A, bottom), as detected by the current produced at the tip of the carbon fibre electrode located very closed to the cell surface. The release signal always lagged the step change in capacitance, with a variable a delay between fusion and bulk release ranging from 10 to 1256 ms. This delay reflects the time taken by the exocytotic machinery to expand the fusion pore (Fig. 1B). Bulk release from the granule matrix occurs once the pore has completely opened as indicated by the drop of pore resistance to a value < 100 MΩ, coinciding with the onset of the amperometric transient (Fig. 1B, second and third traces).

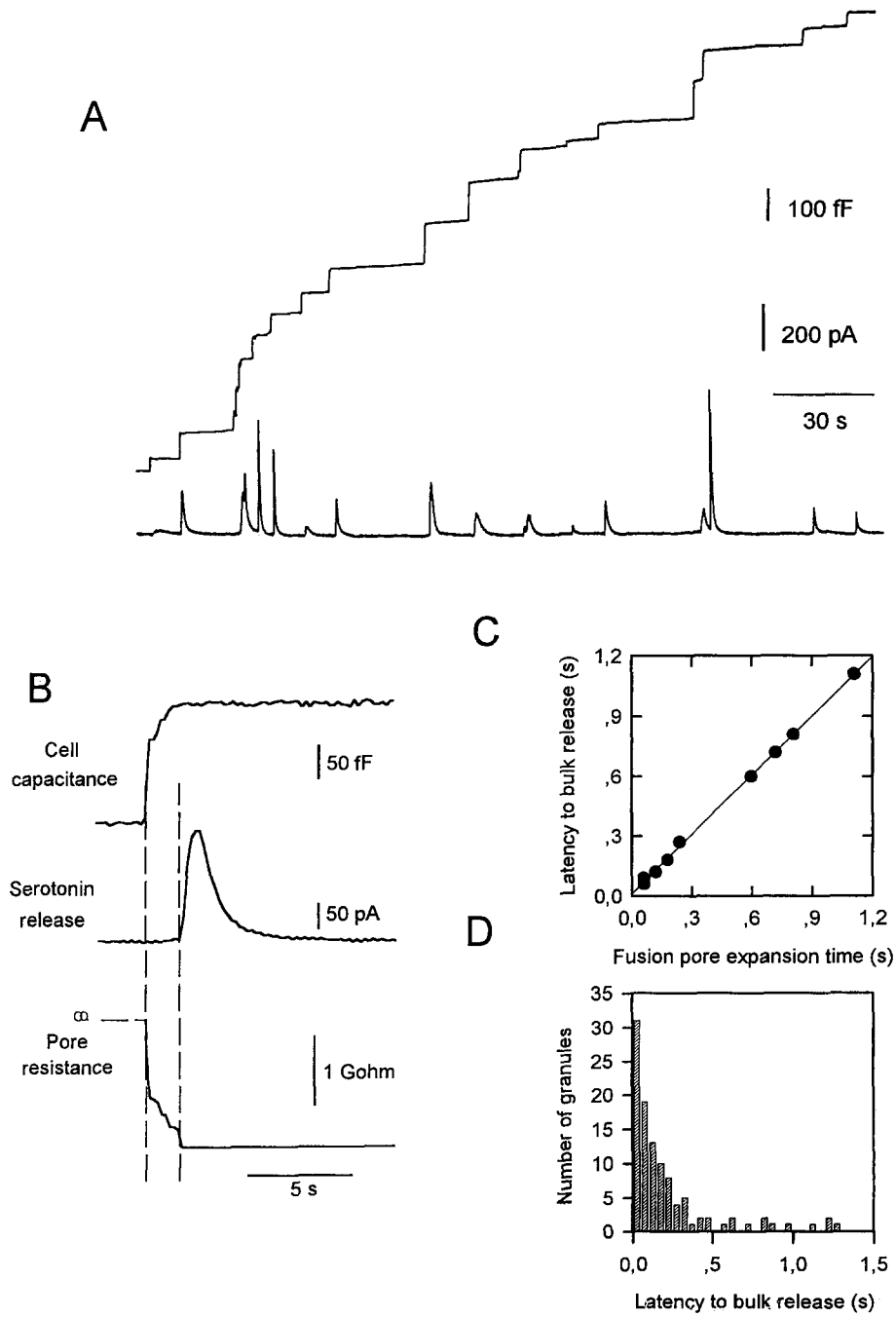

Fig. 1. Single secretory events in a beige mouse peritoneal mast cell. (A) Single vesicle fusion was detected by step-like increases of cell membrane capacitance (top). Every vesicle fusion was accompanied by release of serotonin as detected with amperometry (bottom). The amperometric transients show different characteristics as each granule fuses at different distances from the electrochemical detector. (B) The amperometric transient (second trace) lagged the step change in capacitance (first trace) and occurred after substantial dilatation of the exocytotic fusion pore (third trace). (C) Linear correlation between pore expansion time and lag to massive release. These two independent measurements, therefore reflect the time of fusion pore expansion. The straight line intercepts at 10 ms the y axis, probably suggesting diffusion delay of molecules before reaching the carbon electrode. (D) Distribution of latencies between vesicle fusion and massive release. The latency to bulk release was measured for every event as in B.

The expansion time of the pore as measured by impedance measurements, and the latency to bulk release as detected by amperometry are remarkably well correlated (Fig. 1C), indicating that diffusional delay is considerably shorter compared to the lag period between fusion and release. This lag period is variable and its frequency distribution fits to a single exponential (τ = 205 ms) (Fig. 1D), suggesting that a first order kinetics reaction dominate the pore expansion process.

Based on the results obtained from beige mouse mast cells we used the latency between fusion and onset of the amperometric transient to estimate the time required for pore expansion in rat mast cells. In rat mast cells the fusion pore expansion cannot be assayed by impedance measurements due to the small granule capacitance. However, in this preparation more exocytotic events can be obtained during the course of an experiment, making easier any statistical analysis of data. Fig. 2A, shows a step increase in cell membrane capacitance (upper trace) followed by an amperometric transient (lower trace), showing that a long delay exists also in normal rat mast cells. This suggests that in small granules the fusion pore must also reach a critical size before allowing massive release of secretory products. To test the action of cytosolic calcium on the pore expansion process, we perfused the cells through the patch pipette with solutions containing either 50 nM or 1.1 μM free Ca^{2+} by using a combination of Ca-EGTA buffers. In cells dyalized with GTPγS (25 μM) and low $[Ca^{2+}]_i$, the latency to bulk release was exponentially distributed, as in beige granules, and had a mean value of 231 ms (n = 114, from 18 cells, exponential fit with a τ of 233 ms) (Fig. 2C,E,F). At higher $[Ca^{2+}]_i$, the average latency was 123 ms (n = 303, from 47 cells, τ = 129 ms) (Fig. 2 B,D,F). These two data sets are significantly different, $P < 0.05$ (log rank test). The parameters of the amperometric spike, however, did not change appreciably. For example, the half-width of the spike was 152 ± 14 ms (mean ± S.E.M., n = 116, 18 cells) at low calcium (Fig. 2H) versus 141 ± 8 ms (n = 327, 47 cells) in high calcium conditions (Fig. 2G). Elevated $[Ca^{2+}]_i$ exclusively influenced the delay. Since the characteristics of the amperometric transient reflect the release from the polymer granule that is not in contact with the cytoplasmic compartment, we conclude that intracellular calcium regulates the properties of the fusion pore rather than the release from the granule matrix.

Cytosolic Ca^{2+} is known to control many steps in exocytotic secretion before fusion [12–14]. Therefore, it could be argued that the mechanisms expanding the fusion pore could be directly coupled to a previous step, like vesicle plasma membrane

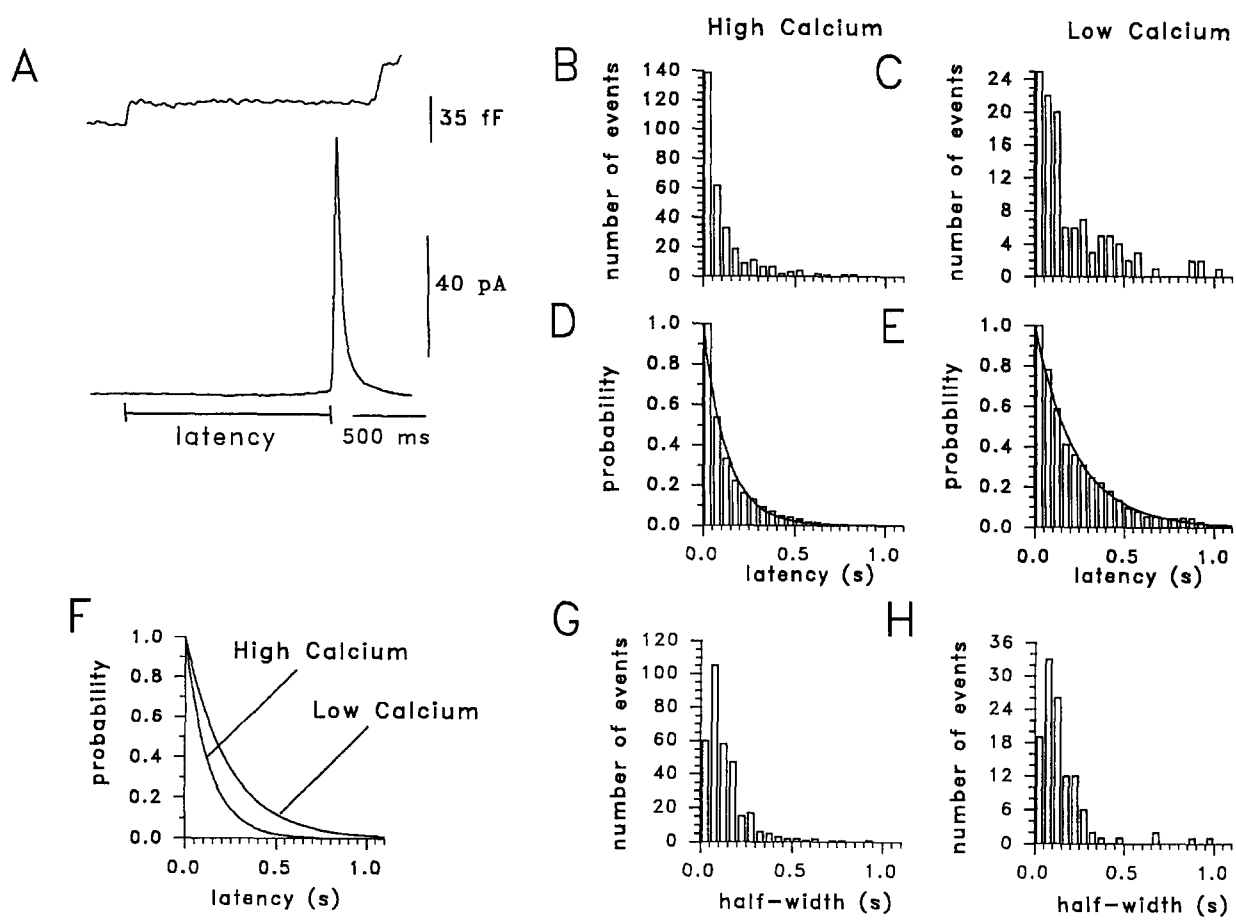

Fig. 2. Effect of cytosolic calcium on the latency to massive release. (A) Step change in capacitance and amperometry in a normal rat mast vesicle. (B) and (C) Frequency histograms obtained in cells perfused with high calcium (1.1 μM) and low calcium (50 nM) pipette solutions respectively. (D) and (E) Probability distribution functions of the histograms shown in B and C. The fits of both probability functions are shown for better comparison in (F). (G) and (H) Frequency histograms of half-widths (duration of amperometric spike at half its height) under high and low calcium conditions. These two frequency histograms are not significantly different.

fusion. As a result, an increase in the rate of degranulation would equally shorten the lag period between fusion and release. To test this idea, we did experiments fixing $[Ca^{2+}]_i$ at 1.1 μM, but decreasing the amount of GTPγS in the patch pipette to reduce the rate of degranulation [15] (Fig. 3). The rate of degranulation was measured in the linear part of every macroscopic capacitance trace. At a concentration of 0.5 μM GTPγS and high $[Ca^{2+}]_i$ we obtained an average delay of 128 ± 16 ms (mean ± S.E.M., n = 107, 12 cells). 5 μM GTPγS did not produce a significant change on the delay (145 ± 20 ms, n = 64, 15 cells), despite that the rate of degranulation increased by 3-fold, from 70 fF/s to 212 fF/s. Higher doses of GTPγS, as expected, increased even further the rate of degranulation. However, the latency to massive release did not change appreciably. Lowering $[Ca^{2+}]_i$ to 50 nM, elevated concentrations of GTPγS (5, 10 and 25 μM) increase the rate of exocytosis, but the delay between fusion and release obtained for all GTPγS concentrations was, on average, higher than the one obtained in high calcium conditions. These results clearly suggest that the action of cytosolic calcium reside in a different site to the one exerted by other secretagogues that induce vesicle fusion, like GTPγS.

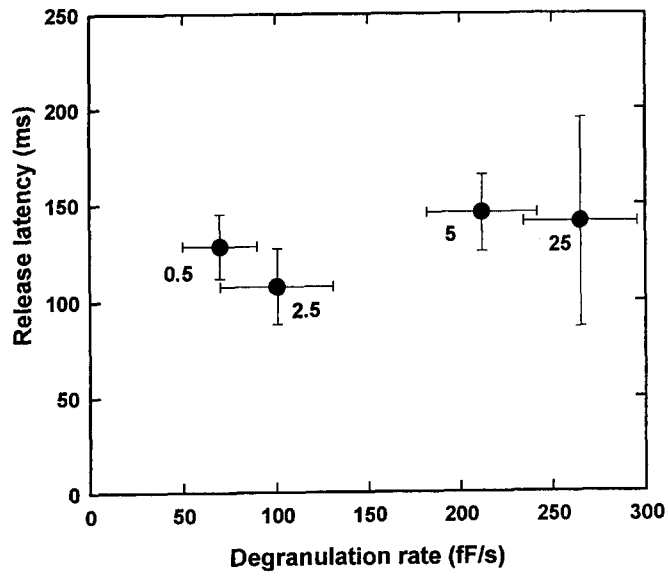

Fig. 3. Effect of the rate of degranulation on the latency to massive release. At high cytosolic calcium the rate of exocytosis increases as GTPγS concentration rises inside the patch pipette (numbers next to the symbols, in μM). However, the lag period between fusion and release did not change significantly. These results show that calcium ions act at a site different from the one that causes vesicle plasma membrane fusion.

4. Discussion

Our results show that vesicle fusion and bulk release are separated processes. There is a lag period between fusion and release reflecting the time required for fusion pore expansion. Although this latency could be explained by a delayed release of secretory material from the polymer granule matrix, once the pore is completely opened, this possibility is unlikely. This conclusion is based on the slight effect of $[Ca^{2+}]_i$ on the characteristics of the amperometric signal, which reflects the dynamics of release of serotonin from the polymer matrix gel [16]. Furthermore, the exponential distribution of lag periods obtained in rat mast cells granule is very similar to the one obtained in beige granules, suggesting that the latency to bulk release occurs during the expansion of the exocytotic fusion pore. These results point to a direct action of calcium on the fusion pore itself. Although the nature of the pore on its very early stage, what it has been called, an early fusion pore [1], is uncertain [17,18], the nature of the late fusion pore, once starts to dilate, is purely lipidic [19]. This conclusion is mainly drawn from the large conductance of the pore at those stages (>1 nS), too large to be mediated by a channel forming protein, like a gap junction or an ion channel. These arguments on the lipidic nature of the pore agree well with the experimental data obtained in rat mast cells, because the minimum estimated pore conductance from a capacitance step with no projection onto the real part of the admittance in a mast cell granule of an average size of 18 fF is 1 nS. Lipidic pores with similar dimensions to exocytotic fusion pores could spontaneously open due to the energy created by membrane curvature [20]. However, micromollar or submicromollar $[Ca^{2+}]_i$ may not be sufficient to modify the shape, and therefore the energy required for pore expansion, of lipidic pores because the calcium dissociation constant of phospholipids is in the millimollar range [21]. This suggests a protein mediated action on the fusion pore expansion process. Increased rates of exocytosis have been observed in neutrophils treated with depolymerizing agents of microtubules [22], and synaptic facilitation has been observed in mutants lacking synapsin I [23]. Both results could be interpreted as vesicles prevented for complete fusion by cytoskeletal elements, but release from their cytoskeletal attachments to fuse fully with the plasma membrane by calcium action. This novel action of calcium ions may be important at active zones at synapses, where elevated cytosolic calcium concentrations are reached within milliseconds. Also, this site could be the target for important physiological mechanisms as facilitation or depression of synaptic terminals.

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References

[1] Breckenridge, L.J. and Almers, W. (1987) Nature 328, 814–817.
[2] Spruce, A.E., Breckenridge, L.J., Lee, A.K., Almers, W. (1990) Neuron 4, 643.
[3] Almers, W. (1990) Annu. Rev. Physiol. 52, 607–624.
[4] Chow, R.H., Rüden, L.v. and Neher, E. (1992) Nature 356, 60–64.
[5] Alvarez de Toledo, G., Fernández-Chacón, R. and Fernández, J.M. (1993) Nature 363, 554–558.
[6] Hamill, O.P., Marty, A., Neher, E., Sakmann, B. and Sigworth, F.J. (1981) Pflügers. Arch. Eur. J. Physiol. 391, 85–100.
[7] Leszczyszyn, D.J., Jankowski, J.A., Viveros, H.O., Diliberto, E.J. Jr.,Nears, J.A. and Wightman, R.M. (1990) J. Biol. Chem. 265, 14736–14737.
[8] Tatham, P.E.R., Duchen, M. and Millar, J. (1991) Pflügers Arch. Eur. J. Physiol. 419, 409–414.
[9] Neher, E. (1988) J. Physiol. 395, 193–240.
[10] Alvarez de Toledo, G. and Fernandez, J.M. (1987) Soc. Gen. Physiol. Ser. 43, 332–344
[11] Fidler, N. and Fernandez, J.M. (1989) Biophys. J. 56, 1153–1162.
[12] Thomas, P., Wong, J.G., Lee, A.K. and Almers, W. (1993) Neuron 11, 93–104.
[13] Neher, E. and Zucker, R.S. (1993) Neuron 10, 21–30.

[14] Heinemann, Ch., Rüden, L.v., Chow, R.H. and Neher, E. (1993). Pflügers Arch. Eur. J. Physiol. 424, 105–112.
[15] Fernandez, J.M., Lindau, M. and Eckstein, F. (1987) FEBS Lett. 216, 89–93.
[16] Curran, M.J. and Brodwick, M.S. (1991) J. Gen. Physiol. 98, 771–790.
[17] Monck, J.R. and Fernandez, J.M. (1992) J. Cell Biol. 119, 1395–1404.
[18] Almers, W. and Tse, F.W. (1990) Neuron 4, 813–818.
[19] Monck, J.R., Alvarez de Toledo, G. and Fernandez, J.M. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 7804–7808.
[20] Nanavati, C., Markin, V.S., Oberhauser, A.F. and Fernández, J.M. (1992) Biophys. J. 63, 1118–1132.
[21] McLaughlin, S. Mulrine, N., Gresalfi, T., Vaio, G. and McLaughlin, A. (1981) J. Gen. Physiol. 77, 445–473.
[22] Nüβe, O. and Lindau, M. (1988) J. Cell Biol. 107, 2117–2123.
[23] Rosahl, T.W., Geppert, M., Spillane, D., Herz, J., Hammer, R.E., Malenka, R.C. and Südhof, T.C. (1993) Cell 75, 661–670.