Glutamate stimulation increases hormone release in rat melanotrophs

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Abstract

In melanotrophs, neuroendocrine cells from the intermediate lobe of the rat pituitary gland, glutamate causes a rise in intracellular $[\text{Ca}^{2+}]$ suggesting the presence of ionotropic NMDA and non-NMDA AMPA/K receptors. However, the $\text{Ca}^{2+}$-dependent release of the major peptide hormone, $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH), in response to glutamate stimulation has not been studied yet in this cell model. Significant spontaneous secretion of the peptide, which results in hormone deposits on the perimeter of the cells, has been confirmed by using confocal microscopy. Co-staining with a membrane area marker FM 1-43, which co-localized with the immunocytochemically marked hormone deposits, showed that fusion-competent sites on the plasma membrane coincided with secretion-competent sites. Stimulation of the cells with glutamate and high K$^+$ saline induced a significant increase in the plasma membrane area covered with $\alpha$-MSH deposits compared to control cells incubated with glutamate and CNQX, a glutamate channel blocker. The optical approach to monitor the secretory activity of a single neuroendocrine cell revealed that glutamate stimulates the release of $\alpha$-MSH at distinct exocytotic membrane domains only.

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Melanotrophs, neuroendocrine cells from the intermediate lobe of the rat pituitary gland, secrete the pro-opiomelanocortin-derived peptide hormones, $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH) and $\beta$-endorphin [10]. Melanotrophs exhibit substantial spontaneous secretion in cell culture [2], which is reduced by the neurotransmitters, dopamine and $\gamma$-aminobutyric acid (GABA), present in the hypothalamic neurons innervating the intermediate lobe [19,20]. Corticotrophin-releasing factor and $\beta$-adrenergic agonists have been shown to stimulate melanotroph secretory activity in rats [6,8]. In addition, excitatory glutamatergic innervation has been suggested to affect the secretion of peptide hormones from these cells [4].

Glutamate is an excitatory amino acid neurotransmitter in the mammalian central nervous system. In addition, evidence is emerging for a role of glutamate in the autocrine and paracrine signalling in peripheral tissues [5]. Glutamate has a differential effect on prolactin release from anterior pituitary cells: it exerts a stimulatory action via ionotropic receptors and an inhibitory effect via metabotropic receptors [14]. In the rat melanotrophs, glutamate and specific receptor agonists have been shown to cause a rise in intracellular $[\text{Ca}^{2+}]$ suggesting the presence of ionotropic NMDA and non-NMDA AMPA/K receptors, but not the metabotropic glutamate receptors [4].

Hormone secretion is mediated by fusion of the secretory vesicle with the plasma membrane. This has been examined earlier in melanotrophs by monitoring rapid changes in membrane surface area as discrete jumps in membrane capacitance [9,23], by amperometry [15,22] and by steryl dye FM 1-43 fluorescence changes [17]. However, the final stage of secretion in rat melanotrophs, the release of the peptide hormones in response to the glutamate stimulation has not been studied yet.

Structures, where fusion of vesicles can occur, have been recently identified for exocytotic fusion of single synaptic vesicles in the presynaptic nerve terminal of retinal bipolar neurons and in adrenal chromaffin cells by total internal reflection fluorescence (TIRF) microscopy [21] and in rat melanotrophs using steryl dye [17]. Multiple vesicles fuse with the plasma membrane within distinct membrane domains and
presumably contribute to a localized release and deposition of vesicle cargo on the cell surface.

In this work we tested the hypothesis that stimulation of exocytosis by glutamate in rat melanotrophs results in hormone release and in the deposition of peptide hormone cargo on the cell surface.

Adult Wistar rats were used for the study, and were euthanized with carbon dioxide. The animals were sacrificed in accordance to the following ethical codes and directives: 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 Republic of Slovenia, Nos. 98/99, 126/03 and 20/04).

Rat pituitary melanotrophs were prepared as described previously [16]. For combined live cell immunofluorescence and FM 1-43 imaging, cells cultured on glass coverslips were incubated in PBS/BSA solution containing anti α-MSH antibodies (1:400 dilution, from Peninsula Laboratories, USA) for 10 min at 4 °C. Cells were given a single wash in PBS/BSA solution and were then incubated in PBS/BSA solution containing Alexa 546 labelled secondary goat anti-rabbit IgG (1:500 dilution, from Molecular Probes, USA) at 37 °C for 2 h. FM 1-43 fluorescence, the external medium was replaced with 5 μM FM 1-43-containing external solution. The cells were imaged on an inverted microscope with ×63, 1.4 NA oil objective, coupled to a Zeiss 510 laser scanning confocal microscope (Zeiss, Jena, Germany). To avoid cross-talk between the fluorescence channels sequential excitation mode has been used (FM 1-43, 458 nm, Ar laser; Alexa 546, 543 nm, He-Ne laser). In addition, to take intensity images, the emissions of FM 1-43 and Alexa 546 were separated by using BP 505–530 nm and BP 565–615 nm emission filters, respectively. Optical sections for spherically shaped cultured melanotrophs were taken at the equatorial plane.

To assess the area covered by depositions of α-MSH on the cell periphery under different physiological conditions, cells were incubated in solutions consisting of the control (external bath solution), high KCl saline, glutamate and glutamate in the presence of the CNQX that blocks the glutamate ionotropic AMPA receptor, and also binds to the glycine site of NMDA receptors, for 2 min. The external bath solution contained (in mM): NaCl 131.8, CaCl2·2H2O 5, KCl 5, MgCl2·6H2O 2, HEPES/NaOH 10, d-glucose 10, NaH2PO4·2H2O 0.5, NaHCO3 5; pH 7.2. For KCl depolarization of the melanotrophs, 70 mM KCl and 67 mM NaCl were used in the bath solution. The glutamate and CNQX concentrations were 500 and 10 μM, respectively. The cells were subsequently fixed in paraformaldehyde (4% in PBS), at room temperature, without permeabilizing the cells with detergent, washed thrice in PBS, followed by incubation for 10 min in PBS/BSA solution containing anti α-MSH-antibodies (1:400 dilution, from Peninsula Laboratories, USA) for 10 min at 4 °C. Cells were washed thrice with PBS and incubated for 20 min in PBS/BSA solution containing Alexa 546 labelled secondary goat anti-rabbit IgG (1:500 dilution, from Molecular Probes, USA), and mounted in Slow Fade (Molecular Probes, USA). To quantify images, these were analyzed with LSM510 software (Zeiss, Jena, Germany) and 3D view of the intensity profile was prepared with Matlab software (MathWorks, Novi, MI, USA).

Previous studies in rat prolactin-secreting cells revealed that the matrix of secretory vesicles is stained by FM 1-43, which is manifested in abrupt appearance of fluorescent spots after secretion stimulation. Some of the spots also immunostain for prolactin [1]. The FM 1-43 staining of lactotrophs enables the real time monitoring of unitary exocytotic events in live cells [1,18]. Similarly, in rat melanotrophs, patches of intense staining by FM 1-43 associated with the plasma membrane were observed (Fig. 1b, middle). In order to understand whether the localized bright spots of membrane area marker FM 1-43 fluorescence relate to the deposits of vesicle content associated with secretory activity, we stained living melanotrophs additionally for α-MSH, by using an antibody. The cytofluorogram in Fig. 1b (right) shows the pixels in which signals related to the α-MSH (Fig. 1, left), and FM 1-43 (Fig. 1, middle) are co-localized. The high intensity labelling by FM 1-43 is associated with local cell surface deposits of secretory vesicle matrix containing α-MSH, and can be surmised to report the end point of secretory activity. In addition, the FM 1-43 also faintly labelled the plasma membrane as reported previously [7,18].

We examined hormone deposits on the plasma membrane of rat pituitary melanotrophs by the staining for α-MSH after fixation without detergent permeabilization. We tested whether discrete regions in the cell perimeter can respond to glutamate stimulation by a change in fluorescence. Prior to immunocytochemical labeling, the cells were stimulated by exposure to glutamate that predominantly activated the ionotropic receptors in the melanotrophs [4]. Cells were also stimulated to undergo release in high K+ saline, and in the presence of glutamate...
along with CNQX, glutamate channel blocker. The control cells showed discrete patches of staining for α-MSH associated with the membrane surface (Fig. 2), indicating that in control conditions a substantial spontaneous rate of secretion was present in these cells, resulting in hormone deposition on the cell surface. This is in line with previous findings of hormone secretion by cultured melanotrophs in the absence of stimulation [2]. The patches of staining for α-MSH were uniformly distributed at the surface of the membrane. To quantify confocal images, optical sections of spherically shaped cultured melanotrophs were taken at the equatorial plane. The amount of α-MSH depositions associated with the membrane surface was normalized with the cross-sectional area of the cell and plotted as percent deposition per cell area.

As shown in Fig. 2a and the histogram in Fig. 2b, stimulation with high K⁺ saline or glutamate caused a significantly higher area of α-MSH patchy depositions compared to control cells co-incubated with glutamate and CNQX. The experiments show that a limited number of discrete patches of α-MSH deposits can be detected on the equatorial perimeter of single cells.

The bright FM 1-43 stained patches in the cell perimeter appeared to be qualitatively different from the fluorescence observed in the rest of the plasma membrane, typically with several fold higher fluorescence intensities. This could be due to FM 1-43 staining of vesicle content during secretory vesicle fusion, as observed previously in pituitary lactotrophs [1,18]. The physiological basis for this observation is consistent with the relatively high basal secretion documented in rat melanotrophs [2]. Peaks of the FM 1-43 fluorescence profiles are shifted toward cell interior in respect to peaks of α-MSH fluorescence profiles. Shifts in locations marked from 1 to 4 in Fig. 1a were 70, 420, 280 and 70 nm, respectively. This may suggest, that a fraction of depleted vesicles, stained with FM 1-43 translocate away from the plasma membrane after exocytosis.

The patchy distribution of α-MSH antibody staining on the cell perimeter may stem from spatially confined areas of glutamate- and KCl-stimulated calcium sources in the plasma membrane [12] that could trigger the release of uniformly distributed secretory machinery in the plasma membrane. However, this is unlikely, since using fura-2 imaging, Ca²⁺ influx in melanotrophs appears evenly distributed (not shown), consistent with observations in acutely isolated neurohypophysial axon terminals [3]. Moreover, dialysis of different calcium buffers into melanotrophs revealed a relatively large distance between calcium sources and calcium sensors of the secretory apparatus [11,13].

Experimental evidence of patchy α-MSH antibody deposits indicates that distinct locales of the plasma membrane in melanotrophs carry secretion competence, termed exocytotic domains. Therefore, this approach can be used to monitor both the spontaneous and stimulated secretion in single neuroendocrine cells in culture.

The staining of the cell membrane by FM 1-43, discrete high fluorescent spots have been seen, and associated with staining of granule content in the pituitary neuroendocrine lactotrophs [1].

Previous work has shown that stimulation of the ionotropic glutamate receptors by both AMPA and NMDA increased

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**Fig. 2. Patchy hormone depositions on resting and stimulated melanotrophs.**

(a) The images on the left show immunofluorescence in cells incubated under different conditions, while the 3D profile of the fluorescence intensity is shown in the right. The fluorescence intensities were sampled (8 bit) and the image was sectioned with an intensity threshold of 40% of the maximal intensity, which is indicated by the horizontal plane (right column). Scale bar: 5 μm. (b) Histogram showing the percent area of hormone deposition per cell area in the four different conditions (Control, [Cont.]; membrane depolarization by high K⁺, [+ K⁺]; stimulation by glutamate, [+ Glut.]; stimulation by glutamate in the presence of an AMPA receptor blocker CNQX, [+ Glut. + CNQX]). A 2 μm thick circular rim around the cell perimeter in the equatorial plane of the cell was analyzed (46–60 cells tested) for fluorescence intensity using confocal microscopy. The area of the cell occupied by pixels above the threshold was measured, normalized by the cell cross-sectional area and plotted as % of deposition area per cell. *P < 0.0001, Student’s t-test.
intracellular calcium, compared to the activation of metabotropic glutamate channels [4]. To test whether activation of these receptors activates secretory activity, monitored by measuring fluorescence at the cell perimeter, we stimulated the cells with glutamate. Cells were also stimulated to undergo release in high K⁺ saline, in presence of glutamate along with CNQX that block the glutamate ionotropic AMPA receptor, and also binds to the glycine site of NMDA receptors. Stimulation with high K⁺ saline and glutamate caused a significantly higher area of α-MSH patchy depositions compared to control cells co-incubated with glutamate and CNQX. These deposits are likely to be due to vesicle fusion activity at the secretion-competent sites in melanotrophs since the depositions of the vesicle content on the receptors critically regulate localized secretory activity in the plasma membrane, rather than unspecific binding. AMPA to vesicle fusion activity at the secretion-competent sites in melanotrophs with glutamate and CNQX. These deposits are likely to be due to vesicle fusion activity at the secretion-competent sites in the plasma membrane, rather than unspecific binding. AMPA receptors critically regulate localized secretory activity in melanotrophs since the depositions of the vesicle content on the membrane surface, was effectively blocked by CNQX (Fig. 2).

In summary, the optical approach to monitor the secretory activity of a single neuroendocrine cell revealed that glutamate stimulation of hormone secretion results in hormone depositions at distinct exocytotic membrane domains.

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