Local electrostatic interactions determine the diameter of fusion pores

Alenka Guček1, Jernej Jorgačevski1,2, Urszula Górska1, Boštjan Rituper1, Marko Kreft1,2,3, and Robert Zorec1,2,*

1Laboratory of Neuroendocrinology-Molecular Cell Physiology; Institute of Pathophysiology; Faculty of Medicine; University of Ljubljana; Ljubljana, Slovenia; 2Celica Biomedical; Ljubljana, Slovenia; 3Biotechnical Faculty; University of Ljubljana; Ljubljana, Slovenia

Keywords: astrocytes, exocytosis, fusion pore, HCN channels, lactotrophs, vesicles

Introduction

In regulated exocytosis vesicular and plasma membranes merge to form a fusion pore in response to stimulation. The nonselective cation HCN channels are involved in the regulation of unitary exocytotic events by at least 2 mechanisms. They can affect SNARE-dependent exocytic activity indirectly, via the modulation of free intracellular calcium; and/or directly, by altering local cation concentration, which affects fusion pore geometry likely via electrostatic interactions. By monitoring membrane capacitance, we investigated how extracellular cation concentration affects fusion pore diameter in pituitary cells and astrocytes. At low extracellular divalent cation levels predominantly transient fusion events with widely open fusion pores were detected. However, fusion events with predominately narrow fusion pores were present at elevated levels of extracellular trivalent cations. These results show that electrostatic interactions likely help determine the stability of discrete fusion pore states by affecting fusion pore membrane composition.
could affect the stability and local curvature of the pore, as proposed by Kabaso et al. 18

Recently, we reported that Hyperpolarization-activated Cyclic Nucleotide-gated (HCN) channels modulate fusion pore properties.19 HCN channels are permeable to cations (Na++, K+ and Ca2++)20-22 and likely affect exocytosis indirectly by increasing the local [Ca2++]i, but may also contribute directly via electrostatic interactions with charged membrane constituents near the fusion pore. Here, we assessed the contribution of electrostatic interactions, mediated by extracellular di- and trivalent cations, to changes in fusion pore conductance, a parameter reporting pore geometry and in particular fusion pore diameter.23 For this we have first modified the extracellular concentration of cations by removing Ca2+ from or by adding Al3+ to the extracellular solution and then studied the discrete states of fusion pore.

Results and Discussion

HCN2 channels modulate exocytosis

It was shown previously that an increase in intracellular second messenger cAMP affects exocytotic events in cultured pituitary lactotrophs19 and that some of the modulations are mediated by HCN channels, which are expressed in the plasma membrane and in the membrane of secretory vesicles.24 If the plasma membrane-resident HCN channels are activated, then this may increase the local [Ca2++]i, a stimulus known to increase the exocytotic activity.25 However, in lactotrophs overexpressing HCN2 channels, the overall [Ca2++]i was lower compared to non-transfected lactotrophs and an increase in intracellular cAMP did not significantly affect [Ca2++]i,24 consistent with previous results.26 A possible explanation is that the activation of sarco/endoplasmic reticulum Ca2+-ATPase (SERCA)27 decreased global [Ca2++]i, as depicted in the model (Fig. 1). How, then, did exocytotic activity increase in the study by Calejo et al.24 It is possible that Ca2+ is still increased locally, in the proximity of the fusion pore. Alternatively, HCN channels could modulate exocytosis through a more general local cloud of positively charged ions that may potentially lead to lipid demixing at the region of the fusion pore.18 To date, the exact proteo-lipidic composition of the fusion pore remains unclear.28 However, it is safe to assume that anisotropic membrane constituents (proteins, lipids or other nanodomains) can attribute to its stability.29,30 The constituents of biological membranes (lipids, glycoproteins, glycolipids, etc.) frequently carry one or more ionized or polar groups17 and are influenced by local cation clouds.31,32 Prime candidates are widespread anisotropic anionic lipids, known to bind di- and trivalent cations, such as phosphoinositides (PI), phosphatidic acid (PA) and, particularly, phosphatidyserine (PS).33 The interaction of anionic lipids with cations (especially Ca2+) can dehydrate anionic lipid head groups and consequently alter local membrane curvature and lipid molecule packing into the local membrane regions (e.g. in the fusion pore region),28 leading to lateral phase separation of membrane components.34 These changes can then affect anisotropic neutral (e.g., cholesterol) and zwitterionic (e.g. phosphatidylethanolamine) lipids,35 known to participate in regulated exocytosis.36 Therefore, changes in cation concentration in the pore area likely influence the membrane fusion process during exocytosis.

Removal of extracellular divalent cations results in fusion pores with relatively wide diameters

HCN2 channels have been shown to reside in or near vesicles in lactotrophs.24 Manipulation of HCN channels, where their presence was either increased by HCN2 overexpression or their rhythmic re-opening was accelerated by cAMP, amplifies HCN-specific In current,24 which likely increases local cytoplasmic cation concentration near fusion pores (Fig. 1A). Simultaneously, the proportion of narrow fusion pores was decreased.24 To assess if the observed effect may in part be attributed to the electrostatic interactions, we designed a conceptually similar experiment, where instead of increasing divalent cation concentration intracellularly, we removed Ca2+ ions from the extracellular space. Then, cell-attached patch-clamp technique was employed to measure reversible discrete steps in the membrane capacitance (Cm), corresponding to unitary, transient fusion events of vesicles with the plasma membrane in real time.36

To test the robustness of our predictions on the general importance of electrostatic interactions in regulated exocytosis, we performed these experiments on a different cell type – astrocytes. Astrocytes are electrically silent and abundant glial cells in the brain, which actively contribute to information processing in the central nervous system by releasing gliotransmitters.37 In astrocytes, reversible discrete steps in Cm were observed in controls with 2 mM Ca2+ (Fig. 2A) and in conditions without Ca2+ (Fig. 2B). Here, we focused only on reversible exocytotic events, which likely represent transient fusion pore openings.38 A fraction of reversible events exhibit a measurable (narrow) fusion pore conductance, which is discerned by the projection between the imaginary (Im) and the real (Re) parts of admittance signals.39 In controls half of the reversible events exhibited projections to the Re trace (Fig. 2A). For these events the average fusion pore conductance of 35 ± 4 pS was calculated, which corresponds to the average fusion pore diameter of 0.73 ± 0.05 nm (n = 12 cells) (see Materials and Methods for details). In contrast, in astrocytes that were bathed in Ca2+-free ECS, reversible exocytotic events exhibited no projections to the Re trace (Fig. 2B), indicating fusion pores with relatively wide diameters. Experimentally determined detection limit for projected exocytotic events with our recording system was determined at ~2.6 nm. Non-projected exocytotic events therefore exhibit fusion pores wider than ~2.6 nm in diameter. Moreover, the frequency of all reversible exocytotic events was significantly lower in Ca2+-free ECS (0.14 ± 0.06 events/min, n = 12 cells) compared to ECS with 2 mM Ca2+ (2.2 ± 0.2 events/min, n = 12 cells, P < 0.001, U-test).

This experiment shows that the removal of extracellular Ca2+ reduces exocytotic activity. However, fusion pores that are formed have relatively wide diameters, which is in line with the proposed model A (Fig. 1). Moreover, compared to HCN2 overexpressing cells, where [Ca2++]i was also shown
to be reduced, the observed effect was even stronger, since none of the events exhibited projected (narrow) fusion pore (compared to the 17% of narrow fusion pores observed in cells overexpressing HCN2).

Extracellular trivalent cations stabilize fusion pores with relatively narrow diameters

In lactotrophs exposed to HCN2 blocker (ZD7288), the HCN-specific $I_h$ current was decreased, indicating a reduction in local cytoplasmic cation concentration (Fig. 1B). In this case the proportion of narrow fusion pores recorded was increased. This effect was even more profound after the addition of cAMP, which likely triggered the activation of SERCA pumps, subsequently decreasing cytoplasmic cation (Ca$^{2+}$) concentration. To further validate our model, we conducted conceptually the opposite experiments, as depicted in model B (Fig. 1). To increase the local cation concentration in the extracellular space, we monitored discrete changes in $C_m$ of lactotrophs bathed in ECS containing 30 μM Al$^{3+}$.

Here, the majority of reversible exocytotic events were projected to the Re trace of the admittance signal (Fig. 3A). Compared to the previous reports, where ~25% of reversible events exhibited projections to the Re trace in conditions where normal ECS was used, Al$^{3+}$-treatment significantly increased the percentage of reversible events to 77% (Fig. 3B), suggesting strong stabilization of narrow exocytotic fusion pores (Fig. 3). Although Al$^{3+}$ has a wide range of modus operandi, electrostatic interactions could, as proposed in the model (Fig. 1B), be responsible for this outcome. The average frequency of all reversible exocytotic events was significantly lower in Al$^{3+}$-treated lactotrophs (0.45 ± 0.09 events/min, n = 8 cells) compared to controls (2.5 ± 0.9 events/min, n = 8 cells; $P < 0.001$, U-test), as previously reported.

Local cation concentration modulates discrete fusion pore state

We propose that changes in divalent cation concentration near fusion pores determines the extent of cation binding with charged membrane constituents, which affects membrane curvature and affects lipid demixing. These changes then, in turn, provide a framework responsible for the stabilization of fusion pore configurations, as summarized in Fig. 4. This is consistent with results acquired on chromaffin cells where an increase in extracellular calcium concentration shifts the mode of exocytosis to kiss-and run and reduces the quantum content of a single exocytotic event. The results presented in this work show a role of electrostatic interactions in affecting the transitions between discrete states of fusion pores. However, they do not argue against the necessity of protein-protein interactions (such as the formation of the SNARE complex) in this process.

In summary, our results show that changes in the extracellular concentration of cations directly modulate fusion pore conductance, a parameter related to the fusion pore diameter. We propose that the fusion pore stability in either a wide or a narrow configuration is affected by electrostatic interactions mediated by cations adjacent to the fusion pore. These observations bear physiological significance, since extracellular calcium concentration is reduced during activity in the nervous system.
system, which may regulate synaptic activity via sensing extracellular Ca\(^{2+}\) via GPCR receptors\(^4\) and as shown in this study by directly affecting the fusion pore properties as well.

**Materials and Methods**

**Materials and solutions**

Extracellular solution (ECS) for astrocytes contained (in mM): 130 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 D-glucose, 10 HEPES and pH 7.2 (with NaOH). In experiments without extracellular Ca\(^{2+}\), CaCl\(_2\) was replaced with NaCl. Extracellular solution used for lactotrophs contained (in mM): 130 NaCl, 5 KCl, 8 CaCl\(_2\), 1 MgCl\(_2\), 10 D-glucose, 10 HEPES (N-2-Hydroxyethylpiperazine-N’-2-ethanesulfonic acid) and pH 7.2 (with NaOH). AlCl\(_3\) was prepared as a stock solution and was added to the growth medium and ECS at 30 µM (final concentration). Osmolarity of solutions was \(\approx\)300 mOsm. All chemicals were purchased from Sigma, unless stated otherwise.

**Cell cultures**

Astrocytes were isolated from cortices of 2–3-day-old Wistar rats\(^3\) and maintained in growth medium (high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% newborn calf serum and l-glutamine at 37°C with 95% humidity and 5% CO\(_2\)).

After the third shaking, the cells were trypsinized and cultured in flat culture tubes until confluence.

Lactotroph primary cultures were prepared as described.\(^3\) After isolation, lactotrophs were plated onto poly-l-lysine coated glass coverslips and kept in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% newborn calf serum and l-glutamine at 37°C with 95% humidity and 5% CO\(_2\).

All experiments were performed within a period of one to 3 d after cell isolation.

Animals were euthanized according to the International Guiding Principles for Biomedical Research Involving Animals developed by the Council for International Organizations of Medical Sciences and Animal Protection Act (Official Gazette of the RS, No. 38/13). The protocol for the euthanization of the animals used in our study was approved by the Veterinary Administration of the Ministry for Agriculture and the Environment of the RS (permit No: 34401–29/2009/2).

**Electrophysiologic measurements**

Cell-attached membrane capacitance measurements (\(C_m\)) were performed with a dual-phase lock-in patch-clamp amplifier (SWAM IIC, Celica, Ljubljana, Slovenia) as described\(^3\) at room temperature. We used fire-polished pipettes, heavily coated with Sylgard (to reduce stray capacitance), and with the resistance of 2–5 MΩ. The bath and pipettes contained ECS. A sine wave (111 mV rms and 1591 Hz for lactotrophs or 6364 Hz for astrocytes) was applied to the pipette and the holding steady state pipette potential was held at 0 mV. During the experiments, the phase angle was adjusted to nullify the changes in the real (Re) trace in response to the manually generated 10 fF calibration pulses.

**Data analysis**

We used custom-made MATLAB (Math Works, Natick, MA, USA) subroutine (CellAn, Celica, Slovenia) to analyze exocytotic events. Fusion event was
considered detectable, if the signal-to-noise ratio exceeded 3:1. We analyzed only reversible exocytotic events, where an off-step in $I_{\text{m}}$ followed an on-step within 3 s. Vesicle diameter was assessed for all exocytotic events by determining vesicle capacitance ($C_v$):

$$C_v = (Re^2 + Im^2)/Im/\omega,$$

where $Im$ denotes the amplitude change in the imaginary part of the admittance trace, $Re$ is the amplitude change in the real part of the admittance trace and $\omega$ is the angular frequency.\(^4\) Vesicle diameter was calculated assuming spherical geometry and using a specific membrane capacitance of 8 fF/µm² (lactotrophs)\(^3\) and of 10 fF/µm² (astrocytes).\(^4\) For transient exocytotic events in the $Im$ that exhibited measurable projections to the Re, we calculated fusion pore conductance ($G_p$):

$$G_p = (Re^2 + Im^2)/Re.$$ $G_p$ was used to estimate the fusion-pore radius by using the equation $G_p = (\pi r^2)/(\rho l)$, where $r$ denotes the fusion-pore radius, $\rho$ is the estimated resistivity of the saline (100 Ωcm) and $l$ is the estimated length of a gap junction channel (15 nm).\(^4\)

SigmaPlot was used for the statistical analyses. Values are presented as mean ± SEM. Differences between samples were tested with the Mann-Whitney U-test, considering $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***)

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the Slovenian Research Agency grants: P3 310, J3 6790, J3 4051, J3 4146, L3 3654; J3 3236, CIPKEBIP, COST Nanonet).
