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Flupirtine Modulates both KCNQ K^+ Channels and $GABA_A$ Receptors in Hippocampal Neurons

Felicia Popovici[1,2], Mario M. Dorostkar[2], Mark S. Shapiro[1], Stefan Boehm[2].
[1]UTHSCSA, San Antonio, TX, USA, [2]Medical University of Vienna, Vienna, Austria.

KCNQ/M-channels are slowly gating and non-inactivating K^+ channels. They are widely expressed in the nervous system and play major roles in regulation of neuronal excitability. Still controversial is the functional role of M-channels in neurotransmitter release. To clarify this issue, we sought to test drugs known to activate or inhibit M-channels for their effects on transmitter release or synaptic transmission, using hippocampal ''micro-island'' autapses or mass cultures. A possible confounding issue are effects of the drugs on excitatory or inhibitory inotropic receptors. Indeed, flupirtine, a drug with poorly understood mechanisms of action had no effect on glutamatergic, but prolonged GABAergic, autaptic currents. In mass cultures, we found direct, but bi-phasic, effects of flupirtine on postsynaptic $GABA_A$ receptors ($GABA_AR$), in which flupirtine (30 μM) reduced by 3-fold the EC_{50} value for GABA-induced currents but reduced the maximal current by 15%. At 100 μM, flupirtine induced an inward current that was $GABA_AR$-mediated, since it was abolished by bicuculline (30 ∈ μM). To differentiate between effects on synaptic and extrasynaptic $GABA_AR$, miniature inhibitory postsynaptic currents (mIPSCs) were analyzed. Flupirtine did not alter the rise time, decay time, nor amplitude of the mIPSCs, but enhanced the bicuculline-sensitive tonic current. When synaptic $GABA_ARs$ were blocked with penicillin G (5 mM), flupirtine potentiated GABA-induced currents, enhancing maximal amplitudes by 43%. These results indicate that flupirtine potentiates GABAergic transmission by distinct effects on synaptic and extrasynaptic $GABA_AR$ subtypes. We are investigating the role of presynaptic KCNQ channels on neurotransmitter release using other KCNQ openers, such as retigabine, or the blockers linopirdine and XE991. To ask if M-type channels localize to hippocampal pre-synaptic terminals, we will perform co-immunostaining of KCNQ subunits and presynaptic markers (e.g. synaptophysin) via confocal microscopy. Supported by NIH and the Austrian Science Fund.

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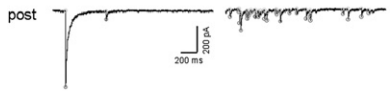
Incontinent Pool of Primed Vesicles in Synaptotagmin-1-Deficient GABAergic Synapses

Silvia Willadt, **Jan C. Behrends.**
University of Freiburg, Freiburg, Germany.

The coupling of neuronal excitation to release of chemical transmitter at synaptic endings is mediated by an electrically gated entry of Ca2+-ions that within a fraction of a millisecond leads to exocytotic fusion of vesicles with the presynaptic membrane. In this rapid process, members of the synaptotagmin (Syt) family of vesicular membrane proteins are likely to function as the Ca2+-sensing molecular trigger. The trigger mechanism employed, however, is currently under debate. Here, using high resolution dual simultaneous pre- and postsynaptic whole cell patch clamp recordings from Syt1-deficient GABAergic neurons, we report a strong potentiation of asynchronous release in the absence of synchronous release. In addition, we show that Syt1-deficient vesicles are hypersensitive to Ca2+-independent osmotic stimulation of exocytosis and that, unlike in controls, chemical priming into a highly fusion competent state directly results in their spontaneous exocytosis. Therefore, the primed vesicle pool is incontinent in these synapses. These findings argue strongly that an inhibitory, latch-like action by Ca2+-free Syt1 on SNARE-function is part of its physiological role in the mammalian nervous system to ensure transmitter release closely timed to the presynaptic action potential.



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Modulation of the Excitatory Synaptic Transmission in Isolated Rat Hippocampus by Direct Current Stimulation

Anatoli Y. Kabakov[1,2], Paul Muller[2], Francis E. Jensen[2,1], Alexander Rotenberg[1,2].
[1]Harvard University Medical School, Boston, MA, USA, [2]Children's Hospital, Boston, MA, USA.

Transcranial direct current stimulation (DCS) is a noninvasive method for changing cortical excitability. When applied to the scalp over the human cerebral cortex, a cathodal direct current stimulating electrode induces a lasting decrease in excitability, whereas an anodal stimulating electrode enhances excitability. However, mechanisms of the DCS effect at cellular and molecular levels are not well understood. To elucidate the DCS mechanisms, we developed methods for DCS in isolated hippocampal slices and tested the effects of current magnitude and polarity on the CA1 field excitatory post-synaptic potential (fEPSP). METHODS: 400 ∈ μm hippocampal slices were prepared from rat pups (P18 - P24). Baseline CA1 fEPSP slope and amplitude were obtained by stimulation of the Schaffer collateral fibers while recording from the CA1 region. DCS (range: −400 to +400 ∈ μA) was applied for 5 minutes via two silver-chloride pellet electrodes located outside of the slice and 1 mm away from CA1 and CA3 regions, respectively. RESULTS: The fEPSPs persisted during DCS (−400 to +200 ∈ μA) and were modulated by current amplitude and polarity. Maximal reduction (by 30%) of fEPSPs amplitude and slope by DCS was achieved with 400 ∈ μA DC directed toward the CA1 region (−400 ∈ μA). Maximal facilitation of fEPSPs (by 18%) was achieved with 100∈ μA DC in opposite direction. The dependence of fEPSP amplitude on DCS amplitude was non-linear with the maximum of the first derivative at 0 ∈ μA. DISCUSSION: We demonstrate for the first time that (1) external DCS can modulate EPSP amplitude and slope, and (2) that the magnitude and direction of the EPSP change is dependent on the magnitude and direction of the direct current. Our data support the hypothesis that DCS effect on EPSPs is due to polarization of the postsynaptic membrane.

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How Does Synaptic Dynamics Regulate Neuronal Encoding?

Jin H. Wang.
Institute of Biophysics, Chinese Academy of Sciences, Beijing, China.

Neuronal spikes and synaptic signals are presumably basic codes in the brain to control behaviors. In terms of their interaction, spike patterns influence synaptic plasticity via presynaptic mechanisms. It is not documented how synaptic dynamics regulate neuronal encodings, especially their quantitative correlations, which we studied at cortical circuits assembled from pyramidal and GABAergic neurons. Our studies at unitary synapses demonstrate that postsynaptic responses are constancy over time, e.g., glutamate receptor-channel currents at GABAergic cells and glutamate transport currents at astrocytes, indicating quantal glutamate release. Moreover, sequential presynaptic spikes up-regulate glutamate release probability and synaptic strength in a linearly correlation. Spike frequency and presynaptic Ca^{2+} levels mediate this increment of release probability, as well as raise the efficiency of probability vs. synaptic facilitation. In terms of their physiological significance, an increase of glutamate release probability improves spike timing precision and capacity at postsynaptic neurons, and the signals integrated from quantal glutamatergic synapses drive spike encodings at postsynaptic neurons to be precisely reliable, which regulates spike encodings at pyramidal cells to be precise via a feedback inhibition. Therefore, the potentiality in releasing presynaptic glutamates and the patterns of quantal release are beneficial to signal transmission at the synapses and spike encodings at the neuron in neural circuits for editing homeostatic brain softwares to manage well-organized behaviors.

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pH-Sensitive Fluorescent Lipids as Novel Probes to Monitor Vesicle Recycling

Martin Kahms, Cora Thiel, Jürgen Klingauf.
Institute for Medical Physics and Biophysics, University of Münster, Münster, Germany.

During synaptic transmission, neurotransmitters stored in presynaptic vesicles are released by excocytosis through fusion of vesicles with the plasma membrane. In a subsequent step, membranes and proteins at the synapse are reinternalized by a reverse process, endocytosis. In the analysis of this synaptic vesicle cycle genetically encoded pH-sensitive fluorescent proteins like the GFP-derivative pHluorin have become indispensable tools. These probes are capable of detecting changes in pH that accompany excocytosis and subsequent reacidification of endocytosed vesicles. Here we describe a new class of fluorescent probes, based on pH-sensitive organic dyes coupled to phospholipids, as promising alternative to genetically encoded fluorescent proteins like pHluorin. Moreover the pH-dependent fluorescence properties of these dyes are opposite to those of pHluorin.

In hippocampal neurons, cell membranes can be stained in a pH dependent manner, and upon quenching of the fluorescence at the plasma membrane by a slightly basic pH, vesicle recycling can be monitored yielding fluorescence transients with kinetics mirroring those of the well characterized pHluorin signal. Furthermore, this approach can be used to study vesicle recycling in acute preparations like bipolar cells of the retina, where application of genetically encoded probes was not possible so far.

This experimental approach using pH-dependent fluorescent lipids has not only the potential of being used in a variety of cellular and slice preparations, but in addition will shed light on an important presynaptic mechanism neglected so far, namely lipid recycling. Comparison of vesicle incorporation of different

dye-labeled lipid moieties will bring new insights into lipid organisation and trafficking at the synapse.

Muscle: Fiber & Molecular Mechanics & Structure I

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Myosin Nucleotide Pocket Thermodynamics Measured by Epr Reveal How Energy Partitioning Relates Speed to Efficiency

Thomas J. Purcell[1], Nariman Naber[1], Kathleen Franks-Skiba[1], Ed Pate[2], Roger Cooke[1].
[1]University of California, San Francisco, San Francisco, CA, USA, [2]Washington State University, Pullman, WA, USA.

We have used spin labeled ADP to investigate the dynamics of the nucleotide-binding pocket in myosins. In actomyosin•ADP the nucleotide-binding pocket is in an equilibrium between closed and open conformations, with the open conformation favored in slow myosins. In rabbit slow skeletal muscle fibers, the $\in''G$ for the closed to open equilibrium is -3.9 kJ mol^{-1}. We found similar values for pig ventricle myosin, chicken gizzard smooth muscle myosin, and chicken myosin V. For faster myosins, the equilibrium shifts to favor a closed conformation, rising to -2.7 kJ mol^{-1} for *Dictyostelium discoideum* myosin II, -1.9 kJ mol^{-1} for pig atrial myosin, -1.1 kJ mol^{-1} for rabbit fast skeletal muscle fibers, and $+2.9$ kJ mol^{-1} for *Drosophila* fight muscle fibers. We believe this represents a destabilization of the open actomyosin•ADP state in the faster myosins, driving ADP release. van't Hoff analysis of the temperature dependence of this equilibrium reveals that the closed to open conformation has a significant positive enthalpy and entropy, with $\in''H$ and $T\in''S$ of 40-50 kJ mol^{-1} for slow myosins. Both components are reduced in this equilibrium for faster myosins, decreasing to $\in''H = 17.7$ kJ mol^{-1} and $T\in''S$ at 25°C $= 18.8$ kJ mol^{-1} for rabbit fast skeletal fibers, and $\in''H = 10.4$ kJ mol^{-1} and $T\in''S$ at 25°C $= 7.5$ kJ mol^{-1}. Our model is that the open actomyosin•ADP state represents a partitioning point between the free energy released during the myosin catalytic cycle. Because of this partitioning, fast myosins destabilize the actomyosin•ADP state, reducing the energy available to do work up until that point, but leaving more free energy in reserve to drive ADP release. This gives a mechanism for the correlation between increased speed and reduced efficiency in muscle.

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Structural Basis for Uncoupling of Force Generation in the F506A *Dictyostelium* Myosin Revealed by Time-Resolved EPR and FRET

Roman V. Agafonov[1], Igor V. Negrashov[1], Sarah E. Blakely[1], Margaret A. Titus[1], Yuri E. Nesmelov[2], David D. Thomas[1].
[1]University of Minnesota, Minneapolis, MN, USA, [2]University of North Carolina, Charlotte, NC, USA.

We have used dipolar electron-electron resonance (DEER) and time-resolved fluorescence resonance energy transfer (TR-FRET) to investigate the role of the myosin relay helix in coupling between the active site and the force-generating region of myosin II. Two double-Cys *Dictyostelium* myosin constructs have been engineered, and the structure of the relay helix was monitored by measuring interprobe distances in MSL/MSL or IAEDANS/Dabcyl-labeled myosin. Experiments were performed on WT myosin and on F506A, a functional mutant that has close to normal enzymatic activity but completely lacks motor functions (e.g. unable to move actin filaments or support cell development). We found that the WT myosin relay helix adopts two distinct states (straight and bent), with the bent conformation populated when ATP and ADP.P_i analogs are bound at the active site. In contrast, binding of nucleotide analogs had very little effect on relay helix conformation in the F506A mutant. In addition, the width of the distance distribution was significantly larger in the F506A compared to WT myosin, indicating loss of structural organization. Our results demonstrate that the relay helix plays a key role in coupling of myosin ATPase and motor activities. Loss of functionality observed in F506A myosin can be explained by the disruption of the relay helix-relay loop interactions that normally stabilize well-defined conformations of the myosin force-generating region allowing it to switch between distinct structural states.

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Subpopulation of Intermediates in Actomyosin Crossbridge-Cycle During Sliding

Eisaku Katayama[1], Yoshitaka Kimori[2], Norio Baba[3].
[1]Institute of Medical Science, The University of Tokyo, Tokyo, Japan, [2]Dept of Ultrastr. Res, Nat'l Inst. of Neuro.& Psychi., Kodaira, Tokyo, Japan, [3]Dept of Inform. Sci., Kogakuin University, Hachioji, Tokyo, Japan.

We have been investigating conformational changes of myosin crossbridges during *in vitro* sliding, utilizing quick-freeze-replication and a novel image analysis to quantitatively compare microscopic images with the atomic models of various conformations. We reported that the leverarm moiety of working crossbridges, is mostly kinked to the opposite side of ADP/Vi-bound structure and such global configuration of myosin head resembles that whose SH1 and -2 are chemically crosslinked. Since the crystal structure of such unusual configuration is not determined, we attempted to reconstruct its 3-D structure by a special version of Single-Particle-Analysis, devised to adapt small-sized particle, utilizing very contrasty feature of metal-replicated images. With a new procedure to manage very few view-angles, we finally obtained the 3-D envelope of the myosin head with oppositely kinked leverarm, analogous to the intermediate structure under sliding conditions. We cut the original atomic model of pPDM-treated-ADP-structure (1L20) into motor-domain and the leverarm, and relocated each module at best-matching position and the orientation, to generate a tentative model that best-fits to reconstructed envelope. We then examined whether all the images of actin-sliding crossbriges can be uniquely explained by that standard SH-crosslinked structure model. The images of actin-attached crossbridges were classified by ''2-D appearance'' and each class-average was compared with 2-D projections of the standard structure as above. By analyzing the orientation of motor-domain and leverarm separately, we found that there could be several sub-populations, some matched to but some deviated from the standard structure. It is known that two reactive thiols can be crosslinked by a variety of bifunctional reagents of different span lengths, implying the distance between them might be flexible. We assume that the extension of new oppositely-kinked configuration would comprise the power-stroke and those sub-populations might correspond to several steps during that structural change.

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Analysis of Conformation of the Skeletal Muscle Myosin Modified by F_2DNB Using FRET

Masafumi D. Yamada[1], Koichiro Ishiyama[1], Eisaku Katayama[2], Yoshitaka Kimori[3], Shinsaku Maruta[1].
[1]Dept. of Eng., Soka Univ., Hachioji, Tokyo, Japan, [2]Div. of Biomol. Imag., Inst. of Med. Sci., The Univ. of Tokyo, Shiroganedai, Tokyo, Japan, [3]Dept. of Ultrastr. Res., Nat'l Inst. of Neurosci., Nat'l Ctr. of Neurol. and Psych., Kodaira, Tokyo, Japan.

Previously biochemical studies have demonstrated that the highly reactive cysteine residues SH1 and SH2 can be crosslinked by variety of bifunctional reagents with different spans (3-14 Å) in the presence of nucleotides, suggesting that the region is highly flexible. The SH1-SH2 region is believed to play a key role in the conformational changes that occur in the myosin head during the force generation coupled to ATP hydrolysis. We have previously shown that the skeletal muscle heavy mero-myosin (HMM), which SH1-SH2 was crosslinked by 1,5-difluoro-2, 4-dinitrobenzene (F_2DNB) in the presence of ADP, have a novel conformation using quick freeze deep etch electron microscopy (QFDE-EM). We have also demonstrated that conformational change of the myosin motor domain during ATP hydrolysis can be monitored by measuring the FRET using fluorescent ATP analogue NBD-ATP. In the present study, we analyzed the conformation of the myosin crosslinked by F_2DNB using FRET between the ATP binding site and the essential light chain (ELC) A1. We prepared skeletal muscle myosin subfragment-1 (S1), which ELC was labeled by 6-bromoacetyl-2-dimethylaminonaphthalene (BD) at the Cys 177. And fluorescent ADP analogue NBD-ADP was trapped within the ATPase site of S1 labeled by BD. The FRET efficiency was estimated by measuring the change of fluorescence intensity of BD comparing with control BD-S1. The FRET efficiency of F_2DNB-S1-NBD-ADP was lower than S1-NBD-ADP state. This suggests that the F_2DNB-S1-ADP states form more kinked conformation than S1-ADP state.

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Switch-2 Dependent Modulation of the Myosin Power Stroke

Daniela Kathmann, Ralph P. Diensthuber, Falk K. Hartmann, Roman Fedorov, Dietmar J. Manstein, Georgios Tsiavaliaris.
Medical School Hannover, Hannover, Germany.

Central to the mechanism of myosins are two conserved sequence motifs, switch-1 and switch-2, which contact nucleotide and Mg^{2+} at the rear of the nucleotide-binding pocket. They act as gamma-phosphate sensors, thus controlling hydrolysis and product release. The movement of switch-2 from a 'closed' to an 'open' conformation is translated via the relay region into a rotation of the converter domain, which drives the force generating power stroke. To investigate the molecular details of this coupling mechanism, we introduced mutation S456Y in switch-2 of the myosin-2 motor domain from *Dictyostelium discoideum* and analyzed the structural and functional consequences. Our kinetic results showed that the S456Y mutant lost the ability to bind effectively ATP, displayed a strongly decreased actin affinity in the ADP-bound state, and moved actin filaments with highly reduced velocities. It has been proposed that the