Myosin II Contributes to Fusion Pore Expansion during Exocytosis

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During exocytosis, the fusion pore expands to allow release of neurotransmitters and hormones to the extracellular space. To understand the process of synaptic transmission, it is of outstanding importance to know the properties of the fusion pore and how these properties affect the release process. Many proteins have been implicated in vesicle fusion; however, there is little evidence for proteins involved in fusion pore expansion. Myosin II has been shown to participate in the transport of vesicles and, surprisingly, in the final phases of exocytosis, affecting the kinetics of catecholamine release in adrenal chromaffin cells as measured by amperometry. Here, we have studied single vesicle exocytosis in chromaffin cells overexpressing an unphosphorylatable form (T18AS19A RLC-GFP) of myosin II that produces an inactive protein by patch amperometry. This method allows direct determination of fusion pore expansion by measuring its conductance, whereas the release of catecholamines is recorded simultaneously by amperometry. Here we demonstrated that the fusion pore is of critical importance to control the release of catecholamines during single vesicle secretion in chromaffin cells. We proved that myosin II acts as a molecular motor on the fusion pore expansion by hindering its dilation when it lacks the phosphorylation sites.

Exocytosis is a fundamental cellular mechanism used by neurons and hormone-secreting cells to interact with each other and to influence their environment through the release of neurotransmitters and hormones. These chemical signals are disposed to the extracellular medium in the form of quanta, as vesicles containing transmitter fuse with the plasma membrane and release their cargo. Release occurs through the exocytotic fusion pore, which is the water channel connecting the vesicle interior to the extracellular space.

The dynamics of the fusion pore have been mainly investigated at the level of single cells by two techniques: patch clamp measure-

ments of the electrical capacitance of the cell membrane (1–3) and the amperometric detection of neurotransmitter with carbon fibers (4, 5). Whereas patch clamp detects changes of cell membrane area and conductance caused by vesicular fusion, the electrochemical method analyzes quantitatively the release of catecholamines from each exocytotic event.

The combination of whole cell capacitance measurements and amperometry showed that during fusion pore opening there is a small release of neurotransmitter preceding the amperometric spike that is directly proportional to the pore conductance (6). Because then, this signal, the prespike foot (PSF) (7) has been considered an indicative of the lifetime of the early fusion pore (7). Later on, higher resolution experiments in rat mast cells by using the patch amperometry technique demonstrated that the size of the fusion pore does not limit release during the upstroke of the amperometric spike (8), and it is accepted that the spike phase corresponds to the fast and massive release of transmitters out of the vesicular matrix after the fusion of cell and vesicular membranes.

In neurons and neuroendocrine cells it has not been possible to report the fusion pore dynamics during the bulk release of the amperometric spike. The role of the fusion pore has been inferred from amperometric recordings alone (9–11), suggesting that many protein candidates control the fusion pore by studying the shape of the PSF or amperometric spike (12–14).

Cytoskeletal proteins play an important role organizing the transport of vesicles to release sites (15). During stimulation, calcium influx induces a dynamic reorganization of the cortical actin network and facilitates exocytosis (16–19). It has been postulated that such transport is based in molecular motors associated with F-actin trails and conduct vesicles along regions with different F-actin organization. More recently, a new role implicating the regulation of single fusion kinetics has been also suggested (20). In that way, myosin II has been postulated to exert a tensional pressure in the F-actin network that could affect membrane tension, fusion pore expansion, or the final extrusion of vesicular contents. In the present work, by using the technique of patch amperometry, we directly determined in chromaffin cells the effect of a protein on the fusion pore dynamics for the first time. This method allowed us to resolve single fusion events from vesicles smaller than 500 nm in diameter by simultaneous measurement of the patch membrane.

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2 The abbreviations used are: PSF, prespike foot; CA, catecholamine; CFE, carbon fiber electrode; RLC, regulatory light chain; GFP, green fluorescent protein; WT, wild type; PKC, protein kinase C.
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capacitance and the release of catecholamines by placing a carbon fiber detector inside the patch pipette. Single-vesicle exocytosis has been determined in rat chromaffin cells infected by modified herpes virus (amplicons) containing the myosin II regulatory light chain (RLC) or an unphosphorylatable form that produces inactive protein (T18A/S19A RLC) chimeras with green fluorescent protein (GFP). Therefore, by direct measurement of fusion pore conductance by admittance methods, we proved that myosin II plays an essential role in fusion pore expansion during exocytosis.

EXPERIMENTAL PROCEDURES

Chromaffin Cell Preparation and Infection—Rat chromaffin cells were obtained from Sprague-Dawley rats (100–200 g) and cultured as described previously (21). The cells were suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 50 IU/ml penicillin, and 50 μg/ml streptomycin. The cells were plated on 35-mm dishes coated with poly-L-lysine (Sigma) and maintained at 37 °C in an atmosphere containing 5% CO2, 95% O2 saturated with water. After 24 h, the primary cultures were infected with a herpes simplex virus (HSV-1) amplicon containing wild type or mutant forms of RLC-GFP constructs obtained as described previously (20). The dilution chosen for the experiments (20–30 μl of virus/1 ml of medium) produced around 10% infection efficiency. GFP fluorescence was observed 1 day after infection and persisted, at least, along the two following days. Patch amperometry experiments were carried out between days 1–3 after culture at room temperature (25 °C).

Reagents and Solutions—The bath solution contained 140 mM NaCl, 2.7 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM HEPES/NaOH, and 5–10 mM glucose. The pH was adjusted to 7.3, and the osmolarity was around 300 mmol/kg. The pipette solution contained 5 mM CaCl2, 5 mM TEA-Cl, 135 mM NaCl, 1 mM MgCl2, 5 mM BaCl2, and 10 mM HEPES/NaOH.

Patch Amperometry—Changes in membrane capacitance and catecholamine release were recorded simultaneously by patch amperometry (22, 23). Briefly, cell-attached patch clamp was achieved with a carbon fiber electrode (CFE) introduced into the patch pipette. A special holder with two Ag/AgCl electrodes was used. The CFE was prepared from 5-

... (both on the vesicle and the extracellular space) of randomly...
distributed molecules in the vesicle (2). The simulation employed dimensions characteristic of the pipette and electrode and those measured of the fusion pore conductance. The diffusion coefficient was that of catecholamines inside the vesicle and in free solution (26, 27). The electrode was evaluated as a disc above the patch inside the pipette, and the concentration at the surface of the disc was set to zero to simulate amperometry. In each instant, a number of oxidizable molecules (given by matrix dissociation) were assumed to diffuse in the vesicle, to escape through the fusion pore, and to diffuse in the surrounding medium. The molecules also were assumed to be reflected, when hitting the cell surface, the pipette, or the cylindrical face of the carbon fiber, or to be absorbed, when hitting the disc-like tip of the carbon fiber. Absorbed particles were assumed to contribute equally to amperometric current.

RESULTS

Delayed Release after Vesicle Fusion in Cells Expressing the Unphosphorylatable GFP-RLC—To simultaneously measure membrane fusion and release of catecholamines in chromaffin cells, we have used patch amperometry (23). This method uses a CFE inside the patch-pipette, detecting exocytotic events occurring at the membrane patch and releasing their contents into the pipette. In addition to the simultaneous measurement of capacitance changes caused by single chromaffin granules and the associated release of catecholamines, patch amperometry allows for a detailed characterization of the dynamics of the fusion pore by off-line admittance analysis (Fig. 1A, bottom panel). We have recorded green fluorescent cells, corresponding to those efficiently infected cells (Fig. 1A, top panel). Sample traces recorded on cells overexpressing the WT RLC-GFP and the unphosphorylatable double mutant T18A/S19A RLC-GFP are shown in Fig. 1 (B and C), respectively. The top traces illustrate step increments in patch membrane capacitance because of the irreversible fusion of single chromaffin granules with the plasma membrane. The bottom traces show amperometric spikes representing CA detection with a CFE placed inside the patch pipette. Every step increase in capacitance was accompanied by an amperometric spike in both experimental conditions, which reflects release of vesicular contents through the fusion pore. In conventional extracellular amperometry, detected spikes originated over the whole cell surface area, giving rise to a wide distribution of spike characteristics caused by CA diffusion. In patch amperometry all of the events detected displayed a rather constant kinetics mostly dependent on the fusion pore lifetime and/or matrix dissociation. Most capacitance changes corresponded to step increases in cell surface area as chromaffin granules fused irreversibly with the plasma membrane. We did not observe differences in the step size distribution in WT RLC-GFP and T18A/S19A RLC-GFP-overexpressing cells (1.11 ± 0.05 fF versus 1.00 ± 0.09 fF, n = 112, eleven cells and n = 73, eight cells, respectively). These values are not either significant different from the average step size for nontransfected cells.
(1.17 ± 0.06 fF, n = 95) (Fig. 1D) and similar to that previously published in rat chromaffin cells (1.13 ± 0.03 fF) (22), indicating that overexpression of either the active form or “null” form of myosin II does not interfere with the formation or ability of chromaffin granules to undergo exocytosis.

The delay between the onset of the capacitance jump and the beginning of the amperometric spike (delay CA) reflects the time required for fusion pore expansion and release of CA. Our experiments showed a longer latency between the capacitance step and the beginning of the amperometric spike in cells expressing the mutant form of myosin II (10.4 ± 1.4 ms) as compared with the wild type protein (7.2 ± 0.7 ms) (Fig. 1E). This effect is more pronounced considering the delay between the onset of the capacitance step and the peak of the spike (delay CP), the time point where the CA concentration reaches the maximum value. The CA signal during fusion pore expansion before bulk CA are released. The delay CA is the stage where the PSF can be observed in the amperometric trace. However, we could hardly detect amperometric feet, probably because of the distance at which the detector is placed from the cell surface in patch amperometry experiments (~5 μm). Moreover, no clear differences were found in the number of PSF detected in WT RLC-GFP and T18A/S19A RLC-GFP cells (5% versus 7%). To quantify the initial phase of fusion pore opening, we performed off-line analysis of the Imaginary and Real traces of the admittance. The spike rise time and the time at the half-height (halfwidth) for the unphosphorylatable mutant were characterized by larger values of 10.38 ± 1.40 and 79.14 ± 5.35 ms, respectively, compared with 7.20 ± 0.69 and 67.60 ± 4.22 ms for wild type RLC-GFP construct. However, there was no significant difference in the mean catecholamine content released in cells expressing both constructs (Q = 4.6 ± 0.36 pC in WT RLC-GFP and 5.2 ± 0.52 pC in T18A/S19A RLC-GFP).

Fusion Pores Are Reluctant to Expand in Cells Expressing the Mutated Form of RLC-GFP—To analyze the hypothesis that the slower rate of vesicle discharge in T18A/S19A RLC-GFP-expressing cells directly depends on the kinetics of the exocytotic fusion pore, we calculated the time course of the fusion pore conductance. Fig. 2A represents the electrical equivalent circuit of a fusion vesicle, in which the fusion pore is represented by a variable conductance (Gp) in series with the vesicle capacitance (Cv) contributed by the vesicle membrane. Current through this circuit flows on fusion pore formation and expansion. The changes in both Gp and Cv can be quantified off-line by analysis of the real and imaginary parts of the admittance (25, 28). Traces in Fig. 2 (B and C) show the fusion pore conductance during two exocytotic events, accompanied by release of CA in a spike-like form in WT RLC-GFP and T18A/S19A RLC-GFP-expressing cells, respectively. In the WT RLC-GFP event (Fig. 2B), the conductance of the pore rises rapidly, reaching a maximum value of ~600 pS in about 15 ms. In the mutant T18A/S19A RLC-GFP (Fig. 2C), the conductance grew at a slower pace (30 ms), reaching only a maximum detectable value of ~200 pS. In both experimental conditions, the fusion pore expanded abruptly and undetectably by our method after reaching the maximum value. The CA signal during fusion pore expansion was slightly different in WT and the double mutant T18A/S19A RLC-GFP. We did not observe a visible amperometric foot during fusion pore expansion. However, the amperometric spike showed a distinct time course as compared with the fusion pore conductance. In both cases, WT and T18A/S19A RLC-GFP, the onset of the amperometric spike occurred when the fusion pore conductance reached ~200 pS. However, the amperometric spike developed at different rates regardless of the fusion pore conductance measured in WT and T18A/S19A RLC-GFP. The time course of fusion pore conductance for 15 WT and 14 mutant vesicles are shown in Fig. 2 (D and G). Most WT cells fusion pores grew up to 300 pS (Fig. 2D). However, cells expressing the mutations consistently showed pore sizes lower than 300 pS (Fig. 2G). In addition, the lifetime of the fusion pore in cells expressing the WT construct has a mean value of 16.9 ± 2.09 ms and in cells expressing the double mutation 23.18 ± 3.2 ms (Log rank test, p = 0.055). The mean maximum pore conductance obtained was ~950 pS in WT RLC-GFP cells and ~200 pS in T18A/S19A RLC-GFP cells. The rate of fusion pore expansion as determined from the slope of Gp in its linear part, was 98.54 ± 55.01 nS s⁻¹ (n = 14) in WT RLC-GFP pores and 14.40 ± 5.34 nS s⁻¹ in T18A/S19A RLC-GFP pores (n = 15). A cumulative histogram of the maximum conductance (Gp max) achieved for all pores in WT cells revealed that more than 50% of the pores displayed maximum conductance values over 300 pS, indicating a continuous expansion of the fusion pore up to 1000 pS, expanding fully to undetectable values of Gp afterward (Fig. 2E). However, mutant cells revealed a cumulative histogram of maximum conductance values under 400 pS (Fig. 2F). The cumulative histogram of pore open times, as measured by the time in which the Gp max value is reached, revealed that over 80% of the pores in WT cells has already opened in 20 ms; meanwhile in mutant cells only a 40% of the pores open in the same time period of 20 ms (Fig. 2, H and I). These results indicate that the expression of T18A/S19A RLC-GFP mutations leads to a slow expansion of the fusion pore with lower conductance values, to finally explode open to release the bulk contents of the secretory vesicle.

To further explore the role of myosin on fusion pore expansion, we analyzed the rates of Gp changes in both experimental conditions (Fig. 3). We characterized the different phases in expansion of the fusion pore during exocytosis. The conductance time course of fusion pores analyzed in Fig. 2 (B and C) are comparatively shown in Fig. 3A. Fig. 3B shows the typical behavior of the fusion pore dynamics from a cell expressing the WT RLC-GFP. We can observe at least two expansion phases. First, there is a low conductance phase. This early stage is well fitted to a linear function. The next stages can be fitted to sigmoid functions with increasing slopes (Fig. 3, B and C). Typically the last phase was absent in cells expressing the T18A/S19A RLC-GFP construct (Fig. 3C), but the first phase (19.77 ± 1.82 ms) and the subsequent expansion phase (13.05 ± 1.24 ms) tended to be longer (Fig. 3D) than those relative to the WT RLC-GFP cells (first phase = 13.02 ± 2.04 ms and second phase = 7.13 ± 1.07 ms). On the other hand, during the initial linear expansion of the fusion pore the net increment of Gp tended to be higher in the mutants (102.16 ± 19.63 pS) (Fig. 3E) than in cells expressing the wild type construct (31.94 ± 14.25...
However, despite the also increased duration of the second phase of pore expansion, the $G_p$ increment was indistinguishable in wild type (127.3 ± 15 pS) and mutated (137.7 ± 22 pS) cells.

**Fusion Pore Expansion Affects the Kinetics of CA Release during the Amperometric Spike**—We could correlate fusion pore conductance with the kinetics of neurotransmitter release from the same fusion event. Recently, it has been reported that the initial fusion pore conductance determines the flux of catecholamines during foot signals (29). However, it is still a matter of debate how the fusion pore affects release during the upstroke of the amperometric spike. To understand how the properties of the fusion pores affect the spikes kinetic, we have compared the maximum pore conductance and the spike rise time in the exocytotic events from the same experiment in cells expressing the wild type RLC-GFP and the T18A/S19A RLC-GFP constructs. We observed that spikes with faster rise time (less than 15 ms) are associated with large pores (Fig. 4A). However, when the fusion pores attains a conductance lower than 500 pS, the spike rise develops at a slower pace. We compare the spike rise time with the lifetime of the fusion pore. Interestingly, we find a linear relation between these two...
parameters in the wild type (Fig. 4B) and in the double mutant (Fig. 4C). These data suggest that the size of the fusion pore and the time required to expand the fusion pore are important determinants of the kinetics of CA release and influence at least the rise phase of the amperometric spike. Because the kinetic parameters of the amperometric spike and the spike rise time in the T18AS19A RLC-GFP type were slower than in the RLC-GFP wild type, our data suggest that myosin II participates controlling fusion pore expansion; the mutation that prevents phosphorylation of the molecule retards the expansion of the fusion pore and, as consequence, delays quantal neurotransmitter release.

Monte Carlo Simulations Explain Experimental Data and Suggest That a Late Fusion Pore Still Controls CA Release during the Decay of the Amperometric Spike—Experimentally it has not been possible to measure the fusion pore conductance during the decay phase of the amperometric spike from a fusing vesicle. To further study the role of fusion pore and myosin II at this stage, we modeled amperometric spikes by entering realistic values of fusion pore expansion in the simulation. Modeling was carried out assuming three different steps in the exocytic process: release through the exocytotic fusion pore, transmitter release from matrix dissociation, and diffusion of molecules into the path pipette.

Visual Basic and Igor software were used to generate Monte Carlo simulation and to account for the relationship between fusion pore expansion time and the amperometric spike kinetics. Simulations of different fusion pores sizes, assuming they do not expand during exocytosis, show that the kinetic properties of spikes are strongly affected (Fig. 5A). To obtain a realistic vision of fusion pore behavior, experimental $G_p$ traces were used to simulate amperometric spikes (Fig. 5B). Simulated currents fitted nicely the time course of the rise time of the amperometric spike until values of fusion pore conductance measured by admittance methods. After the last conductance value measured experimentally, the simulation of the full opening of the fusion pore induced a peak that did not fit with the real spike (Fig. 5B, upper trace). However, when the estimated conductance by Monte Carlo simulation was in the range between the maximum fusion pore size calculated experimentally and 10 times larger, the simulation fitted very nicely with the time course of amperometric data (Fig. 5B, lower trace). Therefore, the simulation can predict what fusion pore sizes account for the time course of the amperometric spike beyond points that could not be measured by admittance measurements. Interestingly, the shape of amperometric spikes experimentally obtained from the mutated version of myosin II can be well fitted by the pure pore properties (Fig. 5C). Additionally, when the calculated fusion pore conductance from a WT and a null mutant fusion event (Fig. 3A) were used as templates to generate the CA release, the resulting amperometric spikes showed kinetics differences similar to those obtained experimentally (Fig. 5D). Because it is improbable that myosin II affects the binding properties of catecholamines to the granule matrix, it is reasonable to assume that any change in spike shape is due to changes in fusion pore characteristics.
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Here, we show a mechanism that can contribute to regulate the speed of neurotransmitter discharge via a post-fusional control of the exocytic fusion pore. This mechanism might be of physiological relevance in neuronal signaling where efficient synaptic transmission is determined by the speed of release of neurotransmitters via rapid opening and expansion of the exocytic fusion pore. The demand for rapid release in chromaffin cells might be also important because these cells secrete epinephrine and norepinephrine, two hormones responsible for the “fight or flight” response. However, the extent of changes reported on this manuscript for the fusion pore by myosin II might not be of physiological relevance in neuronal signaling where efficient synaptic transmission is determined by the speed of neurotransmitter discharge via a post-fusional control of the exocytic fusion pore. This mechanism might be of physiological relevance in neuronal signaling where efficient synaptic transmission is determined by the speed of release of neurotransmitters via rapid opening and expansion of the exocytic fusion pore.

The effect of myosin on fusion pore expansion can be explained by a limited fusion pore expansion in cells expressing mutated form of myosin II; the second one proved that the pore size at which this is attained is smaller as compared with WT cells. Both results indicate that fusion pore expansion is hindered under conditions in which myosin II is not normally phosphorylated. In addition, these results are further supported by the fact that spike parameters are affected accordingly, as it has also been shown previously (19). By using Monte Carlo simulation, we demonstrate that the alterations in spike parameters can be explained by a limited fusion pore expansion in cells expressing the mutated form of myosin II.

The effect of myosin on fusion pore expansion can be explained by two opposing mechanisms. One is that myosin actively contributes to fusion pore expansion, so myosin phosphorylation plays an active role in expanding the fusion pore. Second, myosin acts as a break in a favorable energetically driven process. In such way, the lack of myosin phosphorylation hinders fusion pore expansion.

Exocytosis involves the fusion of phospholipid bilayers subjected to surface tensional forces. In isolated lipid bilayer fusion, surface tension of the membrane of liposomes is sufficient to favor the insertion of a fusing vesicle into a planar membrane. Similarly, in physiological systems, during exocytosis the surface and lateral tension of secretory vesicles can lead to full fusion of the vesicle membrane into the plasma membrane. Therefore, membrane fusion is an energy favorable process, in which the initially formed fusion pore would expand spontaneously depending on the unbalanced surface tension between the plasma membrane and vesicle membrane. This may explain why fusion pores of smaller vesicles expand faster than fusion pores of larger vesicles like peritoneal mast cells or beige mouse.
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FIGURE 5. Modeling the time course of catecholamine release by Monte Carlo simulation. A, simulated currents from a chromaffin vesicle with a 180-nm diameter. Two different sizes of pores were considered (4- and 75-nm radius). B, simulated spikes (red traces) from an experimentally measured fusion pore (solid blue). After the last conductance value measured experimentally, the full pore opening was simulated (yellow trace) by Monte Carlo. The experimental quantal shape (dashed red) can be reproduced adding a theoretical conductance value not measured (2 nS) (dashed blue). A dependence of the decay phase in the amperometric spike on the fusion pore size is thus predicted. C, the simulated current (markers) fits the experimental release obtained experimentally (solid line). A fusion event from a T18A/S19A RLC-GFP cell was taken as a model. D, comparison of simulated amperometric spikes from real data taken into account vesicular matrix dissociation, release through the exocytotic fusion pore and diffusion of molecules into the patch pipette. WT myosin II (red) and null mutant (dashed red) fusion pore events (inset) were used to generate the simulated currents. The spikes were scaled to have similar values for their respective peak.

Mast cells (6). In our experiments fusion pores in chromaffin granules expand gradually and irreversibly. Because we are using cell membrane capacitance measurements in the cell-attached configuration, we can follow pores of up to 10 nS for chromaffin granules (31).

WT chromaffin cells expand their fusion pore in three observable phases, each one corresponding to gradually opened stages. This could account for a cooperative process in fusion pore expansion. The molecular structure underlying this behavior is unknown, although several models have been discussed over the years about the nature of the exocytotic fusion pore (32, 33). In our hands, preventing phosphorylation of myosin II with the mutated form of the molecule slows down all three phases in fusion pore expansion, suggesting that myosin does not play a critical role in the fusion pore itself but acts as a factor contributing to the rapid kinetics of pore expansion. These results point to myosin II as one of the partners of the protein scaffold that leads to membrane fusion during exocytosis (34).

Monte Carlo simulations also suggest that the fusion pore limits release of CA at higher \( G_p \) values, which are beyond the limit of the resolution of \( G_p \) values measured by admittance methods. Experimental data from T18A/S19A RLC and WT RLC cells studied by Monte Carlo model suggest that the fusion pore conductance always limits release during exocytosis. In addition, the fact that fusion pores expand normally in fusing chromaffin granules that lack CA contents (35) suggests that the CA contents or the granular matrix does not play a critical role in fusion pore expansion at this stage of the process. On the whole, these data suggest that the role of myosin II on the exocytosis affects the expansion process because the fusion pore opens until the vesicle membrane becomes fully incorporated into the plasma membrane.

It has been shown that myosin II is associated with the F-actin network and that even the most external docked granules remain associated with filaments of this mesh that otherwise control restricted granule motion in the proximity of releasing sites (19, 35). In fact, F-actin staining with phalloidin-rhodamine demonstrated a clear co-localization of peripheral RLC-GFP and cortical F-actin (supplemental Fig. S1) in cells overexpressing wild type or the unphosphorylatable T18A/S19A RLC-GFP. Therefore the simpler hypothesis to explain how myosin II influences the very final stages of membrane fusion is that the subcortical actin-myosin II network dynamics favor the normal physiological fusion pore expansion. Myosin II phosphorylation by the myosin light chain kinase is required to sustain the F-actin cytoskeletal dynamics controlling vesicle motion (18). The substitution of the myosin II RLC by a nonphosphorylatable form will result in a static F-actin network unable to sustain both vesicle motion (19) and normal pore expansion. In fact, the contribution of myosin phosphorylation on the fusion pore can be related with the influence of treatments favoring protein phosphorylation or dephosphorylation on the exocytotic fusion of secretory granules. Phorbol 12-myristate 13-acetate accelerates expansion of exocytotic fusion pores once they have been formed, but this effect is inhibited when phorbol 12-myristate 13-acetate is applied together with the kinase inhibitor staurosporine (37). However, fusion pores can expand fully even when PKC was inhibited. It has been also reported that site-specific phosphorylation of SNAP-25 and Munc18 by PKC regulates distinct stages of exocytosis (38). If the expansion of the fusion pore is modulated by PKC and cells expressing the unphosphorylatable form of myosin (T18A/S19A RLC-GFP) retard the expansion of fusion pores, myosin could be a target protein for PKC-mediated phosphorylations. Alternatively, myosin II might be able to influence the activity of the molecular machinery of exocytosis. The hypothesis that a scaffold of proteins directs exocytotic membrane fusion (39) was proposed based in morphological analysis in which filamentous structures, as actin, are seen spanning the gap between the secretory granule and cell membranes (40–42). More recently, an association has been demonstrated between the tail of the heavy chain of unconventional myosin V and synaptobrevin II forming part of...
the exocytic fusion complex in synaptic vesicles (43). Therefore, as a second possibility, it is tempting to suggest that different myosins, through interactions with synaptobrevin or other proteins of the fusion machinery, could be modulating the series of transitions occurring in the exocytic fusion complex. The combination of molecular tools (viral infection) and a very sensitive biophysical technique (patch amperometry) has allowed us to understand how changes in fusion pores affect neurotransmitter release and reveals new insights about the role of myosin II. This pioneer strategy opens the possibility to study the function of many proteins identified as key players in exocytosis.

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