Nucleocyttoplasmic Transport

1363-Pos  Board B273
Single-Molecule Studies of Nuclear Transport
Oliver Otto, Anna Huefner, Lorenz Steinbock, Joanne Gornall, Murray Stewart, Ulrich Keyster.

The translocation of single biomolecules through nanopores is one of the most fundamental processes of life. For example, the import of histones into the cell nucleus via the nuclear pore complex (NPC) is crucial for DNA condensation 1-3. We aim to gain a deeper understanding of NPC translocation on a single molecule level by combining optical tweezers with patch clamp techniques. This gives us the unique possibility to analyze the interaction forces between a biological macromolecule and the NPC while measuring the relevant time scales by electrophysiological recordings.

We present preliminary results on the characterisation of the properties of NPCs concerning mode and duration of macromolecular translocation. Nuclei extracted from the syntactical coconut cocos nucifera are one model system. We demonstrate the selectivity of the NPC using an import signal tagged with GFP. We determine the conductance of a single nuclear pore to be approximately 490pS. Our results allow for estimation of the nuclear pore density to be between one and four per μm². From observations of the ionic current across the pores, we are able to observe gating and to derive the translocation time of cargo across single NPCs.

As a technical development towards nuclear pore force spectroscopy we introduce optical fiber illumination for real-time tracking of optically trapped particles. We demonstrate video-based fluctuation analysis of a single colloid at 10,000 Hz in order to study the dynamics of an attached biological macromolecule with picosecond time resolution (3-5).

References
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Single-Molecule Studies of Nucleocyttoplasmic Transport: from 1D to 3D
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The nuclear pore complex (NPC), the sole gateway for nucleocytoplasmic exchange in eukaryotic cells, allows for the passive diffusion of small molecules (< 20–40 kDa) and the transport-receptor-facilitated translocation of larger molecules. However, the precise transport mechanism as to how these two transport modes pave their pathways through the NPC remains in dispute among numerous transport models. By a newly developed single-molecule spectroscopy and a deconvolution algorithm, we advanced the single-molecule imaging of nucleocytoplasmic transport from 1D (one-dimension) to 3D. The 3D routes for both passive and facilitated transport through human NPCs under real-time trafficking conditions have been obtained with a spatial resolution of 9 nm at 400 μs. Our data strongly support a transport mechanism in which transport receptors or transport-receptor-cargo complexes pave their pathways by interacting with the FG repeats at the periphery around a single primary central axial channel for passive diffusion of small molecules.

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Three Dimensional Distinct Nuclear Transport Pathways Illuminated by Single-Molecule Microscopy
Jiong MA, Weidong Yang.

The nuclear pore complex (NPC), the sole gateway for nucleocytoplasmic exchange in eukaryotic cells, allows for the passive diffusion of small molecules and the transport-receptor-facilitated translocation of larger molecules. However, whether these two transport modes spatially separate or joined pathways and whether they functionally conduct uncoupled or coupled translocation remains in dispute. By the single-molecule snapshots of transient diffusion or interactions through native NPCs, we have determined that the three-dimensional transport pathway for each mode is mainly separate. Both pathways can be spatially and functionally regulated by transport receptors. Our data strongly support a transport mechanism in which transport receptors or cargo complexes pave the pathways by interacting with the phenylalanine-glycine repeats at the periphery around a single primary central axial channel for passive diffusion.

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Nuclear Export of mRNA in Living Cells
David Grünewald.

An important step in the life cycle of mRNA is its export from the nucleus to the cytoplasm, which is mediated by nuclear pores, large nano-machines with diameters of roughly 120 nm. As a result, mRNA export occurs over distances smaller than the optical resolution. While there is extensive knowledge of the physical structure and composition of the NPC transport selectivity and dynamics of mRNA export at nuclear pores remain unknown. Major models for nuclear pore function are currently focusing on the central channel of the pore, but we spatially resolved the kinetics of mRNA transport and present a three step model consisting of docking (80ms), transport (5-20ms) and release (80ms), totalling 180 ± 10 ms. Here, access and release from the pore complex are rate-limiting steps with the channel translocation time being in the order of transport dwell times found for protein import to the nucleus.

We developed a super-registration approach using fluorescence microscopy overcoming the current limitations of colorization by means of measuring inter-molecular distances of chromatically different fluorescent molecules with nm precision. With this method we achieve 20 ms time- and at least 26 nm spatial precision, rendering the capture of highly transient interactions in living cells possible.

1367-Pos  Board B277
Tracking Single Nuclear Pore Complexes in Live Cells: Importin β Transport and Beyond
Francesco Cardarelli, Luca Lanzano, Enrico Gratton.

The molecular details of transport through nuclear pore complexes (NPCs) have been well characterized, including the key role of Importin Beta (ImpB) receptor. However, the overall system behavior in intact cells is difficult to analyze because of its highly dynamic nature. Contrary to the common single particle tracking (SPT) approach that tracks an isolated particle as it moves through the pore, we set out to track the center of mass of the entire single NPC. While we track the pore, single molecules pass through it, and their location and dynamics are analyzed by fluorescence correlation spectroscopy (FCS). By this unconventional approach we find that ImpB transport is regulated so as to produce a characteristic bump in the autocorrelation function at the NPC. This regulation is spatially restricted to the pore, dependent on ImpB properties, pore structure, and metabolic energy. Combined to simulations our results suggest that ImpB movement within the pore is likely to be directed instead of unbiased, and that the back and forth components of its shuttling are coupled in time. We use our results to discriminate between existing NPC functional models, and identify key features that must be essential for transport in the intact pore.

Intracellular Channels/IP3 Receptors

1368-Pos  Board B278
TRIC-A Channel and Blood Pressure Regulation
Daiju Yamazaki, Satomi Kita, Shinti Komazaki, Daisuke Naitou, Miyuki Nishi, Hisao Yamamura, Yuji Imaiizumi, Takahiro Iwamoto, Hiroshi Takeshima.

TRimeric intracellular cation (TRIC) channel subtypes, namely TRIC-A and TRIC-B, function as intracellular channels conducting monovalent cations throughout tissues and probably mediate counter-ion movements coupled with Ca²⁺ release from the endo/sarcoplasmic reticulum. Knockout mice lacking both TRIC-A and TRIC-B channels suffer embryonic cardiac failure, and the mutant cardiomyocytes display severe dysfunction in SR Ca²⁺ handling (Yazawa et al., Nature, 2007). In knockout mice lacking TRIC-B channels with neonatal lethality due to respiratory failure, alveolar epithelial cells exhibit compromised Ca²⁺ release and thus insufficiently produce and secret surfactant phospholipids (Yamazaki et al., Development, 2009). The observations indicate that TRIC channels act as counter-ion channels functionally coupled with Ca²⁺ release in various cell types.

Here we report the direct linkage of TRIC channels with hypertension. Knockout mice lacking TRIC-A channels showed significant hypertension and brady-cardia. Ca²⁺ channel antagonists, but not blockers for vasoactive agents, exerted antihypertensive effects in the mutant mice. Moreover, despite retaining normal passive diameters in a Ca²⁺-free bathing solution, the mutant arteries showed narrow diameter results from higher resting Ca²⁺ level maintained by Ca²⁺ influx via L-type Ca²⁺ channel. In vascular smooth muscle cells (VSMCs), spontaneous opening of ryanodine receptor channels generates local Ca²⁺ release called Ca²⁺ sparks, activates cell-surface Ca²⁺-dependent K⁺ channels and reduces membrane potential toward relaxation. Our physiological and pharmacological studies suggested that the loss of TRIC-A channels...
comprises Ca$^{2+}$ spark and spontaneous transient outward currents (STOCs) generation. In membrane potential monitoring using the voltage-dependent dye, the mutant VSMCs exhibited elevated resting membrane potential and prolonged repolarizing phases after high KCl-induced depolarization. These results suggested that the loss of TRIC-A in VSMCs enhanced the basal tonus induced by higher resting Ca$^{2+}$ level results from decrease of Ca$^{2+}$ sparks, STOCs frequency and depolarization of membrane potential.

Mitsugumin23 (MG23) is localized to ER/SR and nuclear membranes in a wide range of cell types. It has recently been linked with apoptosis (Yamazaki et al., Hiroshi Takeshima. 2010). Several studies using NAADP binding assays and Ca$^{2+}$ imaging experiments linked members of the two-pore channel family (TPCN1-3) with NAADP-mediated Ca$^{2+}$ release from intracellular stores at low nanomolar concentrations. NAADP-evoked Ca$^{2+}$-release has been demonstrated in invertebrates and numerous mammalian cell types including invertebrates and numerous mammalian cell types, cardiac and smooth muscle cells, T-lymphocytes, platelets and neurons. Several studies using NAADP binding assays and Ca$^{2+}$ imaging experiments linked members of the two-pore channel family (TPCN1-3) with NAADP-induced Ca$^{2+}$ release from lysosome-like acidic organelles. However, there has been no direct demonstration that TPCNs can act as NAADP-sensitive Ca$^{2+}$ release channels. Recently, we developed a highly efficient method to record ion currents in single isolated lysosomes. These recordings are performed using an automated patch clamp approach that involves the immobilization of isolated lysosomes on a solid matrix planar glass chip. Using this method we have provided direct evidence that TPCN2 is a highly selective Ca$^{2+}$-channel. Furthermore, we identified an amino acid residue in the putative pore region that is crucial for conferring high Ca$^{2+}$-selectivity. Here, we extend our biophysical characterization of TPCN2 and report a detailed analysis of the permeation properties of these channels. Overall, our study lays the groundwork for the understanding of TPCN2 function in lysosomal stores. Our glass chip based method will provide electrophysiological access not only to lysosomal TPCN channels but also to a broad range of other intracellular ion channels.

Molecular Characterization of BkCa Channel in Cardiac Mitochondria Harpreet Singh, Ron Ling, Jean Chrissostome Bopassa, Enrico Stefani, Ligia Toro. Increasing evidence indicates that cardioprotective pharmacological and signaling mechanisms converge on the inner mitochondrial membrane Ca$_{um}$ -sensitive and voltage-dependent K$^+$ channel (mitoBkCa). mitoBkCa has been proposed to be formed by proteins of ~50 to ~125 kDa. Thus, we investigated the molecular characteristics of this channel in isolated mitochondria from murine heart. We present evidence supporting the view that mBKCa, alpha and beta (125 kDa) is the backbone of mitoBkCa and that splicing contributes to its targeting to mitochondria. Labeling of adult mouse cardiomyocytes with specific anti-BkCa antibodies directed against its C-terminus, mitotracker, and wheat germ agglutinin yielded remarkable mitochondrial but not plasma membrane localization. Nanoscale fluorescence microscopy (Stimulated Emission Depletion) revealed 7 to 15 of ~40-50 nm BkCa clusters per mitochondria. Further, Western blot analysis of purified mitochondria showed the presence of a full length ~125 kDa protein with both polycanonical (Amonomote lab) and monocanonical (NeuroMab) antibodies. Systematic RT-PCR exon scanning of isolated cardiomyocyte mRNAs were consistent with a full length ~125 kDa alpha-subunit protein and revealed the expression of three splice inserts. Insertless-BkCa when expressed in CHO-cells robustly localized to the plasma membrane but when a C-terminal splice insert was present BkCa was readily targeted to the mitochondria (protein proximity index was ~1.0 indicating 100% colocalization). Hence, cardiac mitoBkCa is composed by full-length BkCa protein but with splice inserts which may facilitate its targeting to mitochondria. Supported by NIH.

Mitsugumin23, a Protein Associated with Intracellular Calcium Stores, Behaves as an Ion-Channel that can Conduct Calcium Elisa Venturi, Kazuhiro Mio, Miyuki Nishi, Toshihiko Ogura, Toshio Moriya, Samantha J. Pitt, Kazutaka Okuda, Rebeca Sitsapesan, Chikara Sato, Hiroshi Takeshima. Mitsugumin23 (MG23) is localized to ER/SR and nuclear membranes in a wide range of cell types. It has recently been linked with apoptosis (Yamazaki et al. 2010) and its physiological functioning is unknown. Proteolytic assay of purified MG23 predicts three transmembrane segments and chemical crosslinking experiments provide evidence of a multimeric complex. Reconstitution of MG23 into artificial membranes under voltage-clamp conditions revealed that MG23 behaves as a cation channel that is permeable to both monovalent and divalent cations. A characteristic of MG23 channels was the multiplicity of charge to be gated by the ion.

We frequently observed long episodes of the apparent co-ordinated gating of multiple channels but we have not identified any tendency for a preferred number of MG23 channels to gate together in synchrony; this seems to be a random process. MG23 showed distinct voltage-dependence, being more open at negative holding potentials. Electron microscopy and single particle reconstruction techniques indicate that purified MG23 forms two types of structure; large shells of 6-fold symmetry and smaller, asymmetrically-shaped particles. We suggest that the apparent coordinated opening of various numbers of MG23 channels may reflect reversible structural changes in subunit assembly. We propose that MG23 is a homo-multimeric complex which transitonarily forms cation-channels in intracellular membranes, enabling rapid changes in the ionic fluxes across intracellular Ca$^{2+}$-stores.

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Regulation of the Type 1 and Type 2 Insitol 1,4,5-Trisphosphate Receptor Single Channel Open Probability by ATP in On-Nucleus Patch Clamp Recordings Larry E. Wagner II, David I. Yule. The insitol 1,4,5-trisphosphate receptor (InsP$_3$R) can be modulated by ATP. InsP$_3$R are also tightly regulated by intracellular Ca$^{2+}$ (Ca$^{2+}$i). The receptors exhibit modulation by Ca$^{2+}$i, in a biphasic [Ca$^{2+}$i]-dependent manner. As [Ca$^{2+}$i] increases, channel activity also increases until it reaches a saturating [Ca$^{2+}$i] and a maximal Po. Further increases in [Ca$^{2+}$i] inhibits activity. We have previously shown that increasing [ATP] differentially enhances single channel open probability (Po) in patch clamp recordings of plasma membrane InsP$_3$R in DT40 cells. When stably transfected with either the rat Type I S1-S2 (InsP$_3$R-1) or the mouse Type 2 (InsP$_3$R-2) receptors. Increasing [ATP] augments Po of InsP$_3$R-1 in the presence of high (10 uM) and low (1 uM) [InsP$_3$]. However, InsP$_3$R-2 single channel Po is not modulated at low [InsP$_3$]. To further elucidate the mechanism behind ATP regulation of the receptor channel, we examined the effects of ATP on the Ca$^{2+}$i dependency of both rInsP$_3$R-1 and mInsP$_3$R-2. We used the on-nucleus patch clamp recording technique and varied the [ATP]. (InsP$_3$), and [Ca$^{2+}$i] in the patch pipette to measure single InsP$_3$R channel activity of stably transfected DT40 cells. Only recordings which presumably contained a single channel were considered for analysis. In experiments containing rInsP$_3$R-1 channels, increasing [ATP] shifted the biphasic Ca$^{2+}$i dependency curve to the left with both high (10 um) and low (1um) [InsP$_3$]. However, mInsP$_3$R-2 single channels exhibited no ATP-dependent shift in the presence of 10 uM InsP$_3$. There was no shift with 1 um InsP$_3$, but an overall decrease in the maximal Po was observed. These data suggest different mechanisms for modulation of the Type 1 and Type 2 InsP$_3$R by ATP.

Investigations on N-Terminal Chimeras of VDAC Isoforms Vito De Pinto, Simona Reina, Flora Tommasello, Francesca Guarino, Angela Messina. VDAC (Voltage Dependent Anion-selective Channels, [1]) are pore-forming proteins mainly found in the outer mitochondrial membrane. In mammals three isoforms have been described [2]. The proposed VDAC structure has been found to form a [bar]-barrel with the addition of 20 amino acids at the N-terminal that fold as an amphipathic z-helix [3]. We have produced swapping mutants (or chimeras) of human VDAC isoforms carrying exchanged N-termini. In particular the addition of VDAC1 or VDAC2 N-termini to the barrel-forming sequence of VDAC3 transforms this latter protein from a poorly active behaviour into a normally active pore-forming protein [4]. The functionality of chimeras has been assayed in an S. cerevisiae strain lacking the endogenous porin. Then the activity of the chimeras has been proved in terms of its ability to complement the defective growth phenotype of yeast, due to a deficiency in the aerobic metabolism of the host cell [4]. In this work we will present our investigations on the importance of the amino acid replacements expressed in the chimeras. In addition, the characterization of expressed and purified chimeras in reconstituted systems in planar lipid bilayers will be presented. The overall picture emerging from our experiments is that the VDAC N-terminal peptide plays a role in the proper function of this protein during cell life events.