Synaptic Plasticity: Spatio-Temporal Analysis of Actin Dynamics

Olga Kochan[1]* and Robert S. Zucker**
*Molecular & Cell Biology and Psychology Major, College of Letters and Science,
**Department of Molecular & Cell Biology, University of California, Berkeley

Key words: *Synaptic plasticity, synaptic transmission, cAMP dependent enhancement, actin, HCN channels*

ABSTRACT: Glutamatergic synapses are highly modifiable, making them key targets in processes such as learning and memory. In crayfish glutamatergic neuromuscular junctions, hyperpolarization and cyclic nucleotide-activated (HCN) channels and actin cytoskeleton dynamics are critical intermediate factors in hormonal modulation of glutamatergic synapses which lead to cAMP (3'-5'-cyclic adenosine monophosphate)-dependent enhancement of synaptic transmission. Although models have been proposed, there has been a lack of experimental evidence on the relationship between HCN channels and the integrity of the actin cytoskeleton during cAMP-dependent enhancement. The specific goal of this study is to test the sequence of activation of the aforementioned mediators in synaptic enhancement via precisely controlled pharmacological experiments. At glutamatergic neuromuscular junctions of crayfish limb muscles, HCN channel activator, lamotrigine (50 μM), enhanced synaptic transmission about 20%. This enhancement was completely blocked with actin depolymerizer, latrunculin B (3 μM). These results support previous models of the temporal arrangement of events leading to synaptic enhancement, specifically that changes in actin cytoskeleton follow HCN channel activation. Concurrently, we are also using a spatio-temporal marker called phalloidin, a toxin which binds actin filaments, to further test the hypothesis that activation of HCNCs precedes actin cytoskeleton polymerization. This allows for manipulation of HCN channels and visualization of actin that could propose the associated molecular mechanisms. Preliminary evidence suggests actin reorganization.

INTRODUCTION

Synaptic plasticity underlies adaptability of the nervous system to changing circumstances and is involved in learning and memory. One such modifiability includes cAMP-dependent enhancement of synaptic transmission. At crayfish neuromuscular junctions (NMJ), the number of glutamate-containing synaptic vesicles available for release by action potentials at the exciter is increased by the circulating neurohormone serotonin (1) acting in part by production of the presynaptic second messenger cAMP (2). This cAMP-dependent enhancement of transmission, which can also be produced directly by stimulating adenylyl cyclase with forskolin, involves the activation of presynaptic hyperpolarization and cyclic nucleotide-activated channels (HCNCs) by cAMP as well as the integrity of the actin cytoskeleton (3).

In past studies, it was noted that any of three HCNC blockers (ZD 7288, DK-AH 269, or Cs+) or depolymerization of actin by cytochalasin D, latrunculin B, or swinholide A, greatly reduce the forskolin-induced enhancement of transmission (4). A schematic model of the steps involved in induction of serotonergic (cAMP-dependent) enhancement of transmission has been proposed (4). From the model, it is evident that both HCN channels and actin cytoskeleton dynamics are required to mediate synaptic enhancement; however, the relationship between HCNCs and actin still remains elusive and merely hypothesized (Figure 1). Therefore, in this study, we set out to determine the sequential relationship between the actin cytoskeleton and HCN channels in supporting synaptic plasticity and transmission.

We used the crayfish NMJ to study the implicated mediators of synaptic enhancement. This model offers many advantages such as availability, suitability for electrophysiological recording and molecular manipulations, as well as physiological and biochemical properties nearly identical to those of mammalian brain synapse. We investigated the mechanism by which changes in HCN channel activation and actin cytoskeleton dynamics result in synaptic enhancement via two main experimental protocols. The first experimental technique manipulates HCN channels

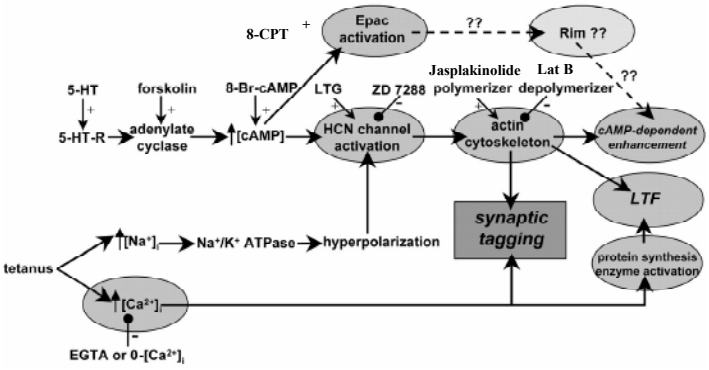

Figure 1: This previously published figure (4) depicts a schematic of the steps involved in induction of serotonergic (cAMP-dependent) enhancement of transmission, tetanic activation of LTF (long term facilitation), and synaptic tagging, which are all mechanisms of synaptic plasticity. cAMP enhances transmission by activating Epac and HCN channels, the latter acting via an actin dependent step. The temporal relationship between HCN channels and actin was still indefinite and needed to be further investigated, leading to the experiments in the present study.

[1] *Corresponding author: University of California, Department of Molecular and Cell Biology, 111 LSA, Berkeley, Ca 94720. Phone number: (925) 286-8491. E-mail: olga54@berkeley.edu.*

with lamotrigine (HCN channel activator) in the presence and absence of latrunculin B, an actin filament depolymerizer. Changes in synaptic enhancement were tracked by means of electrophysiological recordings from the post synaptic muscle cell of the opener muscle bed. The second experimental technique again entails similar pharmacological treatment of HCN channels as mentioned above, however, this time we visually tracked the spatial and temporal dynamics of the actin cytoskeleton in the presynaptic neuron, which was injected with Alexa488-conjugated phalloidin, a toxin from the death-cap mushroom that binds to actin filaments.

Here we show that, as previously hypothesized, HCN channel activation does indeed precede required changes in the presynaptic actin cytoskeleton.

MATERIALS AND METHODS

Preparation: Crayfish (Procambarus clarkii; 5-7 cm) were obtained from either Atchafalaya Biological Supply (Raceland, LA) or Niles Biological (Sacramento, CA) and kept in accordance with institutional guidelines. The first walking legs were removed and immersed in ice-cold low-Ca^{2+}, high-Mg^{2+} modified Van Harreveld's (MVH) solution containing (in mM) NaCl 195, $CaCl_2$ 2.5, KCl 5.4, $MgCl_2$ 13.5 and Na-HEPES 10 (pH 7.4). This solution suppresses synaptic transmission and prevents the spurious induction of synaptic enhancement. Legs were pinned on a Sylgard-lined chamber, covered with 5 ml of ice cold MVH. Removal of the shell and closer muscle exposed the ventral surface of the opener muscle while the leg nerve was dissected from the meropodite. The preparation was then continuously perfused at 2 mL/min with normal Van Harreveld's solution at 15-17°C, containing (in mM) NaCl 195, $CaCl_2$ 13.5, KCl 5.4, $MgCl_2$ 2.6 and Na-HEPES 10 (pH 7.4). Innervation of the dactyl opener muscle was previously described (5,6).

Electrophysiology: Sharp electrodes (electrode resistance 20-25 MΩ) filled with 3M KCl were used to impale and record from proximal muscle fibers. Basal transmission was assessed by stimulating the exciter motor neuron at 1 Hz with a suction electrode containing the axon freed from the meropodite segment of the leg. Electrical signals (excitatory junction potentials, EJPs) were amplified (Neuroprobe 1600 Amplifier, A-M Systems, Everett, WA), filtered at 2 kHz, digitized at 10 kHz, and the average of all EJPs recorded each minute saved to computer using pClamp8.2 software (Axon Instruments, Foster City, CA). EJP amplitudes were measured offline (Clampfit 8, Axon Instruments).

Actin Imaging: The exciter axon was penetrated with a bevelled electrode (50-80 MΩ) filled with f-actin marker phalloidin conjugated to Alexa488 (15 µM in 200 mM KCl). Phalloidin-Alexa488 (excitation 488 nm, emission ≥ 520 nm) was pressure injected into the exciter axon for 15-25 minutes, after which the marker was allowed to diffuse for about 1 hour. Fluorescence emission above 535 nm and excited at 488 nm was monitored in the presynaptic boutons during and after the injection using the BioRad MRC-600 confocal microscope with the standard BHS block, exciting with the 488 nm line of the argon laser.

Drugs: HCN channel activator lamotrigine was a gift from GlaxoSmithKline (Research Triangle Park, NC). Actin depolymerizer latrunculin B was obtained from Biomol (Plymouth Meeting, PA). Epac pathway activator 8-(4-chloro-phenylthio)-2'-*O*-methyladenosine-3',5'-cyclic monophosphate (8-CPT) was purchased from Axxora Biolog Biochemicals (San Diego, CA). Forskolin was purchased from EMD Biosciences (Pasadena, CA). Some stock solutions of drugs (lamotrigine, latrunculin B, forskolin) were prepared in dimethylsulfoxide (DMSO), but dissolved before use in external media to a final concentration containing no more than 0.1% DMSO. Previous control experiments showed that synaptic transmission was not affected by addition of this solvent (7).

Data presentation and statistical analysis: As control EJP amplitudes, taken as the average EJP amplitude over 15-20 minutes of continuous recording in the absence of a drug, were extremely variable from muscle fiber to muscle fiber, results were expressed as percent change from control EJP amplitude. Data are plotted as mean ± S.E. percent change from this control level.

RESULTS AND DISCUSSION

The presynaptic regulation of actin microfilaments, has been shown to affect transmission at some synapses (8,9,10). Actin has been shown to act like a seesaw that tethers reserve pools of vesicles away from the active zones and allowing for their regulated release following high-frequency activity resulting in increased releasable vesicle pool size, and hence the increase in neurotransmitter output (11,12,13). cAMP-dependent enhancement is manifested as an increase in total vesicle pool size (1), making actin a key player. Further studies showed that forskolin-induced cAMP-dependent synaptic enhancement (measured post-synaptically as an EJP in the muscle fiber) was indeed significantly reduced by treatment with actin depolymerizers (2). However, the question still remained whether this actin depolymerization acts upstream or downstream of HCN channels. In order to answer this question, the latter study found that pre-synaptic axonal depolymerization that was used to assay cAMP modulation of HCN channels showed modest, if any, effects in response to actin disruption. Their findings that reduction of forskolin-induced synaptic enhancement by actin depolymerizers was larger than the effect on HCN channels, suggested that actin mainly acts as a step subsequent to HCN

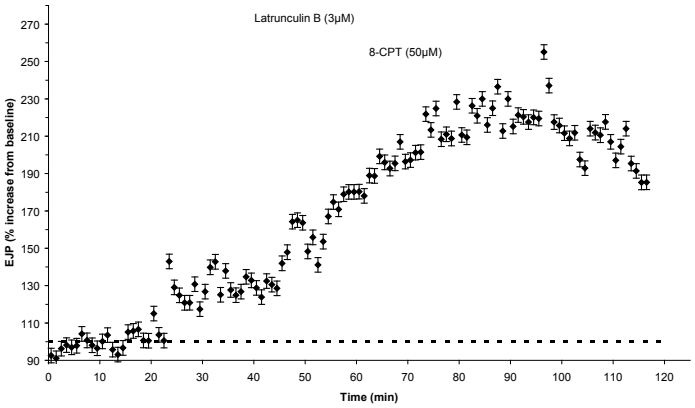

Figure 2: Epac agonist 8-CPT induces a modest increase in EJP amplitude to 139% ± 6% (7). Disruption of actin with depolymerizer latrunculin B (3 µM) in the presence of Epac agonist 8-CPT does not block increased transmission (n=1). The dashed line at the 100% y-intercept marks baseline EJP amplitudes in this and all subsequent figures.

channel activation.

A study by Zhong and Zucker in 2005 found a new target for cAMP, the exchange protein activated by cAMP (Epac), which also led to cAMP-dependent synaptic enhancement (7). They showed that cAMP elevated by forskolin activates both HCN channels and Epac to evoke increases in transmission. This new pathway provides an alternate route to synaptic enhancement by cAMP, paralleling the previously proposed HCN channel activation and actin cytoskeleton involvement. Therefore, previous findings by Beaumont et al, 2002 (2) now became confounded by the Epac pathway and possible actin involvement which may have caused the apparent reduction in forskolin-induced synaptic enhancement.

We felt it was necessary to further investigate the temporal and mechanistic order of HCN channel activation and actin cytoskeleton dynamics to exclude actin involvement in the Epac pathway and to more directly test the intricate relationship between HCN channels and actin.

Actin depolymerization does not reduce cAMP-dependent synaptic enhancement via Epac activation: We used a recently developed agonist of cAMP activation by Epac2 (a type-2 isoform of cAMP-regulated guanine nucleotide exchange factor) called 8-CPT (14,15). 8-CPT has been shown to exclusively activate (independent of HCN channels) the crayfish Epac homologue in regulating transmission at crayfish neuromuscular junctions (7). We treated preparations with actin depolymerizer latrunculin B (3 µM) for about 20 minutes (16) before the addition of 8-CPT, with continued treatment with both drugs thereafter. Figure 2 shows substantial increase in EJP response compared to the control (to 220% ± 4% of initial amplitude). Previous studies have shown that 8-CPT alone can produce only a modest increase in EJPs to 139% ± 6% of the initial amplitude (7). Discrepancies between the relative increase of EJP amplitude between experiment with 8-CPT alone and 8-CPT with latrunculin B are difficult to account for due to the unclear involvement of Epac in the cAMP-dependent synaptic enhancement pathway, although speculations include that Epac exerts its influence on transmitter release by interacting with an isoform of Rim (Rab interacting molecule) to regulate the availability of docked vesicles for release (7).

HCN channel activation precedes actin action in inducing cAMP-dependent enhancement: In order to target HCN channels and actin involvement in the cAMP-dependent synaptic enhancement pathway, and to decouple them from the cAMP target Epac, we used the drug lamotrigine (LGN, 50 µM) to directly activate HCN channels without elevating cAMP levels (7). The preparations were treated with either LGN (50 µM) alone as the control or were first pretreated with an actin depolymerizer latrunculin B (3 µM) for about 20 minutes (16) before the addition of LGN (50 µM), with continued treatment with both drugs thereafter.

Figure 3 shows the control experiments with LGN alone resulted in EJP increase to ~120% ± 4% of initial amplitude (n=1), as compared to previous results published in our lab of an

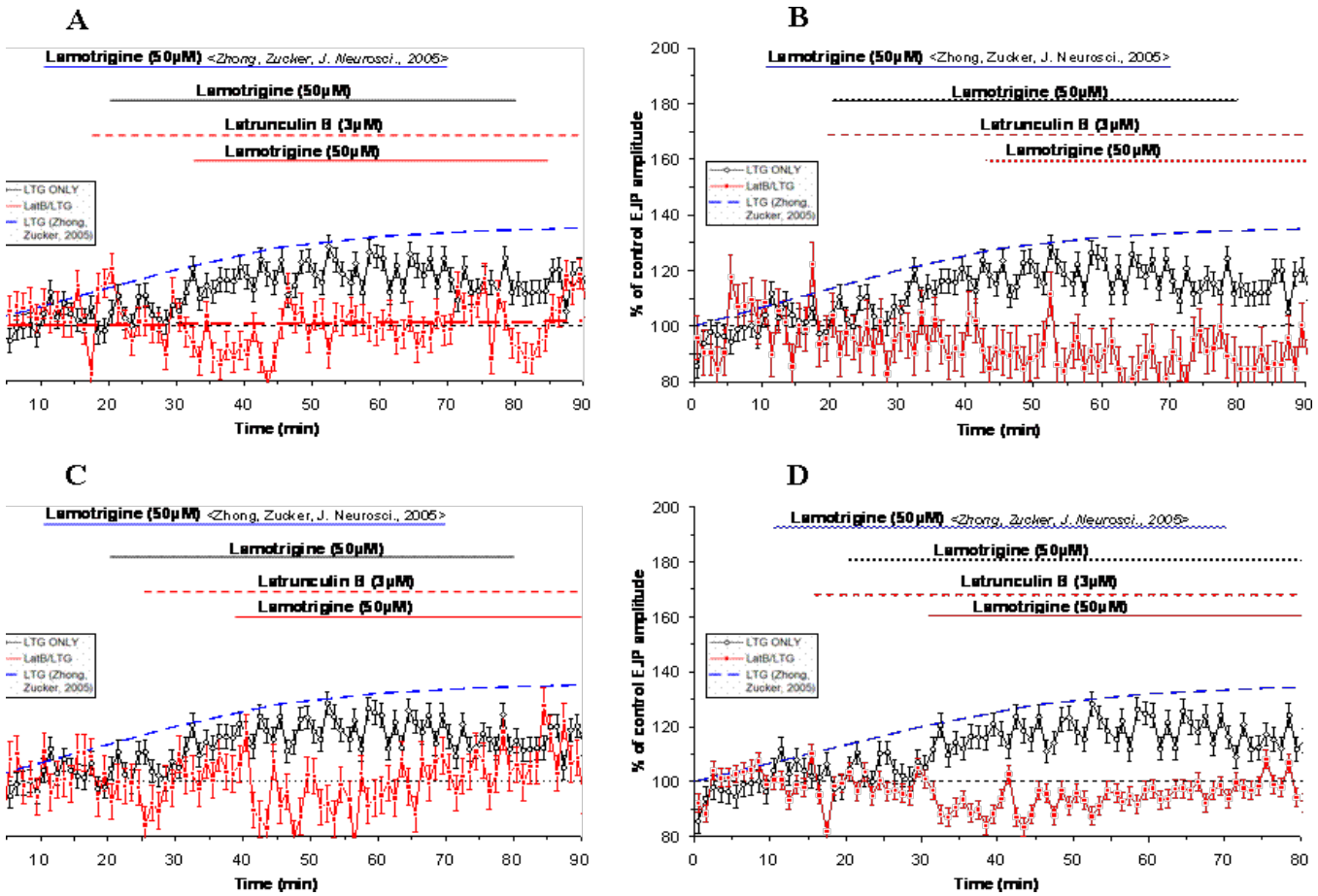

Figure 3: A-D, HCN channel activator Lamotrigine (LTG, 50 µM) alone induced a modest increase in EJP amplitude (in black, to ~120% ± 4% of initial amplitude, n=1). Previously published results (7) have shown similar effects of LTG on EJP amplitude (in dashed blue, 133 ± 0.75%, n=5). In contrast, disruption of actin with depolymerizer latrunculin B (3 µM) in the presence of LTG blocked the increase in EJP amplitude (100% ± max S.E. 8%, n=4), which remained at baseline levels.

EJP increase to 133 ± 0.75% (n=5) when treated with LGN alone (7). The inconsistency in EJP amplitude is most likely due to low sample size in the former experiment.

Figure 3 also illustrates the effects of latrunculin B actin depolymerization during HCN channel activation. EJP response in these experiments (n=3) remained at about 100% ± max S.E. 8% of control EJP amplitudes, indicating no changes from baseline response.

Our findings that depolymerization of actin cytoskeleton by latrunculin B, in all cases, block the effect of LTG, which is known to directly activate HCN channels that in turn lead to enhanced synaptic transmission, suggest that actin acts mainly at a step subsequent to HCN channel activation. These results confirm the current schematic mechanism cAMP-dependent synaptic enhancement (4).

Preliminary results of actin imaging show cytoskeletal rearrangement: In order to further track the dynamics of actin cytoskeleton and its involvement in cAMP-dependent synaptic enhancement, phalloidin was used to track f-actin filaments during forskolin-induced synaptic enhancement. Note that phalloidin recruits the non- or less highly polymerized forms of cytoplasmic actin as well as filamin into stable "islands" of aggregated actin polymers, and it does not interfere with stress fibers, which are thick bundles of microfilaments (17).

Images of phalloidin infused presynaptic boutons were taken at 5-minute intervals for 30 minutes to obtain a control without any pharmacological manipulations. The preparation was continuously perfused with forskolin (30 μM) for 1 hour, with imaging at 10-minute intervals. Figure 4 shows images of the control and forskolin treated actin cytoskeleton in a presynaptic bouton. Visually, there appears to be a qualitative change in the rearrangement of the actin cytoskeleton. During baseline, actin is visualized randomly dispersed in the bouton, however after forskolin treatment, it localizes to the periphery. Peripheral localization suggests that actin filaments may assist in vesicle docking in preparation for neurotransmitter release. Quantitative analysis is not yet conclusive, and will require more experimental data.

Actin Disruption: Changes in the actin cyto-skeleton are a prerequisite for exocytosis, enabling docking and fusion of secretory vesicles with the plasma membrane. In neurons, changes in presynaptic actin modulate vesicle fusion at active zones, increase the movement of actin-tethered synaptic vesicles from the reserve pool to active zones, and promote endocytosis of synaptic vesicles following neurotransmitter release (18). This study suggests that actin disruption by depolymerizers such as latrunculin B would cause massive synaptic disorganization and chaos, rather than being an informative tool to pinpoint actin's role in a specific mechanism of synaptic enhancement. However, it has been found that in well-established synapses (such as our model of a fully mature neuromuscular junction) F-actin becomes increasingly stable and actin depolymerization by latrunculin no longer disrupts basic synaptic structure (19). So, in this study, latrunculin B serves to depolymerize newly formed actin filaments which we believe play a critical role in cAMP-dependent synaptic enhancement. This also explains why treatment with latrunculin B alone shows no change in baseline amplitude of transmission, since the machinery involved in the basic mechanism of neurotransmitter release and synapse integrity is stable and conserved.

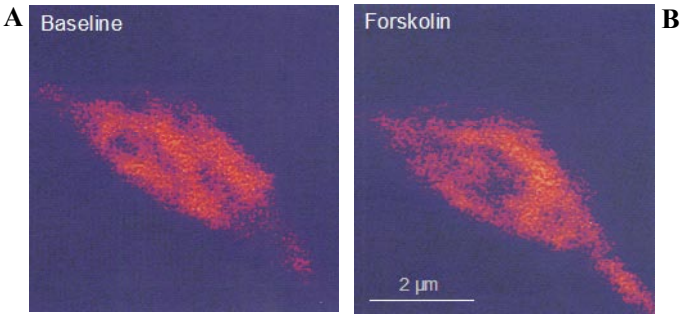

Figure 4. F-actin binding marker phalloidin conjugated to Alexa488 (15 μM in 200 mM KCl) showed A, baseline (drug-free) actin cytoskeleton arrangement in a presynaptic bouton. B, phalloidin-marked actin exhibits relocation and rearrangement in the same presynaptic bouton when perfused with adenylate cyclase activator forskolin (30 μM).

CONCLUSION

Analysis of neuronal activity during pharmacological manipulations of the cAMP-dependent enhancement using single-unit recording has provided new evidence that changes in actin cytoskeleton follow HCN channel activation and function. These results support previous models of the temporal arrangement of events between HCN channels and actin leading enhanced synaptic transmission.

Acknowledgements: We would like to thank the members of the Zucker laboratory, who were extremely helpful in discussions, comments and support during this research project. We also thank Russell English for technical assistance. Funding was provided by Biology Fellows Scholar Program and NSF.

REFERENCES

1. Wang C, Zucker RS, Regulation of synaptic vesicle recycling by calcium and serotonin. Neuron, 21:155-167, 1998.
2. Beaumont, V., Zhong, N., Froemke, R. C., Ball, R. W. and Zucker, R. S., Temporal synaptic tagging by Ih activation and actin: involvement in long-term facilitation and cAMP-induced synaptic enhancement, Neuron, 34:601-613, 2002.
3. Beaumont V. and Zucker R.S., Enhancement of synaptic transmission by cyclic AMP modulation of presynaptic Ih channels, Nature Neuroscience, 3:133-141, 2000.
4. Zhong, N. and Zucker, R.S., Roles of Ca2+, hyperpolarization and cyclic nucleotide-activated channel activation, and actin in temporal synaptic tagging. J Neurosci, 24:4205–4212, 2004.
5. Delaney K., Tank D.W. and Zucker, R.S., Presynaptic calcium and serotonin-mediated enhancement of transmitter release at crayfish neuromuscular junction, Journal of Neuroscience, 11:2631-2643, 1991.
6. Zhong N., Beaumont V., and Zucker R.S., Roles for mitochondrial and reverse mode Na+/Ca2+ exchange and the plasmalemma Ca2+ ATPase in post-titanic potentiation at crayfish neuromuscular junctions, Journal of Neuroscience, 21:9598-9607, 2001.
7. Zhong, N. and Zucker, R. S., cAMP acts on exchange protein activated by cAMP/cAMP-regulated guanine nucleotide exchange protein to regulate transmitter release at the crayfish

neuromuscular junction, J. Neurosci, 25:208-214, 2005.
8. Cole, J.C., Villa, B.R., and Wilkinson, R.S., Disruption of actin impedes transmitter release in snake motor terminals, J. Physiol, 525:579–586, 2000.
9. Kuromi, H., and Kidokoro, Y., Two distinct pools of synaptic vesicles in single presynaptic boutons in a temperature-sensitive Drosophila mutant, shibire, Neuron 20:917–925, 1998.
10. Wang, X.H., Zheng, J.Q., and Poo, M.M., Effects of cytochalasin treatment on short-term synaptic plasticity at developing neuromuscular junctions in frogs, J. Physiol, 491:87–195, 1996.
11. Ceccaldi, P.E., Grohovaz, F., Benfenati, F., Chieregatti, E., Greengard, P., and Valtorta, F., Dephosphorylated synapsin I anchors synaptic vesicles to actin cytoskeleton: an analysis by videomicroscopy, J. Cell Biol, 128:905–912, 1995.
12. Hilfiker, S., Pieribone, V.A., Czernik, A.J., Kao, H.T., Augustine, G.J., and Greengard, P., Synapsins as regulators of neurotransmitter release, Philos. Trans. R. Soc. Lond. B Biol. Sci., 354:269–279, 1999.
13. Humeau, Y., Doussau, F., Vitiello, F., Greengard, P., Benfenati, F., and Poulain, B., Synapsin controls both reserve and releasable synaptic vesicle pools during neuronal activity and short-term plasticity in Aplysia, J. Neurosci., 21:4195–4206, 2001.
14. Christensen, A.E., Selheim, F., de Rooij, J., Dremier, S., Schwede, F., Dao, K.K., Martinez, A., Maenhaut, C., Bos, J.L., Genieser, H.G., Doskeland, S.O., cAMP analog mapping of Epac1 and cAMP-kinase. Discriminating analogs demonstrate that Epac and cAMP-kinase act synergistically to promote PC-12 cell neurite extension, J Biol Chem, 278:35394 –35402, 2003.
15. Kang, G., Joseph, J.W., Chepurny, O.G., Monaco, M., Wheeler, M.B., Bos, J.L., Schwede, F., Genieser, H.G., Holz, G.G., Epac-selective cAMP analog 8-pCPT-2_-O-Me-cAMP as a stimulus for Ca2_-induced Ca2_ release and exocytosis in pancreatic beta-cells, J Biol Chem, 278:8279-8285, 2003.
16. Spector, I., Braet, F., Shochet, N.R., and Bubb, M.R., New anti-actin drugs in the study of the organization and function of the actin cytoskeleton, Microsc. Res. Tech., 47, 18–37., 1999.
17. Wehland, J., Osborn, M. and Weber, K., Phalloidin-induced actin polymerization in the cytoplasm of cultured cells interferes with cell locomotion and growth, Proc. Natl. Acad. Sci. USA, 74:5613–5617, 1977.
18. Dillon, C. and Goda¬, ¬Y, The Actin Cytoskeleton: Integrating Form and Function at the Synapse, Annual Review of Neuroscience, Review, 28:25-55, 2005.
19. Zhang, W. and Benson, D.L, Developmentally regulated changes in cellular compartmentation and synaptic distribution of actin in hippocampal neurons, J. Neurosci., 21:5169–5181, 2001.