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Optical control of calcium-regulated exocytosis

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ABSTRACT

Background: Neurons signal to each other and to non-neuronal cells as those in muscle or glands, by means of the secretion of neurotransmitters at chemical synapses. In order to dissect the molecular mechanisms of neurotransmission, new methods for directly and reversibly triggering neurosecretion at the presynaptic terminal are necessary. Here we exploit the calcium permeability of the light-gated channel LiGluR in order to reversibly manipulate cytosolic calcium concentration, thus controlling calcium-regulated exocytosis.
Methods: Bovine chromaffin cells expressing LiGluR were stimulated with light. Exocytic events were detected by amperometry or by whole-cell patch-clamp to quantify membrane capacitance and calcium influx.
Results: Amperometry reveals that optical stimulation consistently triggers exocytosis in chromaffin cells. Secretion of catecholamines can be adjusted between zero and several Hz by changing the wavelength of illumination. Differences in secretion efficacy are found between the activation of LiGluR and native voltage-gated calcium channels (VGCCs). Our results show that the distance between sites of calcium influx and vesicles ready to be released is longer when calcium influx is triggered by LiGluR instead of native VGCCs.
Conclusion: LiGluR activation directly and reversibly increases the intracellular calcium concentration. Light-gated calcium influx allows for the first time to control calcium-regulated exocytosis without the need of applying depolarizing solutions or voltage clamping in chromaffin cells.
General significance: LiGluR is a useful tool to study the secretory mechanisms and their spatiotemporal patterns in neurotransmission, and opens a window to study other calcium-dependent processes such as muscular contraction or cell migration.

1. Introduction

Exocytosis is the process by which a cell directs the content of secretory vesicles out of the cell membrane. It is a key process in the expression of membrane proteins, in the secretion of soluble proteins and hormones, and in neurotransmission where exocytosis of transmitter molecules at the presynaptic terminal and the subsequent activation of ligand-gated channels at the postsynaptic neuron allow the propagation of the nerve impulse from one neuron to another.

Exocytic events have been studied in great detail in chromaffin cells, because their size and shape provide good experimental access [1], and the molecular machinery involved in secretory granule exocytosis is homologous to that of synaptic vesicles in neurons [2].

Available methods to manipulate exocytosis can act at two stages of the process. First, by depolarizing the membrane of secretory cells in order to activate the native voltage-gated calcium channels (VGCCs) that mediate exocytosis under physiological conditions. These methods include application of depolarizing solutions, depolarizing pulses by means of patch clamp electrodes, and more recently the activation of the cation-selective channel Channelrhodopsin-2 (ChR2) with light. Alternatively, exocytosis can be manipulated by altering the intracellular calcium concentration with Ca^{2+} ionophores (*i.e.* ionomycin) or Ca^{2+} uncaging. These classical methods directly modify intracellular Ca^{2+} concentrations but are not reversible. Thus, new methods are needed in order to control directly and reversibly intracellular calcium levels, thereby triggering regulated exocytosis.

An appealing possibility is controlling the presynaptic Ca^{2+} concentration with light using photoswitchable ion channels like ChR2 [3] or the light-gated glutamate receptor (LiGluR) [4]. Light stimulation is non-invasive and provides spatial and temporal control at the level of individual synaptic terminals (1 μm) and single action potentials (1 ms). LiGluR and ChR2 are cation-selective channels that upon activation produce large inward currents mainly due to Na^+ ions at the cell resting potential [5–7]. Light-gated currents readily depolarize the neuron soma and trigger action potentials that propagate along the processes and have been used to investigate neuronal circuits *in vivo* [3,8–11] and to manipulate astrocyte-to-astrocyte communication [12]. However, the Ca^{2+} permeability of light-gated

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channels has never been exploited to manipulate the intracellular Ca^{2+} concentration without altering membrane potential, which opens up the possibility of directly triggering secretion processes [13]. Here, we demonstrate specific control of Ca^{2+} currents and exocytosis with light using LiGluR and we compare it with exocytosis triggered by VGCCs in the same cells.

2. Materials and methods

2.1. Chromaffin cell culture and infection

Chromaffin cells were isolated from medulla of bovine adrenal glands by enzymatic treatments [14]. Dissociated chromaffin cells were plated at 2.5×10^5 cells $well^{-1}$ density in poly-L-lysine treated coverslips. After 1 day, the cells were infected using an adenoviral construction carrying the fusion protein GluK2-L439C-eGFP. Amperometry and patch clamp experiments in chromaffin cells were performed after 1 to 2 days after infection.

2.2. Conjugation of MAG photoswitch

MAG was synthesized as described [4] and the concentrated stock (10 mM in DMSO) was stored at −20 °C. Before all experiments, the cells were incubated in the absence of light, for 10 min in a Na^+-free and low-Ca^{2+} (0.5 mM) solution with 10–100 μM of MAG (DMSO final concentration <1%) and 0.3 mg mL^{-1} concanavalin A, to block GluKs desensitization [15].

2.3. Carbon microfiber amperometry

Catecholamine release was detected using homemade polyethylene-insulated carbon fiber electrodes of 12-μm diameter [16,17]. Amperometric electrodes were first tested in a solution containing 5 mM ferricyanide in 0.1 M KCl and pH 6.8. Electrodes displaying a current between 1 and 10 nA at a holding potential of +700 mV were selected, and their integrity was verified by voltammetry [18]. Only electrodes showing a symmetric oxidation/reduction current response to a symmetric ramp from +700 to −300 mV (scan rate 100 mV/s) were used to measure exocytosis in chromaffin cells. When necessary, electrodes were freshly cut with a scalpel on a glass surface, and were used for further experiments if the basal current was between 10 and 20 pA (at holding potential +700 mV) in the bath solution. Amperometric current was recorded by applying a holding voltage of +700 mV with an EPC-10 amplifier (HEKA) controlled with Patch Master (HEKA). The sampling rate was 100 kHz and the current was filtered with a Bessel Filter set at 30 kHz. After data acquisition, traces were digitally filtered at 1 kHz. For controls, cells were stimulated by local application of a high-K^+ solution. For light stimulation, illumination was applied using a TILL Photonics Polychrome V monochromator through the side port of an IX70 inverted microscope (Olympus) and using a UApo/340, 40×/1.35 objective. Shutter and wavelength were controlled trough an EtherNet-COM-1 connection to a PC, using TILL Photonics Polychrome V Control (PolyCon) software. The light power measured with a light meter model Newport 1916-C placed next to the objective was 0.9 mW mm^{-2} at 380 nm and 1.7 mW mm^{-2} at 500 nm.

2.4. Voltage-clamp recordings

Recordings of voltage-clamp under whole cell configuration were done using an EPC-10 amplifier and Patch Master software. Pipettes were pulled from borosilicate glass tubing (Harvard Apparatus) with P-97 puller from Sutter Instruments, with a typical resistance of 2–4 MΩ. Membrane capacitance was implemented using the Sine + DC mode of the Patch Master lock-in module. A 1000 Hz, 25 mV sinusoidal wave was applied to a holding potential (V_h) of −80 mV and current was acquired at a sampling rate of 20 kHz. Before each stimulus (depolarization or UV-light) a hyperpolarization of −90 mV was applied to later allow leak subtraction to ion currents. In all voltage-clamp experiments, illumination was set using the same system as for the amperometric. However, light was synchronized with the stimulus, then shutter and wavelength were controlled through photometry module of Patch Master, via Photochromic Manual Control (TILL Photonics) connected to EPC-10 amplifier.

2.5. Data analysis

All analysis was done with IgorPro from Wavemetrics. A macro developed by Eugene Mosharov (http://www.sulzerlab.org) was used for amperometric spike and parameter analysis [19]. Data was exported to Matlab to calculate secretory rate. The membrane capacitance increase was calculated by subtracting the mean basal capacitance to the mean capacitance after stimulus using an IgorPro custom made macro. To calculate the mean membrane capacitance after the stimulus, it was considered a 300 ms time window, starting after 100 ms from the end of the stimulus [20]. All data are expressed as mean ± s.e.m. (standard error of measurement, calculated over the number of n), except for the parameters calculated by curve fittings, where the error is calculated as 95% confidence interval.

We define secretory or exocytic efficacy as the increase of the membrane capacitance divided by the Ca^{2+} charge of the measured Ca^{2+} influx ($\Delta C_m/Q_{Ca^{2+}}$), expressed in fF/pC.

2.6. Solutions

The composition of physiological bath solution (in mM): 140 NaCl, 2.5 KCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose, 2.5 $CaCl_2$ at pH 7.42 and 300 mOsm kg^{-1}. The high-K^+ solution was composed by (in mM): 90 NaCl, 50 KCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose, 5 $CaCl_2$ at pH 7.42 and 300 mOsm kg^{-1}. The Na^+-free and high-Ca^{2+} bath solution contained (in mM): 110 $NMDG^+$, 2.5 KCl, 1 $MgCl_2$, 10 HEPES, 10 Glucose, 30 $CaCl_2$ at pH 7.42 and 300 mOsm k^{-1}. The composition of pipette solution was (in mM): 120 Cesium methanesulfonate, 10 TEA-Cl, 20 HEPES, 3 Na_2ATP, 1 NaGTP and 0.4 BAPTA, pH 7.2 and 290 mOsm kg^{-1}. Except for the experiments with restriction of Ca^{2+}-diffusion, in which EGTA was used instead of BAPTA at (mM): 0.4, 0.6, 0.8, 1.5, 2.7, 5 or 15. In the indicated experiments, the following cocktail of toxins was added to the bath solution: 100 nM ω-agatoxin IVA and 1 μM ω-conotoxin GIVA (Alomone Labs) and 10 μM nifedipine.

All reagents were obtained from Sigma unless otherwise specified.

3. Results

In order to test whether secretion of neurotransmitters can be controlled optically in neurosecretory cells, the receptor-channel GluK2-L439C-eGFP was expressed in bovine chromaffin cells by application of a viral vector. More than 70% of the cells showed GFP expression after 24 h of infection. Prior to the experiments, the receptor was chemically conjugated to the photoswitch Maleimide Azobenzene Glutamate (MAG) as described [4] in order to obtain LiGluR. In 90% of the fluorescent cells evaluated, illumination with 380 nm light resulted in LiGluR opening and visible light of 500 nm closed the channel, indicating that its permeability for Ca^{2+} was susceptible to be controlled by light.

3.1. Expression of LiGluR in chromaffin cells supports optical control of neurosecretion

Exocytosis from chromaffin cells was assayed with amperometry using carbon microfiber electrodes [21,22]. In order to avoid the activation of VGCCs during optical stimulation, cells were bathed in a physiological solution containing nifedipine, ω-conotoxin GVIA and ω-agatoxin IVA, which blocks the three types of VGCCs expressed in