Exo–endocytosis and closing of the fission pore during endocytosis in single pituitary nerve terminals internally perfused with high calcium concentrations

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Communicated by Bert Sakmann, February 9, 1994 (received for review August 30, 1993)

ABSTRACT An increase in free Ca\(^{2+}\) triggers exocytosis in pituitary nerve terminals leading to an increase in membrane area and membrane capacitance. When Ca\(^{2+}\) is increased by step depolarization, an instantaneous capacitance increase during the first 80 ms is followed by a slow increase extending over several seconds. We measured capacitance changes associated with exocytosis and endocytosis in single pituitary nerve terminals internally perfused with high Ca\(^{2+}\). At 50 μM Ca\(^{2+}\) the capacitance increased by up to 2%/s, similar to the slow phase observed during depolarization. Our results indicate that at the site of fusion very high Ca\(^{2+}\) is required. Following exocytosis, large downward capacitance steps were measured, reflecting endocytosis of large vacuoles. These events were not abrupt but reflected a gradual decrease of fusion pore conductance from 8 nS to 30 pS during 500 ms, revealing the dynamics of individual fusion pore closures. Above 300 pS, narrowing of the endocytic fusion pore was ~10 times slower than the previously reported expansion of the exocytotic fusion pore. The transition between 300 pS and 0 pS took ~200 ms, whereas it has been reported that the exocytotic fusion pore measured in mast cells opens from 0 to 280 pS in <100 μs. The time course of closing of the fusion pore may be explained by an exponential decrease in pore diameter occurring at a constant rate.

Neurosecretion of transmitters stored in small vesicles and of peptides stored in dense core vesicles involves cycles of exo–endocytosis. The membrane of secretory vesicles is inserted into the plasma membrane during exocytosis and is subsequently internalized by an endocytic mechanism (1, 2). This function occurs specifically in specialized parts of the neuron, the nerve terminals. Pituitary nerve terminals secrete the peptide hormones vasopressin and oxytocin. Depolarization-induced exocytosis of these hormones is followed by increased endocytosis of vacuoles slightly bigger than the secretory vesicles (3). This uptake is rapid (4) and involves a mechanism that leads to specific internalization of the previous vesicle membrane proteins (5), suggesting that granule fusion may be transient. Following intense stimulation, endocytosis of very large vacuoles was observed in chromaffin cells (6), suggesting endocytosis after full incorporation of granule membrane into the plasma membrane. The changes in plasma membrane area associated with exo- and endocytosis can be measured with high resolution in single cells by time-resolved patch-clamp capacitance measurements using a lock-in amplifier (7). This technique was recently also applied to follow exo–endocytosis in isolated pituitary nerve terminals (8, 9).

After internal dialysis of nerve terminals with high Ca\(^{2+}\) concentrations, we observed exocytosis of secretory vesicles followed by endocytosis of large vacuoles similar to previous results obtained in chromaffin cells (7). When the fusion pore which connects the interior of the endocytic vacuole to the extracellular space becomes very small, the finite pore conductance can be measured to provide an estimate of the size of the pore as described for the reverse process, the opening of exocytotic fusion pores (10, 11). Using these techniques, we have recorded the final 500 ms of fusion pore closure.

MATERIALS AND METHODS

Nerve endings were obtained from the hypophysis of male Sprague–Dawley rats (260–500 g) (12). The neural lobe was removed from the pars intermedia and the pars distalis and was gently homogenized in an Eppendorf tube containing 270 mM sucrose/10 μM EGTA/10 mM Hepes (300 mosmolal, pH 7.25). The resulting suspension was not further purified. One hundred to 200 μl was placed in a Petri dish with a glass bottom (coverslip), and 1–3 ml of medium 199 (Biochrom, Berlin), containing 7 μM glucose, 25 mM glucose, and 5% fetal bovine serum (320 mosmolal, pH 7.25) was added. Each preparation contained hundreds of small nerve endings and also 40–60 nerve endings with a diameter of 8–15 μm. In the experiments described here most nerve endings had a diameter of 8–10 μm and an initial capacitance of 1.5–2.5 pF. The dishes were kept in an incubator with a humidified atmosphere of 10% CO\(_2\)/90% air at 37°C.

Time-resolved patch-clamp capacitance measurements were done with the lock-in amplifier method (7) in the whole-terminal mode with a holding potential of −80 mV by using an EPC9 patch-clamp amplifier (HEKA Electronics, Lambrecht/Pfalz, Germany). The sine-wave command voltage had a frequency of 800 Hz and 10 mV rms amplitude. The time constant of the lock-in amplifier (PAR 5210, EG & G, Princeton, NJ) was set to 10 ms, 12 decibels, and was sampled by the computer every 25 ms. Exocytosis was stimulated by intracellular application of Ca\(^{2+}\) through the patch pipette. Following patch disruption the sine wave was switched on. The phase was determined automatically by phase tracking (13) and the capacitive and ohmic changes were calculated on-line in the computer (14, 15). Pipette solutions contained free Ca\(^{2+}\) at various concentrations [125 mM cesium glutamate/20 mM tetraethylammonium chloride/10 mM Hepes/2 mM MgCl\(_2\)/2 mM Na\(_2\)ATP with 1.1–3.5 μM Ca\(_{\text{II}}\) and 2.0–3.64 mM N-hydroxyethylthioldiaminetetraacetic acid (HEDTA) giving free Ca\(^{2+}\) concentrations of 13–135 μM, 300 millimolar, pH 7.25] buffered by HEDTA and ATP. The free HEDTA concentration varied between 0.9 mM at 13 μM free Ca\(^{2+}\) and 0.1 mM at 135 μM. For 150 μM free Ca\(^{2+}\) the MgCl\(_2\) concentration was raised to 4 mM. To obtain 3.5 μM free Ca\(^{2+}\), we used 5 mM EGTA and 4.8 mM CaCl\(_2\). The Ca\(^{2+}\) concentrations in the pipette solutions were

Abbreviation: [Ca\(^{2+}\)], intracellular concentration of free Ca\(^{2+}\).

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calculated by a computer program and confirmed by measurements with a Ca\(^{2+}\) electrode. Inside the nerve ending endogenous buffers could retard the initial increase in Ca\(^{2+}\). Because of the large volume of the pipette solution these buffers will not affect the steady-state intracellular concentration of free Ca\(^{2+}\) ([Ca\(^{2+}\)].) However, Ca\(^{2+}\) transporters in the plasma membrane might slightly reduce the steady-state [Ca\(^{2+}\)]. The Ca\(^{2+}\) concentrations given here should thus be considered as approximate values. The bath solution was 140 mM NaCl/2 mM CaCl\(_2\)/1 mM MgCl\(_2\)/10 mM Hepes/5 mM KCl/20 mM glucose (275–290 millimolar, pH 7.25). All experiments were done at room temperature.

**RESULTS AND DISCUSSION**

Very High [Ca\(^{2+}\)]\(\text{a}\) Is Required to Stimulate Exocytosis. When nerve terminals were internally perfused with pipette solutions containing EGTA and no added Ca\(^{2+}\), no increase of plasma membrane capacitance was induced, and we determined the specific capacitance of resting nerve terminals to be 7.6 ± 0.5 fF/μm\(^2\), using the capacitance compensation of the patch-clamp amplifier by estimating the membrane area from the terminal diameters under the microscope and assuming spherical shape. [Ca\(^{2+}\)], in the range 50–150 μM in the pipette solution stimulated a capacitance increase which started immediately after the beginning of internal perfusion (patch disruption). Fig. 1 shows the time course of plasma membrane capacitance (upper trace) and conductance (lower trace) in a nerve terminal internally perfused with 50 μM Ca\(^{2+}\), a concentration where the highest rates of capacitance increase were observed. The capacitance changes are not correlated with changes in the conductance trace. The capacitance trace thus accurately reports changes in membrane area and is not distorted by changes in membrane conductance or pipette resistance. The trace starts with a steep, apparently continuous increase with an initial slope of 33 fF/s corresponding to an increase of ~2%/s. Between 3.5 and 30 μM [Ca\(^{2+}\)], no such increase was observed. Peptidergic vesicles have a mean diameter of 180 nm (16). For a specific capacitance of 7.6 fF/μm\(^2\) this corresponds to a membrane capacitance of about 0.8 fF per vesicle. The capacitance increase can thus be converted into an exocytotic rate of 40 vesicles per second. However, since endocytosis of small vesicles may occur simultaneously (4), this value should be considered as a lower limit.

![Fig. 1. Time course of capacitance (upper trace) and conductance (lower trace) measured in a nerve terminal internally perfused with a pipette solution containing 50 μM free Ca\(^{2+}\). The recording started 3-5 s after patch disruption and beginning of internal perfusion with the pipette solution. At the star the capacitance compensation was transiently reduced by 0.2 pF. The upward deflections in the conductance trace were generated by switching a 1-MΩ resistor between ground and the bath electrode for automatic phase tracking.](image)

When [Ca\(^{2+}\)], is elevated by Ca\(^{2+}\) entry through voltage-dependent Ca\(^{2+}\) channels during step depolarization, an instantaneous capacitance increase, which occurs during the first 80 ms, is followed by a second phase with a slow increase extending over several seconds (8). The initial slope of the slow increase during depolarization is 1–2%/s and is thus very similar to the maximal rate which we observed here when [Ca\(^{2+}\)], was elevated by pipette Ca\(^{2+}\) buffers. However, during depolarization [Ca\(^{2+}\)], never exceeds a few micromolar as measured by fura-2 (8). When we introduced Ca\(^{2+}\), in a concentration range between 3 and 30 μM, the slope of the capacitance increase was always <0.1%/s. This apparent discrepancy suggests that the Ca\(^{2+}\) concentration beneath the plasma membrane during depolarization may be >10-fold higher than the average [Ca\(^{2+}\)], in the whole nerve terminal measured by fura-2. The initial fast exocytotic burst observed during the first 80 ms of depolarization (8) could not be detected in the present experiments, since it presumably occurs immediately after patch disruption.

**Downward Capacitance Steps Indicate Endocytosis of Large Vacuoles.** Superimposed on this smooth increase, eight large downward steps having a size of 37–270 fF are seen in Fig. 1, presumably reflecting endocytosis of large vacuoles. Similar observations were reported for bovine chromaffin cells (7), where the formation of large cytosolic vacuoles after intense stimulation was also seen in electron micrographs (6). In our experiments the step size was 114 ± 68 fF (mean ± SEM, n = 17 steps), corresponding to endocytic vesicles with a diameter of 2.1 ± 0.7 μm, indicating that a similar mechanism for endocytosis of large vacuoles exists in pituitary nerve terminals. The downward capacitance steps were not due to bleb formation outside the nerve terminal, since during the recordings no such structures were observed under the microscope. Instead, formation of visible vacuoles inside nerve terminals was seen. These intraterminal vacuoles had the right size to correspond to the downward capacitance steps. However, they were also observed in the absence of large downward steps.

In Fig. 1 all events together represent a capacitance decrease of 1130 fF opposed to an exocytotic increase of 1530 fF, so that exocytosis and endocytosis are nearly balanced. Endocytosis of large vacuoles was observed in 10 out of 72 nerve terminals with pipette solutions containing 3–150 μM free Ca\(^{2+}\). Although endocytosis of large vacuoles was thus rare, these large endocytic events allowed us to examine the fusion process in more detail.

**Time Course of a Single Fission Event.** The third downward step of Fig. 1 is shown on an expanded time scale in Fig. 2A (top trace). It can be seen that the measured capacitance decrease is not abrupt but requires at least 300 ms. During the final stages preceding the fission event, the endocytic vacuole may be connected to the extracellular space by a narrow pore with low electrical conductance. We call this the fission pore, in analogy to the fusion pore which opens during the reverse process of exocytosis (10, 11). The nerve terminal should thus be described by the equivalent circuit of Fig. 2B. During the actual fission event only the pore conductance (G\(_{P}\)) should change while the other elements of the equivalent circuit are constant. The time course of the pore conductance can then be calculated from the time course of the measured capacitance change as previously described for the opening of an exocytotic fusion pore (10, 11, 15).

Knowing the capacitance of the endocytosed vesicle (C\(_V\)) from the size of the capacitance decrease, the deviation of the capacitance trace from an abrupt down step allows us to calculate the pore conductance (10, 15). When the capacitance shows a significant decrease below the initial value (arrowhead in Fig. 2A), the pore conductance becomes measurable (Fig. 2A, second trace). In this example the decrease of pore conductance occurs in phases. Between
these phases the conductance halts at 3.5 and 2 nS for about 25 ms (arrows). The decrease down from 5 nS occurs with an overall slope of about $-16$ pS/ms.

To confirm that the gradual capacitance decrease is indeed due to a gradual decrease of pore conductance, the in-phase output of the lock-in amplifier was examined. For the time course of fusion pore conductance shown in Fig. 2A the predicted shape of the in-phase signal is shown in the third trace. The measured in-phase signal (Fig. 2A, bottom trace) agrees very well with the prediction, confirming that our analysis is correct.

**Average Time Course of Fission Pore Closure.** Seventeen fission events were analyzed in the described way. To compare the time course of the different events we have chosen the time when the pore conductance had a value of 500 pS as the reference time, and the traces are superimposed in Fig. 3A. Although there is some variability the overall shape of all traces is quite similar. Below 400 pS the noise makes it very difficult to determine the time course of fission pore conductance.

To determine the mean time course at improved signal-to-noise ratio, the traces were averaged (Fig. 3B, solid line). Between 4 nS and 300 pS the pore conductance decreases with a slope of $-18$ pS/ms. The final closing occurs with a slope of $-1.5$ pS/ms extending over 200 ms. The question arises whether the change in slope is a property of individual pores or whether it reflects two different populations within the average, with some pores closing rapidly and others slowly. The time course of the fission event of Fig. 2A is thus shown as the dashed line for comparison. This single pore closure resembles the average behavior quite well. Up to $+85$ ms, where the pore conductance is as low as 140 pS, the agreement is very good. The bending and the reduced slope below 300 pS are also apparent in the single event. The fission pore closure of Fig. 2A is thus representative of the average behavior.

To convert the conductance change into a change of the size of the fission pore, certain assumptions have to be made regarding the pore geometry. A decrease in pore conductance may be generated by an increase in pore length, a decrease in pore diameter, or both. The simplest model is a cylindrical pore with a length of 150 Å (15 nm), equivalent to the thickness of two membrane bilayers. For a given pore conductance this assumption provides the time course of the smallest possible pore diameter, as shown in Fig. 3C. The diameter decreases from 80 Å to zero within 500 ms. When the closing is fitted with a single straight line, an average closing rate of 200 Å/s is obtained. However, the slope appears to change, indicating that the diameter decreases from 65 Å to 20 Å within 200 ms at a rate of about 220–280 Å/s, whereas the final closing down to <6 Å takes another 200 ms with a rate of about 80 Å/s. The circumference of the pore correspondingly decreases from 280 Å to 60 Å at $\approx$800 Å/s and down to <20 Å at $\approx$250 Å/s. The size of a lipid head group is about 8 Å. We have followed the pore conductance down to 40 pS, corresponding to a pore size of $\approx$6 Å, which is just about the size of a pore which may be formed by a ring of three lipids, indicating that we are indeed observing the properties of the very final stages of membrane fission. At the two slopes lipids must be removed from the pore circumference at rates of 100 and 30 lipid molecules per second, respectively. A simple model would be to assume that the rate at which lipids are removed from the circumference depends on the number of lipids present and should thus be proportional to the length as well as to the pore diameter. According to this model the pore diameter should decrease exponentially. A single-exponential fit (Fig. 3C, dashed line) fits the overall time course quite well, giving a rate constant of 5–7 s$^{-1}$. This corresponds to a time constant of 150–200 ms and removal of 10% of the lipids in 15–20 ms.

If the decrease in pore conductance were entirely due to an increase in the length of the pore, as would be the case for a vesicle pulled into the nerve terminal and connected to the exterior by a membrane tube of fixed diameter, the increase in length would be proportional to an increase in pore resistance. The time course of average fission pore resistance is plotted in Fig. 3D. This plot shows the time course of the increase in the lengthening rate, which we consider unlikely.
As a third possibility we consider simultaneous lengthening and narrowing of a pore with constant surface area of the cylinder forming the pore wall. If the length increases linearly in time, the pore resistance should increase proportionally to $r^3$ (Fig. 3D, dotted line) which does not fit the observed time course. Again the observed sudden increase in slope indicates that a continuous change in the shape of a membrane tube cannot account for the observed time course of fission pore closure. At some point preceding the separation of the vesicle membrane from the plasma membrane, the resistance should obviously be determined by a short and narrow pore. We suggest that at least during the late phase of fission pore closure ($r > 0$, pore conductance $< 500$ pS) the conductance reflects the properties of a short pore of molecular dimensions.

The properties of the fission pore may be compared with those of the fusion pore that opens during exocytotic granule-plasma membrane fusion. The fusion pore conductance attains an average value of 280 pS within 100 µs and subsequently expands at a rate of 200 pS/ms (11), which is about 10 times faster than the narrowing rate which we observe when the fission pore conductance is $> 300$ pS. The transition between 300 pS and 0 pS is even slower in contrast to the abrupt opening of the exocytotic fusion pore (10). It has been proposed that the initial event in exocytotic fusion may be the opening of a preassembled fusion pore similar to a gap junction (17). It is unlikely that the final fission event involves a similar protein structure. The mechanisms of fission and fusion may thus be quite different. On the other hand, expanded exocytotic fusion pores are able to close again, although at that stage they already allow for transfer of lipid (18).

The processes of fusion and fission have also been observed in pure lipid systems, and attempts have been made to explain fusion and fission by changes in membrane tension and bending energy (19-21). With the right choice of parameters the observed transitions could be reproduced by these models. Although they thus provide a possible explanation, so far no data are available which independently determine the assumed changes in tension and bending energy.

Exocytotic reuptake of membrane following exocytosis may involve several distinct mechanisms (22). It is widely believed that synaptic vesicles or chromaffin granules are fully incorporated into the plasma membrane and are subsequently internalized via coated pits (1, 2, 23, 24). However, there is also evidence that internalization of synaptic vesicles (22), as well as dense core vesicles in pituitary nerve terminals (3-5), may occur immediately after exocytosis without full fusion with the plasma membrane. Endocytosis of the large vacuoles investigated here was previously observed in chromaffin cells after intense stimulation (6, 7) and may be related to clathrin-independent internalization of macropinosomes (25). Very little is known about the mechanism of this endocytic pathway. Electrical measurements of fusion events and the effect of biochemical manipulations of endocytosis on the closing of the fission pores may provide further insight into the endocytic machinery.

We are indebted to Jean Nordmann for his encouragement and participation in the early part of this work. We wish to thank Wolf Almers and Mike Clague for very helpful comments on the manuscript. This work has been supported by the Deutsche Forschungsgemeinschaft (Sfb 312/B6) and by the Deutscher Akademischer Austauschdienst (311-pro-ca).