Comparison of unitary exocytic events in pituitary lactotrophs and in astrocytes: modeling the discrete open fusion-pore states

Doron Kabaso1,2*, Jernej Jorgačevski2,3, Ana I. Calejo2,4, Ajda Flašker2, Alenka Guček2, Marko Kreft2,3,5 and Robert Zorec2,3,6

1 Laboratory of Biophysics, Faculty of Electrical Engineering, University of Ljubljana, Ljubljana, Slovenia
2 Laboratory of Neuroendocrinology-Molecular Cell Physiology, Faculty of Medicine, Institute of Pathophysiology, University of Ljubljana, Ljubljana, Slovenia
3 Celica Biomedical Center, Ljubljana, Slovenia
4 Department of Biologia e CESAM, Universidade de Aveiro, Aveiro, Portugal
5 Department of Biology, Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia

INTRODUCTION

In regulated exocytosis the merger between the vesicle and the plasma membranes leads to the formation of an aqueous channel (a fusion-pore), through which vesicular secretions exit into the extracellular space. A fusion pore was thought to be a short-lived intermediate preceding full-fusion of the vesicle and the plasma membranes (full-fusion exocytosis). However, transient exocytic events were also observed, where the fusion-pore opens and closes, repetitively. Here we asked whether there are different discrete states of the open fusion-pore. Unitary exocytic events were recorded by the high-resolution cell-attached patch-clamp method in pituitary lactotrophs and brain astrocytes. We monitored reversible unitary exocytic events, characterized by an on-step, which is followed by an off-step in membrane capacitance ($C_m$), a parameter linearly related to the membrane area. The results revealed three categories of reversible exocytic events (transient fusion-pore openings), which do not end with the complete integration of the vesicle membrane into the plasma membrane. These were categorized according to the observed differences in the amplitude and sign of the change in the real ($Re$) parts of the admittance signals: in case I events ($Re \approx 0$) fusion pores are relatively wide; in case II ($Re > 0$) and case III ($Re < 0$) events fusion pores are relatively narrow. We show that case III events are more likely to occur for small vesicles, whereas, case II events are more likely to occur for larger vesicles. Case III events were considerably more frequent in astrocytes than in lactotrophs.

Keywords: capacitance measurements, equivalent circuit, transient fusion-pore, modeling, astrocytes

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and electrophysiological measurements (Vardjan et al., 2007; Jorgačevski et al., 2008). In electrophysiological measurements specifically, by determining fusion-pore conductance ($G_p$) and vesicle capacitance ($C_v$) from admittance measurements (Lindau, 1991; Lollike and Lindau, 1999). In these recordings, changes observed in the imaginary ($\Delta Im$) and in the real ($\Delta Re$) parts of admittance signals reflect changes in $C_v$ and $G_p$, which can be used to determine vesicle diameter and fusion pore diameter (Lindau, 1991; Rosenboom and Lindau, 1994). Occasionally, the admittance traces associated with a transient fusion-pore opening exhibit an incremental or a decremental cross-talk in the $Re$ signal (Breckenridge and Almers, 1987; Lindau, 1991; Henkel et al., 2000). However, the underlying mechanisms responsible for these non-zero projections in the $Re$ signal are not fully understood.

In previous studies, the equivalent circuit of the fusion pore was reported (Lindau and Neher, 1988; Scepek and Lindau, 1993; Lollike et al., 1995). It was shown analytically that the incremental cross-talk projection on the $Re$ signal could be due to the fusion-pore opening devoid of complete vesicle membrane integration into the plasma membrane (Lindau, 1991; Henkel et al., 2000). By studying pituitary lactotrophs, an ideal cell preparation to study secretory activity at the single vesicle level (Stenovec et al., 2004; Vardjan et al., 2007), and astrocytes which release gliotransmitters (Parpura et al., 1994) by likely employing regulated exocytosis (Parpura and Zorec, 2010), we compared the properties of unitary exocytic events. Using equivalent circuit analysis, we here demonstrate that the decremental cross-talk projection on the $Re$ signal depends on the $G_p$ as well as on the size of the fused vesicle. Moreover, these results indicate the existence of a very narrow, nearly closed, open fusion-pore state in pituitary lactotrophs and in astrocytes.

MATERIALS AND METHODS

CELL CULTURES

Primary lactotroph and astrocyte cultures were prepared from adult male (lactotrophs) and 2–3 days old female Wistar rats as described previously (Schwartz and Wilson, 1992; Ben-Tabou et al., 1994; Jorgačevski et al., 2008). After the isolation we plated cells on poly-L-lysine-coated coverslips and maintained them in high-glucose DMEM (Invitrogen) medium, supplemented with 10% newborn calf serum and 2 mM L-glutamine in an atmosphere of humidified air (95%) and CO$_2$ (5%). We cared for the experimental animals in accordance with the International Guiding Principles for Biomedical Research Involving animals, developed by the Council for International Organizations of Medical Sciences, and the Directive on Conditions for Issue of License for Animal Experiments for Scientific Research Purposes (Official Gazette of the Republic of Slovenia 40/85 and 22/87). The procedures using animals were approved by the Veterinary Administration of the Republic of Slovenia (approval no. 34401-29/2009/2).

ELECTROPHYSIOLOGY

Cell-attached capacitance measurements on isolated rat lactotrophs and astrocytes were performed with a dual-phase lock-in patch-clamp amplifier (SWAM IIC and SWAM CELL, Celica, Ljubljana, Slovenia) as described (Kreft and Zorec, 1997; Vardjan et al., 2007; Jorgačevski et al., 2010). Briefly, a sine wave voltage (1591 or 6400 Hz, 111 mV) was applied to the pipette, while holding the pipette potential at 0 mV. The phase of the lock-in amplifier was adjusted to nullify the changes in $Re$. A 10 fF calibration pulse was manually generated every 10 s to ensure correct phase angle settings. We used thick-walled, fire polished glass pipettes, which were heavily coated with a resin (Sylgard®184) and had a resistance of 2–5 MΩ.

THE EQUIVALENT CIRCUIT OF A TRANSIENT FUSION PORE

A patch-clamp configuration could be approximated by the series combination of the membrane and the access resistance ($R_A$) of the pipette tip through the patch (Figure 1A). The membrane included a parallel setup of the whole-cell $C_m$ and the membrane conductance ($G_M$). When a small vesicle fused with the patch region, the patch-clamp system could detect the fusion event by the observed changes in the admittance measurement. It has been previously confirmed that the fusion of a vesicle was accompanied by an increase in the measured $C_m$ (Neher and Marty, 1982). The equivalent circuit of the patch-configuration with a fused vesicle is shown in Figure 1B. The vesicle $C_v$ is denoted by $C_v$, and the fusion-pore conductance is denoted by $G_p$.

In the present analysis, all the parameters except $G_p$ were held constant. It was assumed that $C_v << C_m$ or $\omega C_v << 1/R_A$, which are reasonable assumptions, since the vesicle $C_m$ (vesicle surface area) is considerably smaller than the cell $C_m$ (cell surface area), and the patch resistance is considerably greater than the capacitor load of the vesicle ($\omega C_v$). The admittance change ($\Delta Y$) describes the change in the admittance between the open and nearly closed state of the fusion pore. According to the equivalent circuit, the admittance difference is (Lindau, 1991):

$$
\Delta Y = T^2(\omega) \left( \frac{\omega C_v}{1 + \frac{\omega C_v}{G_p}} + \frac{\omega C_v}{1 + \frac{\omega C_v}{G_p}} \right),
$$

where $T^2(\omega)$ stands for the factor $T^2(\omega) = 1/(1 + R_A G_M + i\omega C_m R_A)^2 = |T(\omega)|^2 \cdot e^{i\theta}$, and $i$ is $\sqrt{-1}$. The admittance change

![FIGURE 1](https://www.frontiersin.org) | The equivalent circuit of a patched membrane with a fused vesicle. The cell-attached patch-clamp configuration enables the detection of vesicle fusion in the patched membrane region (A). In the equivalent circuit, the fusion of a vesicle is considered in parallel to the patch membrane (B). Note that $R_A$ is the access resistance, $C_m$ is the whole-cell membrane capacitance, $G_M$ is the membrane conductance, $C_v$ is the vesicle membrane capacitance, and $G_p$ is the fusion-pore conductance.
(ΔY) is an imaginary number, in which the first term in the parenthesis is the ΔRe, and the second term in the parenthesis is the ΔIm. For the sake of simplicity, the fusion pore state is denoted as fully open or incompletely open. In the fully open state, the Gp is infinite (Gp → ∞), and the ΔRe in Equation (1) vanishes, which gives ΔY = iT²(ω)Cv. On the other hand, when there is incomplete fusion, the Gp can be on the same scale as ωCv. As a result, both the ΔRe and ΔIm have a finite value, which can be used for the calculation of the unknown Gp (Breckenridge and Almers, 1987). The Cv and the Gp can be obtained from the real and imaginary parts (Lindau, 1991; Lollike and Lindau, 1999), as follows:

$$C_v = \frac{\Delta Re^2 + \Delta Im^2}{\Delta Im}$$

$$G_p = \frac{\Delta Re^2 + \Delta Im^2}{\Delta Re}. \quad (2)$$

**RESULTS**

**ADMITTANCE MEASUREMENTS OF THE THREE CASES OF TRANSIENT EXOCYTIC EVENTS**

The admittance measurements of repetitive opening and closure of fusion-pores were obtained in lactotrophs (Figures 2Ai,ii) and in astrocytes (Figures 2Biii,iv), where representative transient exocytic events are shown, denoted as cases I, II, and III on Figure 2. The asterisks in these recordings indicate calibration pulses. The recordings in lactotrophs (Ai,ii) and astrocytes (Biii,iv) reveal a zero projection of each event from the admittance records. On Figure 2A (recorded in lactotrophs) the Cv in case I, case II, and case III, was 0.9, 4, and 1.2 fF, respectively. Whereas, on Figure 2B, where representative recordings in astrocytes are shown, the Cv was in case I (0.3 fF), case II (1.6 fF), and case III (0.6 fF). One can note that the amplitude in Im, reflecting Cv, appears larger in events of case II in comparison with case I events. Case I events exhibit an incremental change in Im trace and are devoid of projection on the Re trace. In case II events and increment in Im trace is associated with an incremental projection on the Re trace, whereas, in case III events an increment in Im trace is associated with a decremental projection on the Re trace. In Figure 2C the three cases of transient exocytic events are diagrammatically presented as pre-fused vesicles with an initial narrow fusion-pore (narrower than the detection limit of the recording system), which can reversibly widen to a larger diameter—a state that can be detected electrophysiologically.

The experimental datasets used in our analysis, include ΔRe and ΔIm of cases I–III obtained in lactotrophs (Figures 3A–D) and astrocytes (Figures 3E–H). The Gp of each event is calculated from ΔRe and ΔIm of cases II (ΔRe incremental) and III (ΔRe decremental). Figures 3A–H shows scatter plots of ΔRe and ΔIm as a function of Gp. The relationships between ΔIm versus ΔRe are plotted for the datasets of incremental and decremental ΔRe projections. A strong linear relationship (r = 0.79 in Figure 3C; r = 0.75 in Figure 3G) was revealed between ΔIm and ΔRe in the incremental ΔRe projection group (Figures 3C,G). The slope of this relationship in astrocytes is smaller than one (Figure 3G), however that in lactotrophs is close to unity (Figure 3C), suggesting that ΔRe and ΔIm are of similar size. The non-zero ΔRe can be attributed to the incomplete opening of the fusion-pore, which is accompanied by the increased fusion-pore resistance, whereas, the fully open fusion-pore exhibit a negligible resistance.

On the other hand, in the decremental ΔRe projection datasets, a weak correlation between ΔIm and ΔRe was revealed (r = 0.49 in Figure 3D; r = 0.08 in Figure 3H). We noted that the datasets of decremental ΔRe projections are clustered more in the range of small Gp (Figures 3B,F).

**REPRODUCING THE THREE CASES OF ADMITTANCE MEASUREMENTS**

Next, we considered the mechanisms responsible for the incremental and decremental ΔRe projections in the admittance measurements of the two cell types. According to the equivalent electrical circuit, the change in the real (ΔRe) and imaginary...
\( \Delta \text{Im} \) parts of admittance signals can be employed for the calculation of \( C_v \) and \( G_p \) (Lindau, 1993; Lollike and Lindau, 1999).

In Figure 4, \( \Delta \text{Re} \) and \( \Delta \text{Im} \) are plotted as a function of \( G_p \) calculated for intermediate \( (C_v = 1.2 \, \text{fF}) \), large \( (C_v = 4 \, \text{fF}) \), and small \( (C_v = 0.5 \, \text{fF}) \) vesicles, representing events of case I, II, and III, respectively. The fact that in case I there is no projection on the \( \text{Re} \) trace can be reproduced by a change from a fully closed fusion-pore state \( (G_p = 0 \, \text{pS}) \) to a fully open fusion-pore state (e.g., \( G_p > 500 \, \text{pS} \) in lactotrophs and \( G_p > 70 \, \text{pS} \) in astrocytes) or by a change from a nearly closed fusion-pore state (e.g., \( G_p = 5 \, \text{pS} \) in lactotrophs and \( G_p = 2 \, \text{pS} \) in astrocytes) to an incompletely open fusion-pore state (e.g., \( G_p = 30 \, \text{pS} \) ) (Figure 4A). The positive projection in case II can be due to fusion-pore opening from a fully closed state, or a nearly closed state, to an incompletely open state (Figure 4B). On the other hand, the negative projection in case III can be reproduced only when the pre-fused state is nearly closed (Figure 4C). In addition, the change in \( \Delta \text{Re} \) can be considerably larger than the change in \( \Delta \text{Im} \) (see inset). Overlay of \( \Delta \text{Re} \) \( \text{G}_p \) of cases I and III reveals the effect of vesicle size on the projection type (Figure 4D). By having the same pre-fused state (i.e., the same fusion pore conductance), the incomplete fusion-pore opening of a large vesicle size leads to an incremental projection, whereas, the same incomplete opening of a small vesicle causes a decremental projection in \( \text{Re} \).

\[ \Delta \text{Im} = \frac{1}{C_v} \Delta \text{Re} \text{ (solid lines) and } \Delta \text{Im} = \frac{1}{C_v} \Delta \text{Re} - \frac{1}{C_v} \text{ (dashed lines)} \text{ as a function of } G_p \text{ for the datasets of incremental } \Delta \text{Re} \text{ (A,E) and for the decremental } \Delta \text{Re} \text{ (B,F) datasets.} \]

FIGURE 3 | The experimental datasets of \( \Delta \text{Re} \) and \( \Delta \text{Im} \) of admittance measurements recorded in lactotrophs (A–D) and astrocytes (E–H) incremental (case II) and decremental (case III) \( \Delta \text{Re} \) projections. The distributions of \( \Delta \text{Re} \) and \( \Delta \text{Im} \) as a function of \( G_p \) for the datasets of incremental \( \Delta \text{Re} \) (A,E) and for the decremental \( \Delta \text{Re} \) (B,F) datasets. A scatter plot of \( \Delta \text{Im} \) against \( \Delta \text{Re} \) reveals a strong linear relationship in the incremental \( \Delta \text{Re} \) datasets of projections (C,E), and a weak linear correlation in the dataset of decremental \( \Delta \text{Re} \) projections (D,H).

FIGURE 4 | The relationships of \( \Delta \text{Re} \) (solid lines) and \( \Delta \text{Im} \) (dashed lines) as a function of \( G_p \) calculated for intermediate \( (C_v = 1.2 \, \text{fF}; \text{case I}), \text{large } (C_v = 4 \, \text{fF}; \text{case II}), \text{and small } (C_v = 0.5 \, \text{fF}; \text{case III} \text{ vesicle size). Note that the change in } \Delta \text{Re} \text{ and } \Delta \text{Im} \text{ are drawn by a full and an open arrow, respectively. The zero projection on the } \text{Re} \text{ part is reproduced by a change from a fully closed state to a fully open fusion-pore state (see inset) or by a change from a nearly closed state to an incompletely open fusion-pore state (A). The incremental projection in case II is reproduced by a change from nearly closed to incompletely open state (B). The decremental projection in case III can be reproduced, only when the pre-fused state is nearly closed (C). In addition, the change in } \Delta \text{Re} \text{ can be considerably larger than the change in } \Delta \text{Im} \text{ (see inset). Overlay of } \Delta \text{Re} \text{ (G}_p\text{) of cases I and III reveals the effect of vesicle size on the projection type (D). By having the same pre-fused state (i.e., the same fusion pore conductance), the incomplete fusion-pore opening of a large vesicle size leads to an incremental projection, whereas, the same incomplete opening of a small vesicle causes a decremental projection in } \text{Re}. \]
same nearly closed state (e.g., $G_p = 5 \text{pS}$), the incomplete opening of the fusion pore would lead to a positive projection in the case of the large vesicle and a decremental projection in the $\Delta Re$ in the case of the small vesicle (Figure 4D).

**DISCUSSION**

In the present paper, we analyzed the discrete open fusion-pore states as well as the conditions under which these states existed. Three different cases are categorized, in which the transitions between discrete states of the fusion-pore do not end with the complete fusion (i.e., exocytosis) of a vesicle and the plasma membrane.

These cases are evident from the changes in the real ($\Delta Re$) and imaginary ($\Delta Im$) parts of admittance measurements (Figure 2). In the first case (denoted as case I), the event is characterized by a step increase in $\Delta Im$ and an approximately zero $\Delta Re$. In the second case (denoted as case II), both $\Delta Im$ and $\Delta Re$ are exhibiting a step increase. The third case (denoted as case III) has an incremental $\Delta Im$ and a decremental $\Delta Re$. The underlying assumption of our model is that the non-zero $\Delta Re$ is due to an incomplete vesicle fusion-pore opening, and that the initial status of the fused vesicle may exhibit a non-zero $G_p$ (Figure 2). The equivalent circuit of a patch-clamp configuration was constructed, in which the fused vesicle is considered in parallel to the cell plasma membrane (Figure 1). The fused vesicle is assumed to be connected to the cell membrane via an aqueous channel (the fusion-pore). The $C_p$ and the $G_p$ are derived from the imaginary ($\Delta Im$) and real ($\Delta Re$) parts of the admittance measurements. The experimental data of $\Delta Re$ and $\Delta Im$ are obtained from admittance measurements in lactotrophs and astrocytes (Figure 2). In case II, a strong linear relationship between $\Delta Re$ and $\Delta Im$ suggests that the fusion-pore opening of a vesicle is incomplete (Figures 3C,G). In case III, there is a weak correlation between $\Delta Re$ and $\Delta Im$ (Figures 3D,H). According to the relationships of $\Delta Re$ and $\Delta Im$ as a function of $G_p$, it is demonstrated that the decremental $Re$ projection is more likely to occur for small vesicles (Figure 4C). Finally, the present calculations reveal that while the incomplete opening of the fusion-pore may be accompanied by the same change in fusion-pore conductance, the resulted projection is predicted to be decremental for the relatively small vesicle and incremental for the large vesicle (Figure 4D).

The high bending energy during the formation of a fusion-pore can be overcome by the assembly of curvature membrane constituents (proteins and lipids) (Kozlov and Markin, 1983; Jorgaˇcevski et al., 2010; Kabaso et al., 2012; Jesenek et al., 2012). The stability of the fusion-pore of a fused vesicle can be due to anionic lipids of negative spontaneous curvature, modulating the formation of the fusion pore (Coorssen and Rand, 1990; Chen and Rand, 1997; Churchward et al., 2008; Rituper et al., 2012). It is then possible that the density of these curvature membrane constituents can affect the $G_p$ of the nearly closed fusion-pore state as well as the projection type and amplitude. The possible incomplete fusion-pore state opens a communication venue, in which the passage of small molecules such as ions may be facilitated continuously through the narrow pore. However, larger molecular weight molecules are unable to exit the narrow fusion pore.

What appears interesting is that in both cell types, electrically excitable (pituitary lactotrophs) and electrically non-excitable (brain astrocytes), fusion-pore properties appear to be shared. While vesicles in the lactotrophs exhibit larger diameters and are therefore more accessible to experimentation (Stenovec et al., 2004; Vardjan et al., 2007), secretory vesicles in astrocytes appear to exhibit relatively large and relatively small diameters. The latter ones can be revealed by the higher-resolution cell-attached patch-clamp measurements (Kreft and Zorec, 1997). Interestingly, it is the smaller ones that exhibit fusion-pores with extremely narrow fusion-pore diameters (Figure 4). The probability of observing an increment in $Im$ trace, associated with a decremental change in $Re$ trace, indicates that a relatively large fraction of vesicles, which are already fused with the plasma membrane, exhibit a very narrow fusion pore. These may pass protons as has been reported previously by using a pH-sensitive vesicle luminal fluorophore in lactotrophs (Vardjan et al., 2007) and in astrocytes (Malarkey and Parpura, 2011). However the relatively large abundance of these events recorded in astrocytes, may not mean that fusion-pore openings mediate a productive release of gliotransmitters. A $G_p$ of less than 5 pS means that the fusion pore diameter is less than 0.2 nm, too narrow to pass even glutamate or acetylcholine (Vardjan et al., 2007). These results are consistent with the view that fusion-pores, when they are established, are relatively stable structures (Jorgaˇcevski et al., 2010). The regulation of exocytotic release of hormones and transmitters, thus involves also the regulation at the fusion-pore level, at the level, when the fusion-pore has been already established, but is too narrow to functionally contribute to the exit of secretions form the vesicle lumen. In the present paper we have revealed that fusion-pores may exhibit distinct fusion-pore diameters and the future work will have to address question of how these open fusion-states transit to a release productive state.

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