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Presynaptic Ryanodine Receptor-CamKII Signaling is Required for Activity-dependent Capture of Transiting Vesicles

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Abstract

Activity elicits capture of dense-core vesicles (DCVs) that transit through resting *Drosophila* synaptic boutons to produce a rebound in presynaptic neuropeptide content following release. The onset of capture overlaps with an increase in the mobility of DCVs already present in synaptic boutons. Vesicle mobilization requires Ca^{2+} -induced Ca^{2+} release by presynaptic endoplasmic reticulum (ER) ryanodine receptors (RyRs) that in turn stimulates Ca^{2+} /calmodulin-dependent kinase II (CamKII). Here we show that the same signaling is required for activity-dependent capture of transiting DCVs. Specifically, the CamKII inhibitor KN-93, but not its inactive analog KN-92, eliminated the rebound replacement of neuropeptidergic DCVs in synaptic boutons. Furthermore, pharmacologically or genetically inhibiting neuronal sarco-endoplasmic reticulum calcium ATPase (SERCA) to deplete presynaptic ER Ca^{2+} stores or directly inhibiting RyRs prevented the capture response. These results show that the presynaptic RyR-CamKII pathway, which triggers mobilization of resident synaptic DCVs to facilitate exocytosis, also mediates activity-dependent capture of transiting DCVs to replenish neuropeptide stores.

Keywords

activity-dependent capture; CamKII; dense-core vesicle; *Drosophila* neuropeptide release; synaptic trafficking

Introduction

The function of nerve terminals depends on vesicular delivery of proteins synthesized in the soma to synaptic boutons. Transport vesicles are known to contain channels, active zone constituents and neuropeptides (Zupanc, 1996; Ziv and Garner, 2004). In contrast to synaptic membrane proteins and classical transmitters that are recycled following exocytosis, neuropeptide release is irreversible. Thus, peptidergic transmission depends on replacement of neuropeptide-containing dense core vesicles (DCVs). This is potentially a very slow process because delivery of vesicles synthesized in the soma to nerve terminals by fast axonal transport can take days. However, a cell biological strategy has been discovered that bypasses such delays. Activity-dependent capture of transiting vesicles utilizes a pool of DCVs that rapidly pass through the resting nerve terminal, but that are captured in response to a burst of activity (Shakiryanova et al., 2006). The onset of this capture, which is evident as decreased DCV efflux and increased neuropeptide content in synaptic boutons, occurs over a period of minutes instead of the hours that would be required for conventional steady state DCV replacement. Essentially, the nerve terminal can tap into the transiting DCV pool to rapidly replenish neuropeptide stores without any direct involvement of the soma. Hence, activity-dependent

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capture of transiting DCVs eliminates the delay in delivering nascent DCVs, apportions resources based on activity and places control of synaptic neuropeptide storage at sites of release instead of the site of synthesis (i.e., the soma) (Shakiryanova et al., 2006). A similar recruitment process also occurs with neurotrypsin-containing vesicles, which were concluded to rapidly undergo exocytosis following stimulated capture (Frischknecht et al., 2008). Likewise, vesicle capture appears to be involved in release of presynaptic Wnt/Wingless protein (Ataman et al., 2008). Therefore, activity-dependent capture of transiting vesicles supports synaptic neuropeptide, enzyme and developmental peptide release.

The signaling required for activity-dependent capture of transiting DCVs is unknown. The long duration of this response in *Drosophila* motor neurons (i.e., for ~0.5 hour) coupled with the requirement for electrical activity suggests a potential involvement of Ca^{2+} -induced phosphorylation. In fact, recent experiments have shown that such signaling increases the mobility of resident DCVs in synaptic boutons. Mobilization, which is triggered by Ca^{2+} influx and persists for ~10 minutes (Shakiryanova et al., 2005), requires Ca^{2+} -induced Ca^{2+} release from presynaptic endoplasmic reticulum (ER) via ryanodine receptors (RyRs) (Shakiryanova et al., 2007). Subsequently, Ca^{2+} /calmodulin-dependent protein kinase II (CamKII) is activated as a necessary step for DCV mobilization (Shakiryanova et al., 2007). The overlapping onset of the capture and mobilization responses in the first minutes following a brief tetanus stimulated us to investigate whether the RyR-CamKII pathway participates in activity-dependent capture of transiting vesicles.

Here a GFP (Green Fluorescent Protein)-tagged neuropeptide is imaged at the intact *Drosophila* neuromuscular junction. We report that the rebound in synaptic neuropeptide stores following activity-evoked release, which is caused by capture of transiting vesicles (Shakiryanova et al., 2006), requires RyR-mediated Ca^{2+} efflux from presynaptic ER and activation of CamKII. Therefore, RyR-CamKII signaling initiates both mobilization of resident DCVs within synaptic boutons and capture of DCVs from the rapidly transiting pool.

Materials and Methods

Drosophila melanogaster expressing Emerald GFP-tagged atrial natriuretic factor in neurons (*elav-Gal4 UAS-preproANF-EMD*) were used as previously described (Rao et al., 2001; Shakiryanova et al., 2006, 2007; Levitan et al., 2007). *elav-GAL4 UAS-preproANF-EMD; UAS-Kum170/CyO, Act-GFP* animals express the temperature-sensitive dominant negative SERCA Kum170 mutant in neurons (Sanyal et al., 2005; Shakiryanova et al., 2007). SERCA was inactivated by incubating these animals in Ca^{2+} -free HL3 saline (70 mM NaCl, 5 mM KCl, 20 mM MgCl_2 , 115 mM sucrose, 5 mM trehalose, 5 mM HEPES, 10 mM NaHCO_3 , 0.5 mM EGTA, pH 7.25) at 40°C for 8 minutes.

Female third instar wandering larvae were filleted in Ca^{2+} -free HL3 saline. The ventral ganglion was severed to eliminate central input to the motor neurons that induces muscle contraction. During live imaging and electrical stimulation, animals were bathed in HL3 saline (70 mM NaCl, 5 mM KCl, 1.5 mM CaCl_2 , 20 mM MgCl_2 , 115 mM sucrose, 5 mM trehalose, 5 mM HEPES, 10 mM NaHCO_3 , pH 7.25) supplemented with 10 mM L-glutamate (HL3-Glut). L-glutamate was included to desensitize postsynaptic glutamate receptors and minimize muscle contraction during imaging experiments. Type Ib synaptic boutons located on muscles 6 and 7 in segments A3 to A6 were imaged using a cooled CCD camera on an upright wide-field epifluorescence microscope with 40× or 60× water-immersion objectives. Motor nerves were stimulated with suction electrodes at 70 Hz for 1 minute. Capture was calculated as the percent of recovery of peptide fluorescence four minutes after the cessation of stimulation normalized to release (i.e., the initial drop in fluorescence immediately after electrical stimulation).

Drugs were bath applied after dissection. Ryanodine (high purity, water soluble), KN-92 and KN-93 were obtained from Calbiochem, La Jolla, CA. KN-92 and thapsigargin (Alamone labs, Jerusalem, Israel) were dissolved in DMSO as stocks and subsequently diluted to yield a final concentration of 0.05% DMSO.

Results

Drosophila type Ib boutons were electrically stimulated to induce release and subsequent capture of transiting DCVs. Prior to stimulation, the signal from the GFP-tagged neuropeptide was stable within a bouton. However, 70 Hz motor nerve stimulation for a minute induced a rapid release of ~20% of the neuropeptide (i.e., fluorescence dropped to ~80% of the resting value). This was followed by a rebound in the following 4 minutes to reach ~90% of the baseline content (Fig. 1), which is mediated by capture of transiting DCVs (Shakiryanova et al., 2006). Hence, capture of transiting DCVs resulted in rapid replacement of ~50% of the neuropeptide release by synaptic boutons.

To determine whether CamKII activity is required for activity-dependent DCV capture, we examined the effect of CamKII inhibition on the response to the above electrical stimulation. Specifically, the effect of the CamKII inhibitor KN-93 was compared to its inactive analog KN-92. Both compounds slightly increased acute release (i.e., the rapid drop in GFP fluorescence immediately following stimulation) (Fig. 2). This minor response, which was not seen with a different stimulation protocol (Shakiryanova et al., 2007), must not involve CamKII because it was produced by both analogs. However, a concentration of KN-93 that abolishes activity-evoked DCV mobilization (Shakiryanova et al., 2007) eliminated the subsequent rebound of neuropeptide content, while the capture response remained detectable with KN-92 (Fig. 2). To normalize for potential differences in release, capture was quantified as the percent of released neuropeptide that was replaced in 4 minutes after the cessation of electrical stimulation. Based on this analysis, KN-93 and KN-92 produced statistically different results (Fig. 3, left). Hence, although the KN compounds may have had a shared minor nonspecific effect on release, the KN-93-specific inhibition of the rebound in neuropeptide content implies that CamKII activity is required for activity-dependent capture of transiting DCVs.

Because activation of CamKII for DCV mobilization requires ER Ca^{2+} release mediated by RyRs (Shakiryanova et al., 2007), the role of RyRs in activity-dependent DCV capture was studied. Pretreatment with an inhibitory concentration of ryanodine eliminated capture (i.e., the replacement of released neuropeptide was not significantly different from zero) (Fig. 3). To independently verify that ER Ca^{2+} release was required, ER Ca^{2+} stores were depleted by inhibiting the Sarco/Endoplasmic Reticulum Ca^{2+} -ATPase (SERCA) with Thapsigargin (Tg) in Ca^{2+} -free medium for 20 minutes. Then the preparation was returned to normal Ca^{2+} -containing solution and electrically stimulated. This protocol dramatically reduced the capture response, while a vehicle control had no effect (Fig. 3). Thus, release of ER Ca^{2+} by RyRs is required for activity-dependent capture of transiting DCVs.

The pharmacologic agents used thus far were bath applied and so acted on both the presynaptic neuron and the postsynaptic muscle. To ensure activity-dependent DCV capture is not caused by retrograde RyR-induced signaling, presynaptic ER Ca^{2+} stores were targeted genetically by expressing a temperature-sensitive dominant negative SERCA subunit (Kum170, Sanyal et al., 2005) specifically in neurons. At a permissive temperature, the capture response in Kum170-expressing boutons was intact. However, replacement of released neuropeptide was inhibited after shifting the animals to the restrictive temperature for 8 minutes (Fig. 3, right). In contrast, this treatment did not affect capture in control animals subjected to the restrictive temperature (data not shown). Therefore, presynaptic ER Ca^{2+} release by RyRs is essential for CamKII-mediated capture of transiting DCVs.

Discussion

In vivo imaging has shown that a brief bout of activity elicits prolonged DCV mobilization and capture. These processes are independent because capture requires axonal transport while mobilization does not (Shakiryanova et al., 2005; Shakiryanova et al., 2006). Nevertheless, the onsets of mobilization of resident DCVs and capture of transiting DCVs overlap (i.e., both develop over minutes following seconds of activity). This observation stimulated us to test the hypothesis that these two mechanisms are initiated by the same signaling. Previous studies had established that Ca^{2+} influx triggers DCV mobilization by activating RyR-mediated Ca^{2+} release from presynaptic ER that in turn stimulates CamKII (Shakiryanova et al., 2007). The pharmacological and genetic experiments presented here establish that RyR-CamKII signaling is also required for activity-dependent capture of transiting DCVs.

This finding raises the issue of how a single signaling pathway produces responses with different durations: after seconds of activity, DCV mobilization lasts ~10 minutes, while the capture response lasts ~40 minutes (Shakiryanova et al., 2005; Shakiryanova et al., 2006). One possible consideration is that these kinetic differences could originate in the processes responsible for reversal of mobilization and capture. Specifically, RyR-CamKII signaling could initiate the two processes in parallel, but dephosphorylation of distinct CamKII substrates might occur at different rates, possibly because of the involvement of different phosphatases. This potential explanation suggests that identifying the CamKII substrates that mediate mobilization and capture will be important for understanding the diversity in long-lasting responses initiated by activity-triggered presynaptic RyR-CamKII signaling. Recently, CamKII-dependent phosphorylation of kinesin superfamily protein 17 (KIF17) was found to be essential for unloading NMDA receptor-carrying cargoes from microtubules near the postsynaptic density (Guillaud et al., 2008). Therefore, CamKII might induce capture by triggering dissociation of transiting DCVs from their molecular motor dynactin complex, which would contain both a kinesin-3 family member UNC-104/Kif1 (Park-Chung et al., 2007; Barkus et al., 2008) and a dynein retrograde motor, while mobilization might depend on another CamKII substrate. Alternatively, some process downstream of dephosphorylation might be rate determining for reversal of capture. For example, once captured vesicles are committed to return to the transiting pool, they might need to recruit an unoccupied motor complex to support rapid transiting. If such complexes are rare, then recovery from capture would be very slow. In contrast, recovery from mobilization, which does not require exiting from the bouton, would not be limited in the same way. Regardless of the specific basis for the diverse time courses of mobilization and capture, the use of the same signaling pathway to induce both of these effects is an elegant means to ensure that facilitation of release is coupled to replacement of depleted synaptic neuropeptide stores.

Acknowledgments

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Figure 1.

Activity-dependent neuropeptide capture following electrical stimulation. (A) Pseudo-color images of GFP-tagged neuropeptide fluorescence in a type Ib bouton before and after 70 Hz electrical stimulation for 1 minute. The relative amount of peptide content was quantified as percentage fluorescence prior to stimulation (%F). (B) Time course of neuropeptide content (n=12). Black bar indicates stimulation. Each experimental point was from one bouton in an independent preparation. Error bars represent standard error of the mean (SEM). Note the rebound in neuropeptide content following release, which is caused by activity-dependent capture of transiting DCVs (Shakiryanova et al., 2007).

Figure 2. CamKII inhibition abolishes activity-dependent DCV capture. Neuropeptide content time course following 15-minute pretreatment with 10 μ M KN-92 (filled triangles, $n=4$) or KN-93 (open circles, $n=6$). Black bar indicates stimulation. Error bars represent SEM.

Figure 3.

CamKII and presynaptic Ca^{2+} release by ER RyRs are required for activity-dependent DCV capture. Comparison of capture after inhibiting CamKII with KN-93 ($n=6$) or treating with an inactive analog KN-92 ($n=4$), inhibiting RyRs with 100 μM ryanodine ($n=6$) for 15 minutes versus untreated controls ($n=12$), and ER Ca^{2+} store depletion by the SERCA pump inhibitor Tg (20 μM , $n=5$) for 20 minutes versus its vehicle control ($n=6$). Finally, the requirement for presynaptic ER Ca^{2+} release was tested in animals expressing temperature-sensitive dominant negative SERCA pre-exposed for 8 minutes to 22°C (Kum170, $n=6$) or 40°C (Kum170 + heat, $n=10$). Unpaired t-test results: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. # indicates no significant difference to zero by one sample t-test. Error bars represent SEM.