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Mehanizmi eksocitoze/Mechanisms of Exocytosis

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Abstract: Vesicles are cellular organelles, in which signaling molecules (neurotransmitters or hormones) are stored and are essential for the function of neurons and endocrine cells in supporting the communication between tissues and organs. Upon stimulation the signaling molecules stored inside vesicles are released from cells by exocytosis. This fundamental biological process consists of membrane fusion between the vesicles and the plasma membrane, leading to the formation of an aqueous channel – the fusion pore – through which signaling molecules exit into the extracellular space or blood stream. The vesicle cargo discharge initially requires the delivery of vesicles to the plasma membrane, where vesicles dock and get primed for fusion with the plasma membrane. Classical view holds that stimulation initiates the fusion pore formation and vesicle cargo discharge in an all-or-none fashion. Once formed the fusion pore may close (transient, "kiss-and-run" exocytosis) or expand, leading to the full collapse of the vesicle membrane into the plasma membrane (full fusion exocytosis). However, recent studies indicate that exocytosis may not be as simple. Here we highlight the novel findings which indicate that transient fusion pore is subject to regulations, which affect the release competence of a single vesicle. Our recent studies have shown that in pituitary lactotrophs vesicle release of peptide signaling molecules involves modulation of fusion pore kinetics and fusion pore conductance.

Keywords: exocytosis, vesicle, fusion pore, transient/full fusion, pituitary lactotrophs, peptide hormones

Izvleček: Mešički so celični organeli, v katerih so shranjene signalne molekule (živčni prenašalci, hormoni), ki so nujno potrebne za delovanje živčnih in endokrinih celic, saj omogočajo komunikacjo med tkivi in organi. Po stimulaciji se signalne molekule izločijo iz mešičkov s pomočjo eksocitoze. Eksocitoza je temeljni biološki proces, pri katerem pride do zlitja membrane mešička in plazemske membrane, pri čemer nastane kanal – fuzijska pora, skozi katerega se izločijo signalne molekule v zunajcelični prostor ali krvni obtok. Pred eksocitozo mešički potujejo v neposredno bližino plazemske membrane, kjer se vsidrajo in pripravijo za zlitje s plazemsko membrano. V naslednjih fazah naj bi stimulacija sprožila nastanek fuzijske pore in popolno sprostitev vsebine mešička skozi fuzijsko poro. Fuzijska pora se lahko po odprtju zapre (*t.i.* prehodna, angl. »kiss-and-run« eksocitoza) ali pa razširi, kar vodi do popolnega zlitja membrane mešička s plazemsko membrano (*t.i.* popolna, angl. »full fusion« eksocitoza). Nedavne raziskave so pokazale, da proces eksocitoze ni tako preprost. V preglednem članku se bomo osredotočili na najnovejše raziskave, ki kažejo, da je prehodna eksocitoza lahko uravnavana, kar vpliva na zmožnost izločanja signalnih molekul iz posameznega mešička. Naše nedavne raziskave so pokazale, da v laktotrofih iz hipofize, sproščanje peptidnih signalnih molekul iz mešičkov vključuje tako modulacijo kinetike fuzijske pore kot tudi uravnavanje prevodnosti (premera) fuzijske pore.

Ključne besede: eksocitoza, mešiček, fuzijska pora, prehodna/popolna fuzija, hipofizni laktotrofi, peptidni hormoni

Introduction

Exocytosis is a fundamental cellular process used by eukaryotic cells to secrete different biological compounds. The basic machinery required for exocytosis has been well conserved throughout evolution from yeast to man. However, the precise molecular mechanisms underlying the process of exocytosis are still poorly understood and therefore extensively studied in different cell systems.

The secretory process consists of few different stages. First vesicles packed with the secretory compounds are transported to the plasma membrane. Then the vesicles are tethered or docked to the appropriate sites at the plasma membrane and prepared for fusion (priming). In the last step the fusion of vesicle with the plasma membrane (exocytosis) occurs and the vesicle content is released through the fusion pore (Fig. 1 and 2) and/or the vesicle membrane components are incorporated into the plasma membrane.

Exocytosis occurs in almost all cells in the form of constitutive exocytosis, which serves to release the components of the extracellular matrix, or just to deliver and incorporate newly synthesized membrane lipids and proteins into the plasma membrane (*i.e.* constitutive exocytosis). In many cells an alternative secretory pathway exists, where an extracellular stimulus is required to trigger exocytosis, therefore allowing a controlled release of peptide hormones and neurotransmitters. The stimulus typically triggers an increase in intracellular Ca^{2+} activity ($[Ca^{2+}]_i$), which activates vesicle fusion with the plasma membrane (*i.e.* regulated or Ca^{2+}-triggered exocytosis; Fig. 2).

In recent years new techniques have allowed direct measurements of elementary exocytotic events in real time in neurons and neuroendocrine cells. By measuring membrane capacitance (C_m) one can reveal changes in cell surface area, which are reflecting vesicle fusion and fission (Neher & Marty 1982, Lindau & Neher 1988). Detection of vesicle fusion with amperometry is based on the indirect electrochemical detection of released molecules with a suitable oxidation potential (Wightman & al.1991, Chow & al. 1992). Furthermore optical imaging with different fluorescence markers can provide a direct readout of vesicle fusion and recycling (Betz & Bewick 1992, Miesenböck & al. 1998, Shaner & al. 2005). These multidisciplinary approaches led to the discovery that the rate (Albillos & al. 1997, Stenovec & al. 2004) and the amount of vesicle cargo release (Angleson & al. 1999) are controlled by the stimulus before the vesicle fusion, and more importantly, also after the fusion pore formation (*i.e.* post-fusion regulation of a release, Rahamimoff & Fernandez 1997).

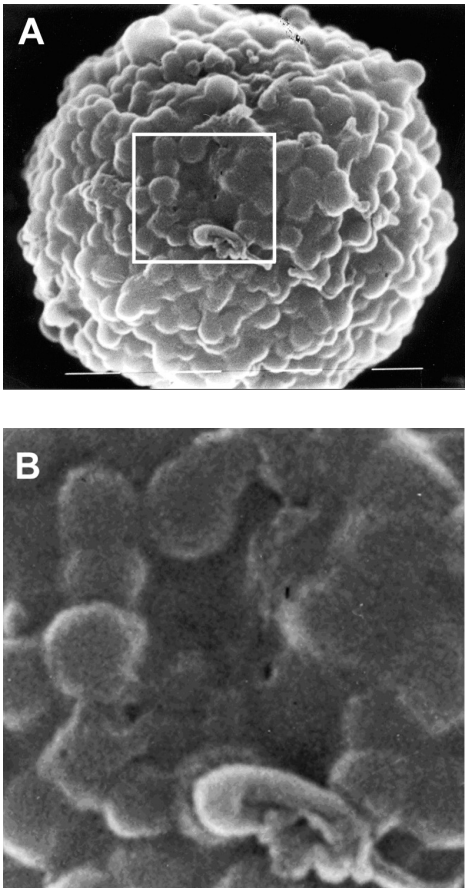

Figure 1: Scanning electron micrograph of the pituitary lactotroph surface exhibits fusion-pore-like formations. (A) Panel shows a view of the whole cell with a diameter of 10 μm. (B) Panel is a magnified view of the framed region in panel A. Several small openings in the cell surface (black) are seen, which may be related to the fusion pore-like structures.

In this article we focus on the mechanisms of regulated exocytosis. We first present an overview

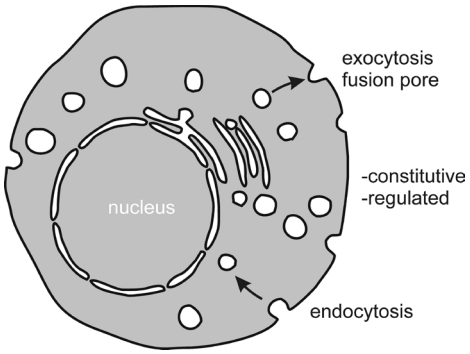

Figure 2: Exocytosis involves fusion of the vesicle membrane with the plasma membrane.
Membrane fusion leads to the formation of the fusion pore – an aqueous channel connecting the vesicle lumen with the extracellular space. Constitutive exocytosis does not require a stimulus to occur, whereas regulated exocytosis is triggered by a stimulus, such as an increase in the activity in cytosolic Ca^{2+}. Endocytosis is a process of plasma membrane retrieval, often balancing exocytosis to keep the surface area of a cell constant over a longer period of time.

of the present knowledge on single vesicle fusion events in lactotrophs, neuroendocrine cells of anterior pituitary, which secrete peptide hormone prolactin. We then discuss mechanisms of post-fusion regulation of vesicle cargo release from neurons and neuroendocrine cells.

Models of exocytosis

One of the first models of regulated vesicle exocytosis, introduced by del Castillo and Katz, predicts that in neurons the vesicles fuse transiently with the membrane and that the vesicle cargo is released in an all-or-none fashion (Katz 1969). In the early 1970s, the first systematic studies on vesicle recycling were performed on frog neuromuscular junction by electron microscopy in the presence of the marker horseradish peroxidase. The studies were performed independently by two research groups, interestingly, their results yielded different interpretations (Ceccarelli & al. 1973, Heuser & Reese 1973).

Heuser and Reese discovered that vesicle fusion is followed by full collapse of the vesicle membrane into the plasma membrane (*i.e.* full fusion exocytosis) emptying the entire vesicle content to the extracellular space (all-or-nothing release). The retrieval of vesicle membrane, which is necessary for maintaining the cell size and integrity, was proposed to occur at the location distal to the fusion site (Heuser & Reese 1973). On the other hand, Ceccarelli and his co-workers proposed that vesicle fusion involves the opening of a small fusion pore, followed by its fast closure at the same site of fusion, without full pore dilation and vesicle membrane collapse into the plasma membrane (Ceccarelli & al. 1973). The model they proposed was similar to Katz's initial model, that vesicles fuse with the plasma membrane only

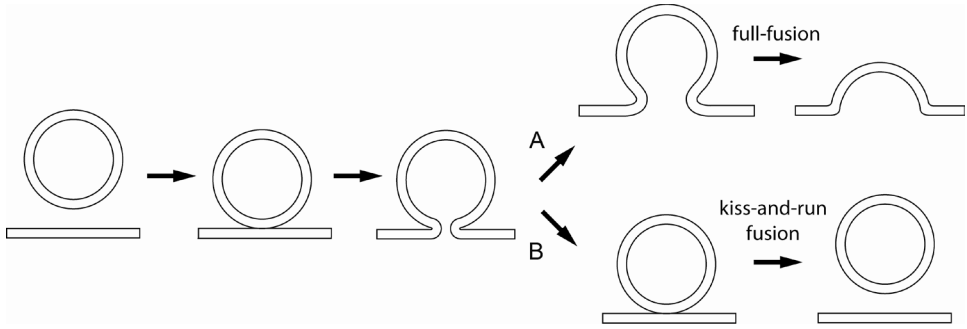

Figure 3: **Models of exocytosis.** Schematic drawing of exocytosis modes. (**A**) Full fusion of a vesicle after the initial formation of a narrow fusion pore. After the fusion pore widening the vesicle membrane is fully incorporated into the plasma membrane. (**B**) An example of transient fusion (a "kiss-and-run" event), where a vesicle forms a transient fusion pore with the plasma membrane and releases only part of its content. After the fusion pore closure, the vesicle lumen is reloaded and vesicle reused.

transiently (*i.e*. transient or "kiss-and-run" exocytosis as proposed by Fesce & al. (1994)). Further studies have revealed that the transient opening of the fusion pore may lead only to the partial release of vesicle cargo (Stenovec & al. 2004, reviewed in Harata & al. 2006) and that once opened, the fusion pore can change its dimensions dynamically and even close and reopen repetitively (fusion pore flickering, the pulsing fusion pore, Fernandez & al.1984, Stenovec & al. 2004, Vardjan & al. 2007). More than 30 years after, these two models (Fig. 3) have been still intensively debated (reviewed in He & al. 2006, LoGiudice & Matthews 2006, Smith & al. 2008).

Stimulus increases the rate of cargo release from a single vesicle in lactotrophs

Figure 4 shows immunolabelled prolactin hormone accumulation at the surface of the plasma membrane following cell stimulation. Recently, it has been observed that in lactotrophs stimulated hormone discharge from a single vesicle is up to 20 times faster than spontaneous peptide hormone discharge (Stenovec & al. 2004). Time-lapse confocal imaging was performed on cells expressing fluorescently tagged peptide ANP.emd (Han & al. 1999) in their vesicle lumen and in the presence of the extracellularly added fluorescent styryl dye FM 4-64 (Betz & Bewick 1992). In lactotrophs, FM-membrane dye stains not only the plasma membrane when in the extracellular medium, but also the matrix of individual prolactin vesicles (Angleson & al. 1999, Stenovec & al. 2005), upon exposure to the extracellular solution. The studies were carried out in resting conditions and following stimulation by exposing cells to a high potassium-containing solution. When exocytotic cargo release occurred, the fluorescence intensity of the ANP.emd probe decreased at the vesicle site and the vesicle was loaded through the same fusion pore with the FM 4-64. In resting lactotrophs, in 50% of spontaneously releasing vesicles, the peptide hormone release and the FM 4-64 loading were slow (~3 min). However, high potassium stimulation triggered hormone release and FM 4-64 loading within seconds, indicating that in lactotrophs stimulation increases the rate of vesicle cargo release, very likely at the stage when the fusion pore is already formed (post-fusion regulation of a release; Rahamimoff & Fernandez 1997, Stenovec & al. 2004).

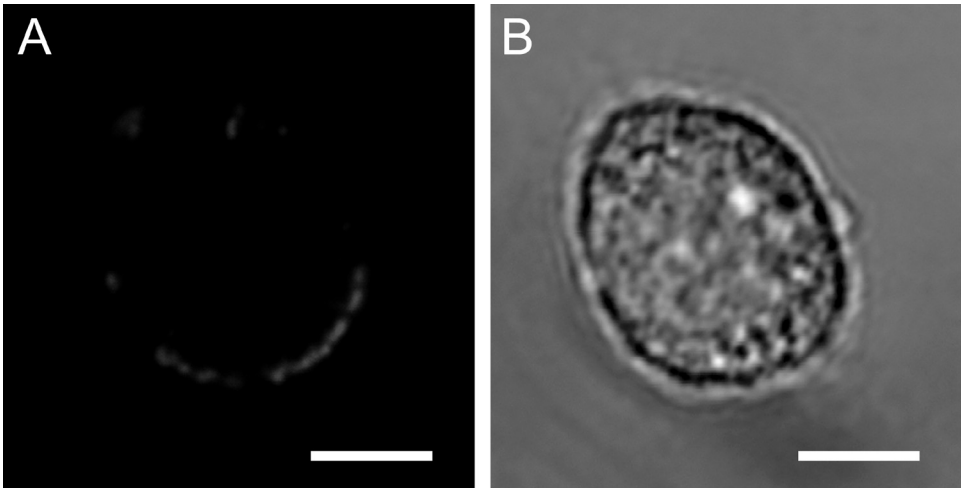

Figure 4: (**A**) Confocal image of secreted rat prolactin (rPRL) visualized on the surface of the plasma membrane by anti-rPRL antibody and fluorescent secondary antibody (red). The cell was exposed to a solution containing 100 mM K^+, to depolarize the membrane and stimulate the cells prior to the immunocytochemical reaction. (**B**) Transmitted light image with DIC contrast of the cell displayed in A. Bar: 5 µm.

Mechanisms of post-fusion regulation of vesicle cargo release in lactotrophs

Recent studies have shown that fusion pores are subject to regulations, which affect the release competence of a single vesicle (Stenovec & al. 2004, reviewed in Harata & al. 2006). In the next two chapters we will discuss two mechanisms that control the rate of vesicle cargo release in lactotrophs: (I) fusion pore kinetics and (II) fusion pore diameter (Fig. 5).

or "kiss-and-run" exocytosis) (Neher & Marty 1982, Fernandez & al. 1984, Scepek & Lindau 1993, Lollike & al. 1995).

The majority of exocytotic events (> 99%) observed by C_m measurements in resting lactotrophs were transient fusion events (*i.e.* transient fusion pore openings). The occurrence of non-reversing steps, representing full vesicle fusion (Neher & Marty 1982), was very low (~0.4% of all events). The fusion pore open-state duration of a single transient event was at rest ~50 ms (Stenovec & al. 2004, Vardjan & al. 2007), similar as in rest-

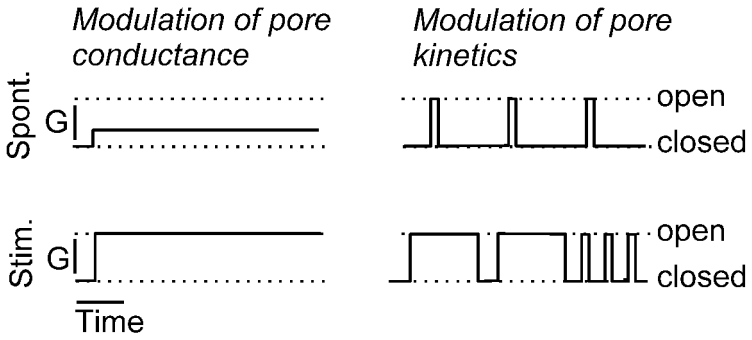

Figure 5: Mechanisms of vesicle cargo discharge modulation.
The permeation of molecules through the fusion pore depends on the fusion pore conductance (*G*; diameter) and/or fusion pore kinetics. A wider fusion pore (higher *G*) and/or faster frequency of fusion pore openings with longer fusion pore dwell-times (faster kinetic of fusion pore openings) leads to increased release of peptides from a single vesicle in stimulated conditions.

I. Fusion pore kinetics

It has been proposed that transient, "kiss-and-run", mode of vesicle fusion can limit or completely prevent peptide hormone release because of the relatively large size of peptide molecules and the consequent low diffusion mobility of these molecules (Barg & al. 2002, Tsuboi & Rutter 2003, Obermüller & al. 2005). To determine, if transient mode of vesicle fusion is slowing the release of vesicle cargo in resting lactotrophs, C_m measurements of single vesicle fusion events were performed by cell-attached patch-clamp technique (Neher & Marty 1982). A stepwise increase in C_m (on-step) indicates full fusion exocytosis. If the on-step is followed by a downward, off-step in C_m, of a similar amplitude to the preceding on-step, the fusion of a vesicle with the plasma membrane is transient (transient

ing synapses of calyx of Held (Sun & al. 2002). These transient events appeared repetitively in bursts lasting for as long as 760 s ("the pulsing pore"; Stenovec & al. 2004, Vardjan & al. 2007), some of them were more complex with multiple amplitude on- and off-steps representing repetitive compound vesicle-to-vesicle-to-plasma membrane fusion events (Vardjan & al. 2009). Regular repetitive fusion pore openings and slow, but synchronous, loading and unloading of fluorescent probes in lactotrophs, likely indicate that in resting lactotrophs the slow probe exchange through the fusion pore may be constrained kinetically by regular fusion pore openings (fusion pore flickering, also termed fusion pore gating; Stenovec & al. 2004). Amperometric studies on neurons revealed that fusion pore flickering can limit the release of dopamine from synaptic terminals (Staal & al. 2004). This indicates that flickering transient

fusion pores may also be involved in the regulation of small molecular weight transmitter release in synapses despite the relatively fast diffusion mobility of small chemical molecules.

Transient and full fusion exocytosis were reported to coexist in some systems and it has been suggested that switching between the transient to full fusion mode due to cell stimulation can result in the modulation of the amount of cargo release from a single vesicle (reviewed in Harata & al. 2006). Interestingly, we have observed by C_m measurements that transient fusion pore openings with estimated mean burst duration of >100 s were the predominant mode of exocytosis not only in resting but also in high potassium stimulated lactotrophs (Stenovec & al. 2004, Vardjan & al. 2007). Full vesicle fusion occurred with practically the same relatively low incidence before and after stimulation (Vardjan & al. 2007). However, transient events occurred fourfold more frequently in stimulated compared to resting lactotrophs. Moreover, the fusion pore dwell time was twofold longer after stimulation. Stimulus thus prolongs the effective open time of the transient pores, facilitating hormone secretion without full fusion (Vardjan & al. 2007). More frequent transient events with prolonged fusion pore open state dwell-time were observed also in lactotrophs after hypotonic stimulation (Jorgacevski & al. 2008). These results are in contrast to previous studies in chromaffin cells where low levels of stimulation triggered "kiss-and-run" exocytosis, whereas with stronger stimulation the predominant mode of exocytosis was full fusion (Elhamdani & al. 2001, Fulop & al. 2005).

II. Fusion pore diameter

Recently, it has been shown that the fusion pore diameter can be the limiting factor preventing the permeation of molecules through the fusion pores (Barg & al. 2002, Takahashi & al. 2002, Tsuboi & Rutter 2003, Fulop & al. 2005). Therefore, the release of vesicle cargo from vesicles undergoing transient, "kiss-and-run", exocytosis may depend also on the diameter of the fusion pore. We used electrophysiological and optical methods to measure the size of the effective fusion pore diameter in exocytic vesicles in lactotrophs before and after stimulation (Vardjan & al. 2007).

The effective diameter of a fusion pore can be calculated from the fusion pore conductance (G_p; Spruce & al. 1990, Lollike & Lindau 1999). Cell-attached patch-clamp technique can be used to monitor the time course of the G_p by modelling a vesicle fusing with the cell membrane as a conductance (the fusion pore) in series with a capacitor (the vesicle membrane) (Zimmerberg & al. 1987). We have measured the G_p as described in Lollike & Lindau (1999) and then we calculated the effective fusion pore diameters from the G_p values (described in Spruce & al. 1990). At rest, the G_p values in transient events ranged from 8 to 200 pS (the average 53 pS), corresponding to fusion pore diameters of 0.4 nm to 2.0 nm. After stimulation, the G_p values of transient events increased to an average measurable G_p of 81 pS. The maximal measurable G_p value after stimulation was ~530 pS (*i.e.* 3.2 nm), indicating that the diameter of fusion pore in the majority of stimulated events had effective pore diameters >3.0 nm. In >98% stimulated events the G_p values increased after stimulation to an unmeasurable final conductance, representing fusion pore expansion, and then decreased again to measurable values, indicating fusion pore narrowing/closing. This is consistent with previous results observed in different cell systems, where fusion pores enlarge their diameters several folds and close completely afterwards (Fernandez & al. 1984, Monck & al. 1990, Spruce & al. 1990, Melikyan & al. 1995, Larina & al. 2007). Events with the measurable size of the fusion pore were more frequent in resting than in stimulated conditions (25% vs. 2%), indicating that the stimulus increases the size of the transient fusion pore (Vardjan & al. 2007).

To confirm our observations, we performed optical fusion pore studies. Permeation of FM 4-64 (molecular diameter ~ 0.9 nm) and HEPES (molecular diameter ~ 0.5 nm) through spontaneously forming fusion pores was studied in lactotroph vesicles expressing synaptopHluorin (spH; Vardjan & al. 2007). spH is a pH-dependent protein consisting of the vesicle membrane-targeted protein VAMP2 (synaptobrevin-2) with a pH-sensitive enhanced green fluorescent protein (superecliptic pHluorin) fused to its luminal side (Miesenböck & al. 1998). At the acidic pH of resting vesicles, spH fluorescence is quenched by protons because of the H^+-ATPase activity. After

fusion with the plasma membrane, the vesicle interior becomes accessible from the relatively alkaline extracellular pH environment, allowing the protons to escape. The fluorescence intensity of spH increases rapidly and remains elevated until the pore closes and the vesicle is reacidified (Miesenböck & al. 1998).

Confocal imaging showed that half of the spontaneous exocytotic events exhibited fusion pore openings associated with an increase in spH fluorescence, indicating permeation of protons, however the pores were impermeable to FM 4-64 and HEPES molecules. Together with the results on G_p measurements these findings indicate an open fusion pore diameter in resting peptidergic vesicles of <0.5 nm. This is much narrower than the size of neuropeptides stored in these vesicles (prolactin molecular diameter = ~5.2 nm; Vardjan & al. 2007), indicating that a narrow open fusion pore (lower conductance - G_p) prevents or slows down the release process in resting lactotrophs. Probes used in the earlier fusion pore permeability studies in resting secretory cells (FM-dyes, horseradish peroxidase, antibodies) were of relatively large size >0.9 nm (Malgaroli & al. 1995, Ryan & al. 1997, Sara & al. 2005), leading to the underestimation of fusion pore diameter and the extent of spontaneous fusion.

In stimulated lactotrophs, >70% of exocytotic events exhibited a larger, FM 4-64–permeable pore (>0.9 nm) consistent with previous fusion pore permeation studies (Barg & al. 2002, Takahashi & al. 2002, Tsuboi & Rutter 2003, Fulop & al. 2005) and our G_p measurements (Vardjan & al. 2007, Jorgacevski & al. 2008). Stimulation-induced fusion pore widening is consistent with a facilitated vesicle cargo discharge in the majority of stimulated fusion events recorded (Vardjan & al. 2007). Similarly, stimulation led to the fusion pore expansion in chromaffin cells, therefore increasing the efficiency of release of small classical transmitters (Elhamdani & al. 2001, Fulop & al. 2005).

Summary

Transient fusion was considered to be an event mediating complete discharge of the vesicle content (Katz 1969), however recent findings indicate that vesicle content may be emptied incompletely in transient fusion events. Partial vesicle discharge appears to occur at the post-fusion stage. Transient fusion pores are subject to physiological regulation which affects the amount of vesicle cargo discharge from a single vesicle. Current knowledge of regulatory mechanisms at the post-fusion stage, involving fusion pore diameter and/or fusion pore open-time modulation, is still limited (Rahamimoff & Fernandez 1997, Vardjan & al. 2007). However, in lactotrophs the transient "kiss-and-run" mode of exocytosis is a robust physiological phenomenon and thus represents a model for further studies towards the unravelling the nature of the exocytotic fusion machinery. Based on the current results the release of vesicle cargo may be restrained kinetically and/or due to a narrow fusion pore. Under stimulation, the pre-formed fusion pore may retain the transient nature, but with a longer dwell-time, increased frequency of re-openings and a wider effective fusion pore diameter. All of these changes will facilitate the vesicle cargo release (Fig. 5; Vardjan & al. 2007). Regulation of fusion pore dynamics is important, as any modulation of release processes could have an impact on specific physiological functions of cells.

Membrane fusion involves high energy barrier, therefore it is unlikely that transient fusion events represent cycles of fusion/fission of a single vesicle. Transient fusion events may represent fluctuations of an open fusion pore between states where the pore is extremely narrow. A narrow fusion pore is likely composed of molecules with highly negative curvatures (Churchward & al. 2008). It was reported recently that the structure of plasma membrane sites where prolactin vesicles undergo exocytosis are distinct from the plasma membrane areas devoid of docked prolactin vesicles (Gonçalves & al. 2008). In the future the determination of the structure of the fusion pore and of the cellular mechanisms underlying the nature of the repetitive transient fusion events will be critical for a better understanding of exocytosis of peptidergic vesicles.

Povzetek

Dolgo je veljalo, da se pri procesu prehodne eksocitoze vsebina mešička popolnoma izloči skozi fuzijsko poro (Katz 1969), vendar pa so novejše raziskave pokazale, da se lahko vsebina mešička pri prehodni eksocitozi sprosti le delno. Delno izločanje vsebine mešička je najverjetneje uravnavano po zlitju mešička s plazemsko membrano oz. na stopnji že oblikovane fuzijske pore. Fuzijska pora je torej prehodna struktura, ki je po samem nastanku lahko fiziološko uravnavana, kar vpliva na sproščanje količine vsebine mešička. Dosedanje znanje o postfuzijskem uravnavanju izločanja vsebine mešička na ravni modulacije premera fuzijske pore in časa odprtja fuzijske pore je pomanjkljivo (Rahamimoff & Fernandez 1997, Vardjan & al. 2007). V laktotrofih je prehodna eksocitoza robusten fiziološki fenomen, zato lahko celice laktotrofov uporabljamo kot model za študij prehodne eksocitoze, kar bi v prihodnje pripomoglo k razjasnitvi osnovnih mehanizmov eksocitoze. Na podlagi dosedanjih rezultatov je izločanje vsebine mešička lahko ovirano kinetično in/ali zaradi ozke fuzijske pore. Po stimulaciji lahko fuzijska pora ohrani svojo prehodno naravo odpiranja, vendar pa je prehodna fuzijska pora po stimulaciji dlje časa odprta, se pogosteje odpira in zapira in ima večji premer. Vse te spremembe pospešijo izločanje vsebine mešička (Fig. 5; Vardjan & al. 2007). Uravnavanje dinamike izločanja vsebine mešička skozi fuzijsko poro je fiziološko zelo pomembno, saj ima lahko vsaka modulacija izločanja vpliv na specifične fiziološke funkcije celic.

Fuzija membran je energijsko neugoden proces, zato prehodna narava odpiranja fuzijske pore najverjetneje ni posledica ponavljajoče fuzije/fisije posameznega mešička. Prehodno odpiranje fuzijske pore je bolj verjetno posledica fluktuacije odprte fuzijske pore med stanji, ko je fuzijska pora skrajno ozka in stanji, ko se le-ta prehodno bolj odpre. Ozka fuzijska pora je zelo verjetno sestavljena iz molekul, ki imajo močno negativno ukrivljenost (Churchward & al. 2008). Nedavno je bilo pokazano, da se struktura plazemske membrane na področjih, kjer poteka eksocitoza prolaktinskih mešičkov razlikuje od plazemske membrane, kjer ni pripetih prolaktinskih mešičkov (Gonçalves & al. 2008). V prihodnje bo določitev strukture fuzijske pore in pa razjasnitev celičnih mehanizmov, ki uravnavajo prehodno odpiranje fuzijske pore, kritično pripomoglo k boljšemu razumevanju mehanizmov eksocitoze peptidnih hormonov.

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