M-AM-SymI-1

THE N-TERMINAL DOMAIN OF THE U1A PROTEIN BINDS TO AN RNA HAIRPIN. ((Kathleen B. Hall)) Dept. of Biochem & Mol. Biophysics, Washington University School of Medicine, St. Louis, MO 63110.

The U1A protein is one of the unique proteins associated with the U1 snRNP particle, where it specifically recognizes an RNA sequence in stem/loop II of the snRNA. U1A includes two domains which could potentially bind to nucleic acids: of these, only the amino-terminal domain is necessary for sequence-specific binding to the RNA hairpin. We have used this 102 amino acid protein domain, together with a short 25 nucleotide RNA hairpin, to study the biophysical details of the RNA:protein interaction. We have found that the binding is extremely tight, with a $K_D \approx 10^{-11}$ M in 250 mM NaCl, 1 mM $MgCl_2$, pH 7, with a measured free energy of association ΔG°=-14 kcal/mol. 102A will bind with lower affinity to a single-stranded RNA ($K_D \approx 10^{-6}$ M) and to a DNA hairpin ($K_D \approx 10^{-6}$ M) which contain the wild-type loop sequence; we do not know if the binding sites are the same for the RNA and DNA hairpins. Binding affinity is sensitive to mutations in the loop sequence of the RNA hairpin, but apparently not to the stem sequence. The structure of the RNA hairpin is being investigated using NMR, which has shown that while the stem is an A-form duplex, the loop is quite floppy. We are now using ^{13}C, ^{15}N, and ^{31}P heteronuclear NMR methods to further define the complex structure of the RNA loop.

M-AM-SymI-2

RNA SECONDARY STRUCTURE ELEMENTS FROM NMR SPECTROSCOPY. ((Gabriele Varani)) MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, England.

We have investigated by NMR several RNA structural motives, including hairpin loops and internal loops. While the double helical stems adopt A-form geometry with C3'-*endo* sugar pucker, the loops contain non-Watson-Crick base pairs. Unusual sugar-phosphate backbone conformation and C2'-*endo* sugar pucker are also found, to accommodate sharp turns and non-Watson-Crick pairs. Using only ^1H NMR, high resolution structures can be obtained for RNAs of 3-5,000 da or less. For larger RNA molecules (up to 8,000-10,000 da), the overall three-dimensional structure is not well determined, but many local structural features can be identified with confidence. Both precision and accuracy are greatly improved when the sugar-phosphate backbone conformation is constrained by scalar coupling measurements. Heteronuclear couplings have been measured with high precision on ^{13}C labeled samples, further restraining the backbone conformation. The structure of a 27-nucleotide RNA containing the internal loop E from eukaryotic 5S ribosomal RNA and a single nucleotide deletion mutant revealed several unexpected features that may be common in internal loops. Non-Watson-Crick pairs, a G•A and a Hoogsteen A•U pair, close the native loop sequence, but no stable base pair was found instead for the mutant. A guanosine is bulged into the major groove, and may be interacting with the Hoogsteen base pair forming a base triple. The non-Watson-Crick base pairs are accommodated by C2'-*endo* riboses for the bulged G and a neighboring A, and by an altered backbone conformation.

M-AM-SymI-3

RNA-PROTEIN INTERACTIONS IN MODEL SYSTEMS FOR HIV TAR-TAT RECOGNITION. ((Joseph D. Puglisi, Ruoying Tan, Barbara J. Calnan, Alan D. Frankel, and James R. Williamson)) Department of Chemistry, MIT and Whitehead Institute, Cambridge, MA 02139.

TAR is an RNA secondary structure element near the 5' end of HIV mRNA that contains a six nucleotide hairpin loop and a three nucleotide bulge. TAR is the binding site for the transcriptional activator protein, Tat. Mutational and chemical modification studies have determined that nucleotides in the bulge region of TAR are required for Tat recognition. Short, basic peptides derived from Tat bind specifically to TAR, as do peptides containing a single arginine. In addition, free arginine also binds to TAR in a manner similar to that observed for peptides. Arginine and Tat-peptides can serve as models to understand how the RNA is recognizing the protein.

We have performed detailed NMR studies on wild-type and mutant TAR RNAs in complexes with arginine and arginine containing peptides. Based on these experiments, we propose a detailed structural model for the interaction of arginine with TAR. There is a significant conformational change in TAR upon arginine binding that brings functionally important groups above and below the three nucleotide bulge into proximity. Features of the complex include a base triple interaction, hydrogen bonding of arginine in the major groove, and arginine-phosphate contacts. The structural model is consistent with both mutation and chemical modification data on TAR. The modest specificity observed for the tat-TAR interaction is fully consistent with this simple recognition motif. The tertiary structure adopted by the RNA plays a crucial role in providing a binding site for Tat.

M-AM-SymI-4

Solution Structures of RNA: RNA Folding Motifs and Ribozymes ((Arthur Pardi, Fiona M. Jucker, Pascale Legault and Edward P. Nikonowicz)) Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309-0215. (Spon. by A. Pardi)

We have recently developed efficient methods for production of ^{13}C and ^{15}N labelled RNA oligomers. With labelled molecules, it is possible to apply multi-dimensional heteronuclear magnetic resonance techniques to the structure determination of RNAs. These 3D and 4D NMR experiments are revolutionizing the solution structure determinations of proteins and these techniques will have a similar effect on NMR structure determinations of RNAs. The resolution of the NMR spectrum of an isotopically labelled RNA is enormously improved by application of multi-dimensional heteronuclear magnetic resonance experiments. These advances make it possible to determine the solution structure of larger more biologically relevant RNAs (1). We are currently using multi-dimensional NMR to study the structure of several catalytic RNAs including the hammerhead ribozyme and a lead-dependent self-cleaving ribozyme. NMR structural studies are also being performed on RNA folding domains such as RNA hairpins containing tetranucleotide loops, and RNA duplexes containing nonstandard base pairs. Progress on structure determinations of these systems will be presented.

1. E.P. Nikonowicz and A. Pardi, *Nature*, **355**, 184-186 (1992)

STRUCTURE AND FUNCTION OF THE CYTOSKELETON

M-AM-SymII-1

PROJECTIN, FLIGHTIN, AND MP20: THREE "NEW" PROTEINS ASSOCIATED WITH THE CONTRACTILE APPARATUS. ((M.L. Pardue[1], A. Ayme-Southgate[2], J. Vigoreaux[3], J.Saide[4], and G. Benian[5]))[1]Mass. Inst. of Tech., Cambridge, MA 02139, [2]Lehigh Univ., Bethleham, PA 18015, [3]Univ. of Vermont, Burlington, VT 05405, [4]Boston Univ. Medical School, Boston, MA 02118, [5]Emory School of Medicine, Atlanta, GA 30322.

Although all Drosophila muscles are striated, there are several muscle types which differ in function. These functional differences are reflected in ultrastructure and in muscle type-specific proteins or isoforms. Understanding the molecular bases of these differences can give clues to muscle structure in general. We have characterized three proteins that are associated with the contractile apparatus in different muscle types and appear to have novel functions. Flightin is found only in stretch-activated flight muscle and is localized in the A band. mp20 is found in all muscles except the stretch-activated muscles and is also localized in the A-band. Flightin and mp20 do not appear to be related to each other, however each may be involved in regulation of contraction, one in stretch-activated (asynchronous) muscle and the other in synchronous muscle. Projectin is the Drosophila member of the titin/twitchin family. The projectin isoform in stretch-activated muscle is in the I band; in other muscle the isoform is detected only in the A band. Although Drosophila has only one projectin gene, the gene products appear to have distinctly different functions in the different muscle types.

M-AM-SymII-2

FORCE GENERATION BY THE MICROTUBULE-BASED MOTOR PROTEIN KINESIN. ((J. Howard)) Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195.

Kinesin is a force-generating enzyme, a motor protein, which converts the free energy associated with the hydrolysis of ATP into mechanical work. The work is used to power the transport of intracellular organelles along microtubules, cytoskeletal polymers of the protein tubulin. We are attempting to understand the workings of this tiny motor which weighs less than 1 ag: how much force can it generate and how far does it move per ATP hydrolyzed?

We have developed cell-free assays in which the movement of individual microtubules across a kinesin-coated surface is directly observed under the microscope. By decreasing the density of kinesin on the surface we have shown that a single molecule suffices to move a microtubule. By raising the viscosity of the solution through which the microtubule moves 100-fold, we estimate that the maximum force that a single motor can exert is ~2 pN. In a second approach to measuring the force we are analyzing the kinesin-driven bending of microtubules of calibrated flexural rigidity.

Through manipulation of the quaternary structure of microtubules, we have elucidated the path which kinesin follows across the surface of the microtubule. Microtubules grown *in vitro* with 12, 13, or 14 protofilaments rotate in the motility assay with a pitch and handedness matching the helical paths of the protofilaments around the cylindrical surface of the microtubule. Thus the distance between consecutive kinesin-binding sites must be a multiple of 4.1 nm, the spacing of the tubulin monomers along the protofilament.

This work was supported by NIH AR40593, the Pew and Sloan Foundations, and the AHA (Washington Affiliate).

M-AM-SymII-3

THE STRUCTURE OF MYOSIN SUBFRAGMENT-1 AT 2.8A RESOLUTION.
((I. Rayment)) University of Wisconsin.

M-AM-SymII-4

MUTATION ANALYSIS OF THE MOLECULAR MOTOR MYOSIN.
((Kathleen M. Ruppel, Taro Q.P. Uyeda, and James A. Spudich)) Departments of Biochemistry and Developmental Biology, Stanford University, Stanford, CA 94305.

We are using site-directed mutagenesis and oligo-directed random mutagenesis to explore regions of the myosin head (Subfragment-1 or S1, the motor unit of the molecule) thought to be important for ATP and actin-binding, as well as to delineate functions of other regions of currently unknown function. Mutated myosins are expressed in *Dictyostelium* cells lacking the endogenous myosin heavy chain gene (created by gene replacement). Resultant *in vivo* phenotypes are used to identify functionally defective myosins. The altered myosins are then purified from these cells using a rapid new purification scheme, and they are characterized by a variety of methods including *in vitro* motility assays. Early mutagenesis efforts concentrated on specific amino acid changes of residues thought to be important for ATP and actin interactions. This approach yielded mutated myosins that are no longer able to bind nucleotide but which bind actin tightly (rigor-binders), myosins that have decreased affinity for nucleotide or decreased ATP turnover, and myosins with decreased stability *in vivo* and *in vitro*. More recent efforts have involved creating banks of random point mutations over short (about 20 a.a.) stretches of highly conserved myosin sequence throughout the myosin head. Initial analysis of two such banks of mutations, both centered in a block of strikingly high conservation within the 50-kDa domain, has revealed mutations that show defects in ATP hydrolysis, actin-activation of ATPase activity, or of coupling of ATPase and motility *in vitro*. Another effort has involved altering the highly variable loop structures between the 50/20-kDa junction in order to assess its importance for regulation of the rate of ATPase and speed of contraction. Sequences corresponding to smooth, cardiac and skeletal muscle myosin loops have been used to replace the *Dictyostelium* sequence, and their effects are being assayed. In collaboration with Dr. Ivan Rayment (University of Wisconsin, Madison), we are placing each of these changes on the high-resolution three dimensional structure of S1 (recently determined by Dr. Rayment and his colleagues). We are also collaborating with Dr. Ken Holmes' group (Max-Planck-Institute, Heidelberg) and Dr. Dietmar Manstein (NIMR, Mill Hill, London) to analyze the position of these mutations in relation to the docked structure of S1 bound to actin in the actin-S1 complex formed in the absence of ATP. These studies should provide a framework for understanding how myosin functions to link ATP hydrolysis to movement and force production.

POTASSIUM CHANNELS I

M-PM-A1

A G_q-LIKE G PROTEIN TRANSDUCES THE BRADYKININ ACTIVATION OF A CALCIUM-DEPENDENT POTASSIUM CURRENT IN NG108-15 CELLS.
((M.A.Wilk-Blaszczak, S.Gutowski, P.Sternweis and F.Belardetti)) Dept. Pharmacology, Univ. TX Southwestern Med. Ctr. Dallas, Dallas, TX 75235.

In differentiated NG108-15 neuroblastoma x glioma cells, bradykinin (BK) stimulates PLC, which breaks down PIP_2, generating IP_3 and DAG. By triggering Ca^{2+} release in the cytoplasm, IP_3 activates an outward $I_{K(Ca)}$. We have examined the role of a recently discovered PTX-insensitive G protein, G_q, in coupling the BK receptor to this cascade. We first studied the PTX-sensitivity of this action using the whole-cell patch-clamp technique (V_h -40 mV). After overnight incubation either with PTX (200 ng/ml, n=12) or with boiled PTX (n=7), pressure applied BK (10 nM) evoked an outward $I_{K(Ca)}$ of similar amplitude in both cases. We next perfused an antibody (AB) raised against the common C-terminal peptide of G_q and G_{11} inside the recording pipette. After 20-30 min of AB perfusion, BK evoked an outward $I_{K(Ca)}$ which was only 37% (n=7) of that evoked after perfusing preimmune IgG (n=19) or peptide-blocked AB (n=6; preimmune or peptide-blocked IgG did not affect BK responses). Finally, we examined the site of action of the AB by evoking the same $I_{K(Ca)}$ with pressure application of the calcium ionophore A23187 (20 μM). Under these conditions, intracellular AB perfusion (n=9) did not reduce the ionophore-evoked current compared to preimmune IgG perfusion (n=9). These data indicate that G_q and/or its homologs couple the BK receptor to the activation of $I_{K(Ca)}$ through activation of PLC.

M-PM-A2

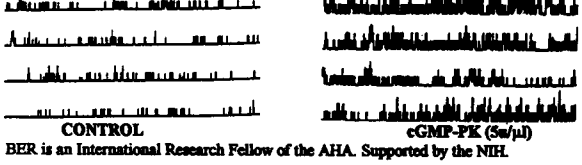
NIFLUMIC ACID ACTIVATES LARGE CONDUCTANCE K_{Ca} CHANNELS.
((L. Toro, M. Ottolia, R. Olcese and E. Stefani)) Dept. Molecular Physiology & Biophysics. BCM. Houston, TX 77030

Large conductance K_{Ca} channels are important modulators of smooth muscle contractility. Their blockade produces depolarization and contraction of several smooth muscles. Conversely, their activation should lead to hyperpolarization and relaxation. Thus, the finding of drugs that selectively open this type of K channels is of significant pharmacological and therapeutical relevance. We found that niflumic acid {(trifluoro-methyl-3-phenylamino)-2-nicotinic acid}, a drug known to inhibit Ca-activated Cl channels, activates large conductance K_{Ca} channels in a dose dependent manner. Niflumic acid (20 μM) greatly increased K_{Ca} channel open probability from coronary smooth muscle incorporated into lipid bilayers. When niflumic acid was added to the external side of the channel its action was immediate; however, when the drug was added to the internal side the effect was less potent and required much more time to take place. This suggests that niflumic acid is capable to diffuse through the lipid environment and reach its site of action on the external side. The niflumic binding site is not located in the conduction pathway since high internal K could not "knock out" its action and the fast blockade induced by external TEA was not prevented by the drug. The opening of K_{Ca} channels by niflumic acid seems to be specific since activation of other K channels (DRK1 and Shaker H4) was not observed. Analogs of niflumic acid were tested; flufenamic acid {2-{{3-(trifluoromethyl)phenyl]-amino]benzoic acid} mimicked its action, while mefenamic acid {2-[(2,3-dimethylphenyl)amino]-benzoic acid} did not at equivalent concentrations. In conclusion, niflumic acid is an activator of large conductance K_{Ca} channels and an important pharmacological tool to determine the importance of these channels in cell function. Supported by grants HL47382, HL37044 and AHA-Natl. Center 900963.

M-PM-A3

NITRIC OXIDE (NO) AND cGMP-DEPENDENT PROTEIN-KINASE ACTIVATION OF LARGE CONDUCTANCE Ca^{2+}-ACTIVATED K^+ (K_{Ca}) CHANNELS IN RABBIT CEREBRAL ARTERY SMOOTH MUSCLE CELLS.
((Blair E. Robertson, Rudolf Schubert, Jürgen Hescheler and Mark T. Nelson)). Univ. of Vermont, Dept. of Pharmacology, Medical Research Facility, Colchester, VT 05446.

K_{Ca} channels help regulate arterial tone (Brayden & Nelson; Science, 256, 532-535 1992). We tested the hypothesis that arterial dilation to NO may involve activation of K_{Ca} channels. Single K_{Ca} channels in cell-attached patches from rabbit basilar artery smooth muscle cells were activated approx. 2 fold following application of 50 μM of the NO donor SIN-1 to the bathing solution. Furthermore, a membrane permeable analogue of cGMP (8PCPT-cGMP, 400 μM) also increased channel activity in cell-attached patches by approx. 3 fold. In inside-out patches, internally applied cGMP-PK (5u/μl) had no effect on its own, but in the presence of ATP and cGMP it activated the channels 9 fold (Fig. 1). ATP and cGMP also had no individual effects, suggesting cGMP activates the kinase leading to ATP hydrolysis and ultimately channel phosphorylation. These results suggest that activation of K_{Ca} channels by cGMP-PK may be involved in arterial relaxation to NO.
Fig. 1. (Conditions: Membrane Potential; + 10 mV. t = 10s. unitary current = 6.5 pA)



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M-PM-A4

IN THE ABSENCE OF EXTRACELLULAR CALCIUM, ADENOSINE (ADO) AND RELATED PURINES DECREASE THE OPEN PROBABILITY OF BK CHANNELS FROM BASILAR ARTERY SMOOTH MUSCLE.
((G. Alexander West and J. Marc Simard)) UTMB, Galveston, Texas 77550 and Univ. Washington, Seattle, WA 98104.

Purine compounds, notably ADO and ATP, have significant effects on the cerebral vasculature. We studied the effects of ADO, ATP and related compounds on large conductance Ca^{2+}-activated (BK) channels in isolated basilar artery smooth muscle cells (n=43). Whole cell, cell-attached, and inside-out patches were studied. In the absence of extracellular Ca^{2+}, ADO produced a dose-dependent decrease in macroscopic I_{BK}, with an IC_{50}=5 μM. Analysis of single BK channels in the whole cell mode showed a dramatic reduction of $N \cdot P_o$, whereas single channel conductance was minimally reduced (see Figure). In contrast, there was no significant effect on BK single channel activity in either cell-attached or inside-out patches. There was no effect of intracellular Ca^{2+} on the response. Similar results were obtained with ATP, GTP, and inosine. Pinacidil was without effect on macroscopic I_{BK}. In the presence of 1.8 mM Ca^{2+} extracellularly, the effect of ADO (5-100 μM) on I_{BK} was completely eliminated. These results suggest that there is an extracellular binding site for Ca^{2+} that is important in the regulation of BK channels and in conferring sensitivity to ADO and related purines. These results may have importance for the regulation of BK channel activity in general.