auto- and cross-correlation. We are focusing on determining the flux through parallel assembly pathways in the 3′-domain of the 30S subunit. Binding rates of proteins to the full-length 16S rRNA were obtained from FCS data, with 1-second resolution.
Assembly of the 3′-domain is initiated by binding of protein S7, followed by parallel binding of three proteins: S9, S13, and S19. These known dependencies are thermodynamic, and there is no information about the flux of the assembling ensemble through these parallel pathways. To begin to develop a kinetic map for 30S assembly, we initiated these fluorescence studies of the early 3′-domain assembly. FCS has provided measurement of the binding rate for individual proteins, and Two and Three-Color FCS spectra provides an as-yet qualitative look at the evolution of multiple intermediates, and a glimpse at how 30S assembly can proceed in parallel.

49-Plat
Visualizing tmRNA after its accommodation in the Ribosome
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In eubacteria, translation of an mRNA that lacks a stop codon produces defective polypeptide that stalls on the ribosome. Transfer-messenger RNA (tmRNA), a molecule in eubacteria that possesses functions of both mRNA and tRNA, rescues the stalled ribosome by ''trans-translation,'' a process by which the tmRNA is recruited to the ribosome with the help of EF-Tu and small protein B (smpB). Tanslation is resumed on the open reading frame of the mRNA-like domain (MLD) of the tmRNA. Several structures of tmRNA in complex with ribosome, in the accommodating and the accommodated states, have been studied by cryo-EM single-particle reconstruction (Gillet et al., 2007; Kaur et al., 2006; Valle et al., 2003). However, the structures of the complex in the subsequent stages of trans-translation remain unknown. Here, using mutagenesis, we have been able to trap the complex in different stages of trans-translation by substituting one of the sense codons of the MLD open reading frame with a stop codon. Structures of these complexes were obtained by the cryo-EM single particle reconstruction technique. To address the sample heterogeneity, we used the maximum-liklihood classification method (Scheres et al., 2007). The resulting density maps were analyzed by rigid body fitting in combination with biochemical data. We discovered that part of the tmRNA molecule maintains a relative defined structure during tran-translation. Also, we identified several possible binding sites of the tRNA like domain (TLD) of tmRNA and smpB on the ribosome.
Gillet et al. (2007) J. Biol. Chem. 282: 6356-6363.
Kaur S. et al. (2006) Proc. Natl. Acad. Sci. USA 103:16484-16489.
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Valle M. et al. (2003) Science 300: 127-130.

50-Plat
Coupling Of Ribosome And tRNA Dynamics During Translation
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Comparisons of X-ray crystallographic and cryogenic electron microscopic structures of ribosomal complexes have led to the hypothesis that conformational dynamics of the ribosome, its transfer RNA (tRNA) substrates, and associated translation factors play important mechanistic and regulatory roles throughout all stages of protein synthesis. Using fluorescently-labeled components within a highly-purified in vitro translation system, we are directly characterizing structural changes of the translational machinery in real time using single-molecule Förster resonance energy transfer (smFRET) in order to elucidate the mechanisms through which these dynamics direct and regulate the individual steps of translation. Here we report new ribosome-ribosome, ribosome-tRNA, and tRNA-translation factor smFRET signals that have allowed us to fully characterize the intrinsic conformational dynamics of a ribosomal domain, the L1 stalk, as well as the coupling between L1 stalk and tRNA dynamics, throughout protein synthesis. Our data reveal that the translating ribosome can spontaneously and reversibly fluctuate between two global conformational states, and that transitions between these two states involve coupled movements of the L1 stalk and ribosome-bound tRNAs, accompanied by ratcheting of the ribosomal subunits. Furthermore, we find that elongation, release, and ribosome recycling factors uniquely recognize these global states of the ribosome and differentially affect transition rates between the two states. Thus, translation factor-mediated recognition and control over intrinsic dynamics of the ribosome plays a major mechanistic role during the elongation, termination, and recycling stages of translation. Our results support the view that specific regulation of the global state of the ribosome is a fundamental characteristic of all translation factors and a unifying theme throughout protein synthesis.

51-Plat
Regulation of the Protein-conducting Channel by a Bound Ribosome
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The evolutionarily conserved protein-conducting channel, or translocon, is a transmembrane protein which has the dual functions of allowing nascent proteins to cross the membrane or to insert into the membrane. These functions are carried out in concert with a partner which feeds the nascent protein into the channel. In many cases, this partner is the ribosome. The specific interactions between ribosome and protein-conducting channel have recently come into focus due to the availability of cryo-electron microscopy maps of the ribosome in complex with a channel monomer. We have used a method recently developed in our lab, molecular dynamics flexible fitting (MDFF), to fit atomic-scale structures into these maps. Using our fitted atomic-scale model of the ribosome-channel complex, we have carried out large (2.7 million atoms) equilibrium molecular dynamics simulations in order to investigate how the ribosome induces channel opening, as suggested by recent experiments. We find that the channel-blocking plug becomes more mobile under the ribosome's influence. By performing simulations of protein translocation through the ribosomal protein exit tunnel and into the translocon channel, we have determined what elements of the ribosome interact most strongly with the nascent chain and in what orientation the growing protein inserts into the channel.

Platform E: Excitation-Contraction Coupling

52-Plat
Impaired Sarcoplasmic Reticulum Calcium Release In Skeletal Muscle Fibers From Myotubularin-Deficient Mice
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X-linked myotubular myopathy (XLMTM) is a disease characterized by severe skeletal muscle weakness leading to death during childhood. XLMTM results from mutations in the *MTM1* gene, coding for Myotubularin, a phosphoinositide phosphatase believed to play a role in plasma membrane homeostasis. The mechanisms responsible for muscle function impairment in XLMTM are unknown. Here we studied the properties of excitation-contraction coupling in skeletal muscle fibers isolated from a mouse model of the disease. Experiments were performed under silicone-voltage-clamp conditions using indo-1 as Ca^{2+} indicator. In muscle fibers from 5-week-old *MTM1*-deficient mice, the amplitude of the voltage-activated Ca^{2+} transient was strongly reduced. For instance, in response to a 10 ms-long pulse from -80 to +10 mV, the peak $\Delta[Ca^{2+}]$ was 0.52 ± 0.1 μM (n=14) in *MTM1*-KO fibers as compared to 1.4 ± 0.14 μM in WT fibers (n=14). Conversely, the rate of $[Ca^{2+}]$ decay after the end of the pulses was similar in the two strains suggesting overall preserved myoplasmic Ca^{2+} removal capabilities. The SR calcium content was also found to be unaltered, as estimated from indo-1 signals measured in fibers equilibrated with high intracellular EGTA and in the presence of a SR Ca^{2+} pump blocker. The reduced amplitude of the Ca^{2+} transient in *MTM1*-deficient fibers was associated with a twice reduction in the peak density of the voltage-activated slow Ca^{2+} current with no apparent concurrent change in the density of intramembrane charge movement. Finally, confocal imaging with di-8-anneps revealed local disruptions in the typical fluorescence banded pattern, indicative of alteration of t-tubule membrane. Overall results unravel a critical role of MTM1 in the proper function of E-C coupling and strongly suggest that defective RyR1-mediated SR Ca^{2+} release is responsible for the failure of muscle function in myotubular myopathy.

53-Plat
Changes of EC-coupling and RyR Calcium Sensitivity in Dystrophic mdx Mouse Cardiomyocytes
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Duchenne muscular dystrophy represents a severe inherited disease of striated muscle, caused by a mutation in the dystrophin gene and characterized by progressive loss of skeletal muscle function. Most patients also develop dystrophic cardiomyopathy resulting in dilated hypertrophy and heart failure. On the cellular level, absence of dystrophin affects mechanical membrane stability and intracellular Ca signaling in cardiomyocytes. Cellular mechanisms leading to deterioration of cardiac function remain elusive. We tested whether defective excitation-contraction (EC) coupling contributes to impaired cardiac performance. EC-coupling gain, a measure for the effectiveness to amplify the Ca signal by Ca release from the sarcoplasmic reticulum (SR), was determined from control and dystrophin-deficient *mdx* hearts. Ca currents were measured with the whole-cell patch-clamp technique, while Ca transients were simultaneously recorded with confocal imaging of fluo-3. Initial findings indicated subtle problems of EC-coupling in *mdx* cells despite matched SR Ca loading. However, lowering extracellular Ca, a maneuver used to unmask latent EC-coupling problems, was surprisingly much better tolerated by *mdx* myocytes. Normalized to control conditions, the EC-coupling gain in *mdx* cells reached 112% compared with 31% in control cells, suggesting hypersensitive EC-coupling. Further investigation of this apparent increase in Ca sensitivity by inducing slow elevations of intracellular Ca resulted in Ca oscillations after a much shorter delay in *mdx* cells, consistent with enhanced Ca sensitivity of SR Ca release channels (ryanodine receptors, RyRs). Elevated cellular reactive oxygen species (ROS) generation in dystrophy suggests redox-modifications on the RyR, enhancing its Ca sensitivity. Preincubation of *mdx* cells with a ROS scavenger normalized the EC-coupling hypersensitivity back to control cardiomyocytes. Our data suggest that in dystrophin-deficient cardiomyocytes, EC-coupling mechanisms are altered, partly due to potentially arrhythmogenic changes in Ca sensitivity of redox-modified RyRs.
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54-Plat
The Skeletal L-type Ca^{2+} Current is a Major Contributor to Excitation-Coupled Ca^{2+} Entry (ECCE)
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The term Excitation-Coupled Ca^{2+} Entry (ECCE) designates the entry of extracellular Ca^{2+} into skeletal muscle cells which occurs in response to prolonged depolarization or pulse trains, and which depends on the expression of both the 1,4-dihydropyridine receptor (DHPR) and the type 1 ryanodine receptor (RyR1). The ECCE pathway is blocked by pharmacological agents that also block store-operated Ca^{2+} entry, is relatively insensitive to nifedipine (1 μM), and is permeable to Mn^{2+}. We have examined the effects of these agents on the L-type Ca^{2+} current conducted via the DHPR. We found that the non-specific cation channel antagonists 2-APB, SKF 96356, La^{3+} and Gd^{3+} all inhibited the L-type current. In addition, complete (>97%) block of the L-type current required concentrations of nifedipine >10 μM. Like ECCE, the L-type channel displays permeability to Mn^{2+} in the absence of external Ca^{2+} and produces a Ca^{2+} current that persists during prolonged (~10 s) depolarization. This current appears to contribute to the Ca^{2+} transient observed during prolonged KCl-evoked depolarization of intact myotubes because (*i*) the transients in normal myotubes decayed more rapidly in the absence of external Ca^{2+}, (*ii*) the transients in *dysgenic* myotubes expressing SkEIIIK (a DHPR α_{1S} subunit pore mutant thought to conduct only monovalent cations) had a time course like that of normal myotubes in Ca^{2+}-free solution and was unaffected by Ca^{2+} removal, and (*iii*) after block of SR Ca^{2+} release by ryanodine, normal myotubes still displayed a large Ca^{2+} transient whereas no transient was detectable in SkEIIIK-expressing *dysgenic* myotubes. Altogether, these results indicate that the skeletal muscle L-type channel is a major contributor to the Ca^{2+} entry attributed to ECCE. Supported by NIH NS24444 and AR44750 to K.G.B., and MDA 4155 to R.A.B.

55-Plat
Post-Tetanic Calcium Transients In Adult Skeletal Muscle Fibers Are Frequency-Dependent And Fiber Type Specific
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Isolated adult *Flexor digitorum brevis* fibers from 4-6 weeks-old mice, loaded with Fluo-3 were stimulated with trains of 270, 0.3 ms pulses at different frequencies. We observed a fast calcium tetanus (associated with contraction) and a second, slower signal, similar to those previously described in cultured myotubes. The slow signal (more than the fast one) was inhibited by 25 micro M nifedipine, suggesting a role for DHPR in its onset and by the IP_3R inhibitor Xestospongin-C (5 micro M). The amplitude of post-tetanic calcium transients depended on both stimulus train frequency and duration; a bell shaped curve frequency was obtained with a maximum at 10-20 Hz. Likewise, signal amplitude was proportional to stimulus train duration. Fibers isolated from *soleus* muscle completely lack slow calcium transients. Using immunofluorescence, we have found that all three IP_3R isoforms are present in adult muscle at different levels and that IP_3R-1 is differentially expressed (with a mosaic pattern) in different types of muscle fibers, being higher in a subset of fast-type fibers. ERK 1/2 phosphorylation of adult muscle fibers after tetanic stimulation appears to relate slow calcium signals to transcription-related events. These results support the idea that different calcium kinetics for the slow signals mediated by IP_3R may exist in different types of muscle fibers and participate in the activation of specific transcriptional programs of slow and fast phenotype. FONDAP 15010006, Bicentenario-PSD24, FONDECYT 1080120

56-Plat
Negative Relationship Between Fractional SR Ca^{2+} Release and Stimulation Rate
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Typically, contraction amplitude in rodent myocardium is inversely related to stimulation rate. We calculated Ca^{2+} fluxes across the sarcolemma and the sarcoplasmic reticulum (SR) membrane, and SR Ca^{2+} content to estimate the fraction of the SR Ca^{2+} load released at a twitch (FR) during steady-state (SS) stimulation at 0.2-2 Hz in intact rat cardiomyocytes. Cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured with indo-1. SR Ca^{2+} content was determined from the transient in response to 10 mM caffeine in Na^+,Ca^{2+}-free medium. From Ca^{2+} buffer parameters, and $[Ca^{2+}]_i$ values and decline kinetics during transients in which SR Ca^{2+}-ATPase and/or Na^+-Ca^{2+} exchanger (NCX) were inhibited, SR- and NCX-mediated Ca^{2+} fluxes at each rate were estimated and integrated up to attainment of SS diastolic $[Ca^{2+}]_i$. Assuming equal inward and outward Ca^{2+} fluxes at SS, Ca^{2+} influx was considered as the NCX-mediated flux, whereas SR Ca^{2+} release and uptake fluxes were considered equivalent. FR was taken as the ratio of the integrated SR-mediated Ca^{2+} flux and SR Ca^{2+} content. Increasing rate did not affect significantly SR Ca^{2+} content (128 ± 4 and 133 ± 4 μM at 0.2 and 2 Hz, respectively), but decreased ($p<0.01$) both Ca^{2+} influx (16.9 ± 1.4 vs. 6.7 ± 0.3 μM at 0.2 and 2 Hz) and SR-dependent Ca^{2+} flux (93 ± 4 vs. 77 ± 3 μM at 0.2 and 2 Hz). Estimated FR showed a negative relationship with the stimulation rate (0.73 ± 0.03 vs. 0.58 ± 0.02, $p<0.01$), possibly due to decreased Ca^{2+} influx, although action potential duration was increased (APD90= 76 ± 9 vs. 130 ± 11 ms, $p<0.01$). Alternatively, incomplete time-dependent recovery from inactivation of SR Ca^{2+} channels might contribute to depress FR. These results support the proposal that changes in FR underlie the negative force-frequency relationship in rodents. (FAPESP, CNPq, FAEPEX).

57-Plat
Flecainide Inhibits Cardiac Ryanodine Channels And Spontaneous Sarcoplasmic Reticulum Calcium Release In Casq2 Null Myocytes
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Background: Catecholaminergic polymorphic ventricular tachycardia (CPVT) is an inherited arrhythmogenic syndrome due to cardiac Ca^{2+} release channel (*RYR2*) or cardiac calsequestrin (*CASQ2*) mutations. VT is caused by spontaneous Ca^{2+}-release from the sarcoplasmic reticulum (SR) that generates afterdepolarizations and triggered beats during catecholamine surge. We recently found that flecainide, a class 1c Na^+ channel blocker, suppressed ventricular arrhythmia in *Casq2 null* (*Casq*$^{-/-}$) mice, a model of CPVT. Here, we investigated the effect of flecainide on on cardiac Ca^{2+} handling in *Casq2*$^{-/-}$ myocytes loaded with Fura-2 AM, and on sheep RyR2 RyR2 channels reconstituted in lipid bilayers.
Results: In isoproterenol-stimulated *Casq2*$^{-/-}$ myocytes, flecainide (6 μmol/l) reduced triggered beats by over 70% ($p<0.001$). Unexpected for a Na^+ channel blocker, flecainide also reduced SR Ca^{2+} leak (Ca^{2+} fluorescence ratio: vehicle: 0.12 ± 0.01 vs. flecainide: 0.08 ± 0.01, n=54 per group, p=0.02) and suppressed the rate of spontaneous Ca^{2+} releases (SCRs) from the SR (SCRs/min: vehicle: 48 ± 5 vs. flecainide: 29 ± 5, n=45 per group, p=0.006), suggesting flecainide directly inhibits SR Ca^{2+} release. Lipid bilayer experiments confirmed a direct action of flecainide on RyR2 SR Ca^{2+} release channels: Flecainide induced brief partial closures of channels to a substate with a conductance equal to 20% of the fully open state. On average, flecainide as low as 5 μmol/L caused a 4-fold increase in the frequency of closed events and caused a significant reduction in the open probability from control levels. The effect of flecainide was concentration dependent (IC_{50} ~ 50 μmol/l) and fully-reversible upon washout. Flecainide also inhibited RyR2 channels activated by high luminal Ca^{2+}.
Conclusion: We report a heretofore unrecognized inhibitory action of flecainide on RyR2 channels, which together with flecainide's inhibition of Na^+ channels may explain flecainide's effectiveness in preventing CPVT.
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