Cell Calcium 54 (2013) 105–110

Contents lists available at SciVerse ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Snapin accelerates exocytosis at low intracellular calcium concentration in mouse chromaffin cells

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ARTICLE INFO

Article history:
Received 22 March 2013
Received in revised form 30 April 2013
Accepted 3 May 2013
Available online 30 May 2013

ABSTRACT

Snapin associates with SNAP-25 and with assembled SNARE complexes, stabilizing the coupling between Synaptotagmin-1 and SNAP-25. Deletion of Snapin reduces releasable pools of vesicles in chromaffin cells and reduces synchronous release of neurotransmitter in cortical neurons. Snapin deletion leads to a deficit in exocytosis at low calcium concentration with no change in the threshold calcium concentration for exocytosis in chromaffin cells. In order to determine whether Snapin deletion alters release rates or calcium dependence, we examined the effect of overexpression of wild type Snapin on readily releasable pool kinetics and pool size in mouse chromaffin cells. Modest increases in intracellular calcium induced by flash-photolysis unmasked a rapidly releasing component of secretion which was enhanced when Snapin was overexpressed. This result indicates that Snapin allows rapid release at lower intracellular calcium levels at which release of the remaining RRP occurs more slowly.

1. Introduction

Snapin was discovered in a yeast two-hybrid screen searching for binding partners for SNAP-25. Snapin promotes binding of Synaptotagmin-1, the calcium sensor for synchronized transmitter release [1], to the assembled SNARE complex [2,3]. Phosphorylation of Snapin by protein kinase A at serine 50 is required for activity [4]. Deletion of Snapin in mouse chromaffin cells reduces the readily releasable pool without altering the sustained rate of release or docking parameters, leading to the conclusion that primed vesicles are more stable in the presence of Snapin. Overexpression of Snapin in Snapin knockout mice rescues this release defect [3]. In cultured hippocampal neurons, Snapin overexpression increases release probability [5] while its deletion leads to a reduction of the readily releasable pool and a dramatic loss of synchronous transmitter release [6]. These authors suggest that stabilization of the RRP and synchronization of synaptic release in neurons occurs by different mechanisms.

Snapin interacts with SNAP-23, a SNAP-25 analog which is ubiquitously expressed and which is involved in many types of membrane fusion events in a variety of cell types [7], and Snapin may play a role in lysosomal and endosomal fusion in neurons as well [8]. Thus, loss of Snapin is not neuron or synapse specific and its loss may lead to a number of developmental changes as a result of changes in membrane trafficking. These developmental effects complicate interpretation of results from knockout cells.

Loss of synchronization of evoked release may result from failure to position releasable vesicles near calcium channels, lowered release probability or altered calcium sensitivity. When a slow increase in calcium concentration (calcium-ramp) was applied [3], chromaffin cells lacking Snapin exhibited reduced secretion at low calcium concentrations (up to 5–6 μM) with no change in the threshold for stimulated release. Since these experiments used calcium uncaging, proximity of release sites to calcium channels cannot explain this result. The lack of a change in threshold does not rule out a shift in calcium sensitivity of some vesicles. Alternatively, vesicles releasing more rapidly with the same calcium sensitivity (e.g. a highly calcium sensitive pool [9]) will also lead to an apparent shift in calcium-dependence during calcium-ramp stimulation, since the calcium concentration changes over time.

In order to distinguish between changes in calcium sensitivity and changes in release rate we have examined how Snapin affects release following stepwise increases in global $[Ca^{2+}]_i$ to low μM concentrations. To avoid developmental effects we have used overexpression of Snapin in wild type cells. Our results indicate that overexpression of Snapin increases rapid release at low calcium levels.

2. Materials and methods

2.1. Chromaffin cell preparation and infection

All electrophysiological experiments were performed on mouse chromaffin cells in primary culture. The cells were prepared on

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http://dx.doi.org/10.1016/j.ceca.2013.05.003

day 0–6 after birth as previously described [10]. Briefly, mice were anesthetized with CO_2, decapitated, and the adrenal glands were rapidly removed and placed in cold Locke's solution. The glands were then incubated for 20 minutes in a DMEM solution containing 20 U/ml papain (Roche, Mannheim, Germany). After removal of the papain solution the glands were rinsed twice with DMEM and treated for five minutes with an inactivating solution (DMEM plus 10% BSA), rinsed and then triturated and plated on glass cover slips. DMEM (3 ml) was added to each well 30 minutes after plating, and the cells were incubated for up to 4 days at 37 °C in DMEM gassed with 8% CO_2. For all overexpression experiments, isolated chromaffin cells were infected with 100 μl of activated virus (pSFV1-Snapin-IRES-GFP, pSFV1-Snapin-S50A-IRES-GFP, pSFV1-Snapin-S50D-IRES-GFP, pSFV1-IRES-GFP or pSFV1-ComplexinII-GFP) following a protocol described earlier [11].

2.2. Electrophysiology

Whole-cell recordings were performed using pipettes with resistances of 3–6 MΩ and recorded using an EPC-9 patch-clamp amplifier controlled with Pulse software (HEKA, Lambrecht, Germany). The extracellular solution contained 145 mM NaCl, 2.4 mM KCl, 10 mM HEPES, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose (pH 7.5). The intracellular solution contained 100 mM Cs-glutamate, 2 mM Mg-ATP, 0.3 mM Na_2-GTP, 40 mM Cs-HEPES, 5 mM nitrophenyl-EGTA (NP-EGTA), ~4 mM $CaCl_2$, 0.4 mM Furaptra and 0.4 mM Fura-4F (pH 7.2). Calcium was added to reach a saturation of the NP-EGTA of 80% or less. Capacitance measurements were performed using the Lindau–Neher technique implemented as the 'sine + dc' mode of the software lock-in extension of the PULSE software [12]. A 1-kHz, 70 mV peak-to-peak sinusoid stimulus was applied at a holding potential of −70 mV. All experiments were performed at room temperature. Data are shown as mean ± SEM. We used the Mann–Whitney U test for comparison of differences between groups. Curve fits were done using IGOR Pro (Wavemetrics, Lake Oswego, OR, USA).

2.3. Measurements of $[Ca^{2+}]_i$ and photolysis of caged Ca^{2+}

$[Ca^{2+}]_i$ was measured using a mixture of two indicator dyes, Fura-4F and Furaptra. The dyes were excited with UV-light alternated between 350 and 380 nm using a monochromator-based system, and fluorescence was measured using a photomultiplier (T.I.L.L. Photonics, Planegg, Germany). To convert the ratio R of the fluorescence at both wavelengths into $[Ca^{2+}]_i$, an in vivo calibration curve was used [13]. To obtain step-wise increases in $[Ca^{2+}]_i$ short flashes of ultraviolet light from a Xenon arc flash lamp (Rapp OptoElectronics, Hamburg, Germany) were applied. The monochromator was used to maintain calcium levels after flashes by applying pulses of illumination at 350 and 380 nm resulting in additional photolysis to offset cellular extrusion/confinement. This allowed simultaneous ratiometric measurement of $[Ca^{2+}]_i$.

3. Results

3.1. Snapin enhances the exocytotic burst generated by photolysis to low μM $[Ca^{2+}]_i$

Flash photolysis produced a $[Ca^{2+}]_i$ jump to ~6 μM which was maintained by illuminating the cell at 350 and 380 nm wavelengths (upper traces, Fig. 1A). The stimulus produced an exocytotic burst measured as a capacitance increase (lower traces, Fig. 1A). A subsequent train of depolarizations produced additional secretion. Cells overexpressing Snapin (gray trace) secreted significantly more in the first 300 ms (107.3 ± 11.4 fF $n = 15$ vs. 55.3 ± 7.3 fF, $n = 15$, $p \leq 0.01$) though the subsequent secretion was similar to that observed in control cells. Total secretion was greater in Snapin expressing cells (181.8 ± 24.7 fF) than in controls (108.6 ± 18.2 fF, $p \leq 0.01$). The secretion is modest, due to use of perinatal mouse cells and moderate calcium loading.

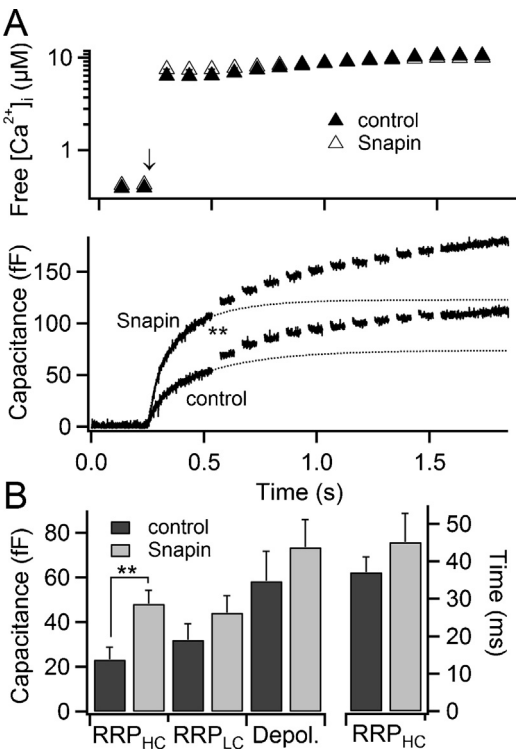

Fig. 1. Snapin enhances responses to $[Ca^{2+}]_i$ jumps in the low μM range. (A) The photolysis of caged Ca^{2+} (↓) was followed 300 ms later by a train of depolarizations to assess the residual RRP. The record shows the mean $[Ca^{2+}]_i$ (±SEM) for control cells (upper panels, control, $n = 15$) and cells overexpressing wild type Snapin ($n = 15$). There was a significantly greater increase in capacitance due to calcium photolysis (lower panel) in the Snapin expressing cells (107.3 ± 11.4 fF) than that in control cells (**$p \leq 0.01$, 55.3 ± 7.3 fF). Total secretion was also greater in Snapin overexpressing cells (181.8 ± 24.7 fF) than in control cells (108.6 ± 18.2 fF, $p \leq 0.01$). Depolarizing pulses induced additional secretion in both cell groups. The initial secretory burst was well fit with a double exponential (shown as dashed line extended to the end of the record). (B) The fast component (RRP_{HC}) as well as the remaining RRP and depolarization are shown (mean ± SEM). The RRP_{HC} of cells overexpressing wild type Snapin (48.2 ± 6.1 fF) was significantly larger than that of control cells (23.2 ± 5.6 fF, $p \leq 0.01$). The time constant of the RRP_{HC} of Snapin expressing cells (37.6 ± 4.1 ms) was estimated from the exponential fits and did not differ significantly from the control cells (44.1 ± 7.1 ms).

The burst of exocytosis prior to the first depolarization was well fit with a dual exponential (Fig. 1A, dashed lines, extrapolated over the entire trace duration). The faster component, which we refer to as RRR_{HC} (RRP, highly calcium sensitive), was significantly larger in cells expressing Snapin (Fig. 1B, left panel, 48.2 ± 6.1 fF vs. 23.2 ± 5.5 fF in control cells; $p \leq 0.05$). There was no difference in the remaining secretion. The remaining component which we refer to as the RRP_{LC} (low calcium sensitivity) was 32.1 ± 7.2 fF and 44.2 ± 7.7 fF for control and Snapin overexpressing cells, respectively. The $[Ca^{2+}]_i$ values reflect global averages and do not reflect local calcium domains. The time constant for the RRP_{HC} was similar in control cells and cells overexpressing Snapin (37.6 ± 4.1 ms and 44.1 ± 7.1 ms, respectively) and was faster than that expected for the RRP at the $[Ca^{2+}]_i$ reached after the first flash in our experiments [14]. We have not shown the values for the time constant for the slower component of the burst, since the short measurement interval (300 ms) renders estimates of this time constant unreliable.