Instituto Juan March
de Estudios e Investigaciones

88

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Structure and Mechanisms of Ion Channels

Organized by

J. Lerma, N. Unwin and R. MacKinnon

R. Aldrich
S. Choe
Y. Fujiyoshi
J. E. Gouaux
K. Keinänen
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R. H. Spencer
D. L. Stokes
N. Unwin
A. Villaroel
B. A. Wallace
M. Yeager
W. N. Zagotta

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Introduction

N. Unwin, J. Lerma and R. MacKinnon

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We met in November 1998 to discuss how ion channels are designed and work. Most meetings on ion channels over the last ten years or so have centred around biophysical measurements at the single molecule level and site-directed mutagenesis experiments combined with electrophysiological study of function. And powerful though these techniques may be, the amount of clear-cut information obtained is limited compared with the amount we can learn from viewing three-dimensional structures directly. What set this channel meeting apart from the previous ones is that there was a shift in focus, with structure obviously taking centre stage. Indeed, the meeting seemed to portend a new era of ion channel investigation in which we will be obtaining fresh insights simply by examining the new three-dimensional structures and relating their specific features to their specific functional roles.

Ion channels are integral membrane proteins. Their transmembrane character, and the fact that cells do not in general make many channels, have in the past represented obstacles too severe for any structures to be solved. But several strategies have been developed successfully over the last few years to circumvent the problems of hydrophobicity and availability in small quantities. One is to work with bacterial homologues of the usually more complex eukaryotic protein, and make use of bacterial expression to obtain the large amounts of protein required for crystallisation trials and eventual X-ray structure determination. It was this approach that led to our first high resolution picture of an ion channel, the K^+-selective ion channel of *Streptomyces lividans*, and next, to the structure of the pentameric mechanosensitive channel from *Mycobacterium tuberculosis*. Another strategy has been to engineer soluble parts of the channel protein, which are more tractable experimentally, and gain from them valuable information about critical functional regions. The crystal structures of the ligand-binding core of the glutamate receptor, the cytoplasmic tetramerisation domain of the voltage-gated K^+ channel and an amino-terminal part of the Herg K^+ channel provide elegant examples based on this approach. A third strategy has been to determine the whole structures by electron microscopy of frozen crystalline sheets or tubes, taking advantage of the propensity of channels to organize in two-dimensions – as in the lipid bilayer – rather than three. Resolutions approaching the atomic level are now being obtained from aquaporin and the nicotinic ACh receptor by this means.

Many of us marvelled not only at the rapid progress made over the last two years, but also at the diversity of structures invented by Nature to transport ions (and small molecules) rapidly and selectively across the membrane. The FhuA protein somehow makes use of a globular plug, enveloped by a β–barrel, to translocate ferric ions across the membrane; the homologous Ca^{2+}– and H^+– ATPases each use ten membrane-spanning α-helical segments, arranged in a complex bundle, to pump their respective ions across the membrane; aquaporin is a tetramer in which each subunit makes a transmembrane pore, lined by hydrophobic and polar side-chains, specifically for water molecules to pass through;

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gramicidin is a helical dimer extending across the bilayer, in which the carbonyl groups of the polypeptide backbone, rather than the side-chains, face inwards creating a central cation-selective pore; the gap junction channel is a hexamer of protein subunits, each essentially a four-helical bundle, delineating a central non-specific pathway for the ions.

Several basic principles underlying the structure and operation of ion channels were illuminated beautifully at the meeting through the details of the structures which have just been determined. In the case of the K^+ channel, for example, the K^+ selectivity over Na^+ is explained by the presence along the 4-fold axis of a 12Å long filter lined by backbone carbonyl groups which are geometrically constrained so that a dehydrated K^+ ion fits with good coordination but a Na^+ ion is too small. Also in this structure, helix dipoles and a water-filled cavity overcome the electrostatic energy barrier facing an ion in the membrane interior; on the other hand, such features are not apparent in the aquaporin pore, which is designed to prevent the transport of ions – i.e. destabilize their presence in the membrane interior – and only let water molecules through.

Another major theme of the meeting was the molecular mechanisms of ion channels: we ultimately need to understand how ion channels are regulated by ligand-binding and/or by changes in membrane potential. Essential insight into these processes is now emerging from several kinds of biophysical technique. Site-directed spin labelling and EPR spectroscopy is shedding light on the nature of the structural rearrangements underlying gating of the K^+ channel. Small-angle X-ray scattering is telling us about the conformational changes that take place on the binding of glutamate to the glutamate receptor ligand-binding domain. Combined mutational and electrophysiological studies are providing valuable new information about the conformational changes and the subunit interactions involved in a number of channel types, including K^+ channels, cyclic nucleotide-gated channels, glutamate and NMDA receptors. And finally, experiments done on kainate receptors emphasise the importance of functional studies, which link channel mechanisms with other cellular processes and place channels properly in the context of the living organism.

This meeting involved only a small group of individuals. But the amount of fundamental new information presented in the talks and the posters, and also the excitement and discussion they inspired, was amazing. Substantial advances are taking place now in the ion channel field. We can realistically look forward to viewing in the near future several more ion channel structures. Some of these might reveal more complex (and unsuspected) architectures and others might illuminate structural principles underlying the physiological events. What is certain is that all will deepen significantly our understanding of how ion channels are designed and work.

N. Unwin, J. Lerma and R. MacKinnon

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Session 1: Structural basis of membrane transport

Chair: Jurg P. Rosenbusch

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Domain structure of membrane proteins

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The folding patterns of membrane proteins currently comprise three types: 1. The alpha-helical bundles. We have crystallized bacteriorhodopsin into 3D-crystals using lipidic cubic phases. This yielded a structure to a resolution of 2.4Å, including the position of several water molecules in the proton pathway. 2. The beta-barrels, for which bacterial porins are the prototypes. Unspecific porins (OmpF from E. coli) allow an evaluation of the effects of charge distribution and insight into the mechanism of channel closing. Specific porins (maltoporin) yields a solute pathway the structure of which helps to design mutants, and to interprete the results of noise analysis. 3. Mixed structures. The solution to 2.7Å of a ferric ion translocator (the FhuA-protein), with or withjout the siderophore ferrichrome as ligand, reveals three domains: a beta-barrel, containing a globular plug that cloggs the channel, and a disordered N-terminal segment of 18 residues. The plug domain contains five short alpha-helices and six beta-strands, four of which form a beta-sheet (slightly tilted relative to the membrane plane). This protein is ligand-gated, with the small, local conformational change at the binding site (on the extracellular face of the membrane) transmitted and amplified to the periplasmatic face. The significant allosteric change in that location apparently predisposes the FhuA-protein to interact with the energy-transducing complex (TonB-protein). This protein may be viewed as paradigm of ligand-gated channel proteins.

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Cryoelectron Microscopy of the ATP-dependent Calcium Pump from Sarcoplasmic Reticulum

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The calcium pump from sarcoplasmic reticulum (Ca^{2+}-ATPase) is an archetype for the family of P-type ion pumps. It uses ATP to sequester calcium within the sarcoplasmic reticulum membrane, release from which is responsible for initiating muscle contraction. Other well studied members of this family are Na^{+}/K^{+}-ATPase from most eukaryotic plasma membranes, H^{+}-ATPase from fungi, H^{+}/K^{+}-ATPase from the gastric mucosa and Kdp from *E. coli*. More recently, a large subfamily has been identified that is responsible for transport of various metals, such as copper, mercury and cadmium, presumably by a similar reaction mechanism (1). The hallmark of this mechanism is formation of a covalent phosphoenzyme as a reaction intermediate, which is used to harness the energy of ATP for a conformational change. This conformational change is thought to be rather large and to affect the transport of cations across the membrane. Similarly, calcium binding at transport sites induces a conformational change which activates the nucleotide binding sites for nucleophilic attack on ATP, thus producing the phosphoenzyme (2). So far, no crystals have been produced that are suitable for x-ray crystallography, so we have been using electron microscopy to characterize the structure and to try to relate it to the mechanism of ion transport. In particular, we have two types of crystals, which we believe represent conformations before and after calcium binding, based on the conditions used for crystallization (i.e., the requirement for saturating calcium concentrations in one case and for the absence of calcium in the other case). Our ultimate goal is to solve structures from both types of crystals and thus to try to understand how calcium binding at transport sites can effect the nucleotide site that is believed to be 40-50 Å away.

The first step in this goal has been to study tubular crystals that form in the absence of calcium within the native sarcoplasmic reticulum membrane. These are essentially two-dimensional, membrane-bound crystals that roll up into a tubular structure, thus producing helical symmetry. These tubes have been imaged in their frozen-hydrated state and helical reconstruction methods have been used to determine their structure at 8 Å resolution (3). Ten helices were fit to the transmembrane domain together with 4 helices in a "stalk" that connects the transmembrane domain to a cytoplasmic head where ATP binding is thought to occur. Assignments for these transmembrane and stalk helices in relation to the amino acid sequence were deduced by considering a wide variety of experimental evidence, such as site-directed mutagenesis, cysteine crosslinking, residue variability, and physical connectivity within the 3D map. In addition, a pathway was observed that could provide aqueous access from the lumenal side of the membrane to the putative calcium binding sites in the middle of the membrane. More recently, we have expanded our interpretation of this map. First, we have solved an independent structure from reconstituted Ca^{2+}-ATPase at a similar resolution and have compared the details of the density distribution to assess the reliability of our previous efforts at helix fitting and identification of helix connectivity. The details of the two structures agree exceedingly well and completely support our original proposals. Second, we have built an atomic model for the transmembrane helices involved in calcium binding, using helical axes defined by the density maps and side-chain orientations suggested by site-directed mutagenesis, by cysteine

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crosslinking, and by residue variability. This model makes very specific predictions for the path of calcium ions through the protein and for residues involved in helix-helix packing, thus suggesting further site-directed mutagenesis and cysteine crosslinking experiments. Third, we have attempted to identify the three domains that compose the cytoplasmic head of the molecule. These attempts have been aided by a recent association between the family of P-type cation pumps and a broad family including haloacid dehalogenases, phosphoserine phosphatases, and phosphomanomutases (4). All of these enzymes proceed by nucleophilic attack of an aspartyl residue on their substrate, resulting in a transient covalent bond (analogous to a phosphoenzyme) during the reaction cycle. These homologies in function and in sequence suggest that a similar fold is employed, thus providing a putative atomic structure for the phosphorylation domain of Ca^{2+}-ATPase. Finally, we have obtained preliminary view of the conformational change induced by calcium binding to Ca^{2+}-ATPase. This comes from a projection map based on our second crystal form grown in the presence of saturating calcium concentrations (5). The projection map reveals large rearrangements of the cytoplasmic part of Ca^{2+}-ATPase, which is broadly consistent with the cytoplasmic densities revealed in a recent structure of H^{+}-ATPase from *Neurospora* (6). Based on this preliminary view, we propose a particular rearrangment of cytoplasmic domains that may be responsible for this conformational change and thus for activation of the nucleotide site by calcium.

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Structure of the plasma membrane H+-ATPase at 8 Å resolution

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Determining the structure of membrane proteins at any level of detail is still a difficult challenge, because crystals are an essential prerequisite. 2D crystals of membrane proteins require less material than 3D crystals and often form more readily. 2D crystals are perfect specimens for electron crystallography. This is an especially promising technique for the analysis of large and complex eukaryotic membrane proteins which do not yield highly ordered 3D crystals readily.

We have used electron crystallography to determine the structure of the plasma membrane H+-ATPase from Neurospora crassa. 2D crystals of the stable, detergent-solubilized hexamer of the enzyme were grown by a new method directly on the carbon support film, without reconsitiution into lipid membranes. Images of 2D crystals were recorded by electron-cryomicroscopy and processed, yielding a 3D map of the ATPase at 8Å in-plane resolution.

The H+-ATPase consists of a single ~100 kd polypeptide chain. The 3D map shows a large cytoplasmic region, comprising ~75% of the protein, connected at several sites to the membrane domain. The membrane domain contains 10 membrane spans, arranged in a bundle with a right-handed twist. Within the cytoplasmic part, 4 major domains were distin-guished. Comparison of the H+-ATPase map with that of the related Ca-ATPase (see abstract by D. Stokes) suggests that the cytoplasmic domains undergo a substantial conformational change as a result of ligand binding.

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Structure of a water channel, aquaporin-1

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The existence of water channels has been postulated almost half a century ago, because the lipid bilayer cannot account for osmotically driven water flow observed in red blood cells [1]. This activity exhibits mercury sensitivity and an erythrocyte integral membrane protein of relative molecular mass 28 kD is the first member of the growing major intrinsic protein (MIP) family with a demonstrated water channel function, and is termed aquaporin-1(AQP1) [2]. Its propensity to form two-dimensional (2D) crystals upon reconstitution in lipid bilayers has promoted electron microscopic analyses. We have reported the 3D structure of AQP1 at 6 Å resolution [3]. Higher-resolution was pursued and a three-dimensional map of AQP 1 at 3.5 Å resolution was analysed from 107 images and 92 electron diffraction patterns based on electron crystallography. The AQP 1 has six hydrophobic sequence stretches that suggest six trans-membrane helices which form a right handed helical bundle. The MIP family share the signature of a repeated NPA (Asn-Pro-Ala) motif in the two most prominent loops. From opposite membrane sides, two loops containing the NPA motifs projected into the membrane inside surrounded by the six trans-membrane α-helices. A short α-helix was formed in each of the two loops whose contrast was indicated by 'X' in our previous paper [3]. The two short helices faced together with at around center of the membrane surrounded by the six long helices inside of which the water channel was located. This structure of AQP 1 will give insight into mechanism of water channeling by which large water volumes pass across specialized membranes in plant, invertebrates and vertebrates, amounting to hundreds of liters per day in humans.

This study was performed in collaboration with T. Hirai, K Murata, K. Mitsuoka, T. Walz, J. B. Heymann, B. L. Smith, P. Agre and A. Engel

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Session 2: Ligand gated ion channels I

Chair: Eric Gouaux

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A biophysical analysis of GluR ligand binding

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Synaptic communication between neurons occurs when neurotransmitters released by one cell are recognized by appropriate receptors on another. In the central nervous system, glutamate receptors (GluR) are the predominant mediators of excitatory synaptic signalling. GluR appear to be structurally distinct from the acetylcholine receptor family, which includes most ligand-gated neurotransmitter receptors.

The GluR contain an interrupted domain with weak homology to bacterial periplasmic binding proteins, which trap their ligands between two lobes (the "Venus flytrap"). Expressed as a soluble fusion protein, this domain has been shown to reproduce the pharmacology of the intact GluR, at least for members of the AMPA receptor subfamily. Unlike the PBP, however, the GluR ligand-binding domain does not close down upon agonist, as monitored by small-angle X-ray scattering. Furthermore, when the peptides connecting the ligand-binding domain to the transmembrane segments are included in the construct, the resulting protein is highly elongated (axial ratio ca. 3:1), indicating that the transitional sequences are accommodated at the two ends of the molecule, distant from the putative ligand-binding site. A kinetic analysis of agonist binding to the soluble domain shows, however, that association and dissociation rates are comparable to those of the PBP, both qualitatively and quantitatively. As a result, we propose that the GluR ligand-binding domain has adopted a mechanism in which high affinity is achieved without substantial cleft closure, similar to that seen with the maltodextrin-binding protein. A disulfide bridge that is conserved across GluR subfamilies, and that is implicated in redox modulation of NMDA receptor affinity, may be responsible for inhibiting cleft closure. These findings have implications for the coupling of agonist binding and channel gating and desensitization.

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Recombinant Expression of a Soluble Ligand Binding Domain from Glutamate Receptor Subunit GluR6

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Our knowledge of the structure of ionotropic glutamate receptors is currently extremely limited. While a consensus topological model with three transmembrane domains and a re-entrant channel lining loop is now generally accepted, other questions, such as the receptor stoichiometry, remain contentious. In the absence of an atomic resolution structure of an entire glutamate receptor, the best chance for a structure lies in the expression of receptor domains, as illustrated recently for the potassium channels. The ligand binding domain of ionotropic glutamate receptors is a good candidate for such work, as two non-contiguous domains, S1 and S2, have been shown to be sufficient and necessary for ligand binding. When joined by a short linker, S1S2 polypeptides from the GluR2 and GluR4 subunits have been shown to be soluble, and to bind the appropriate ligands.

We have similarly overexpressed the agonist-binding domain of the kainate-selective subunit GluR6 as a soluble polypeptide. The construct was generated in the baculovirus expression vector pFASTBac, and consisted of the signal peptide from GluR6 to direct expression to the culture media, the S1 and S2 regions separated by a short linker peptide, and a hexa-histidine sequence added to the C-terminus to aid purification. The resulting GluR6S1S2 polypeptide is soluble, and runs as a monomer in gel filtration. The protein binds [^{3}H]-kainate with nanomolar affinity, and this binding is displaced by the kainate receptor ligands CNQX, Domoate and Quisqualate, but not AMPA. The pharmacology is similar to that seen for recombinant GluR6 homomers expressed either in HEK293 cells or insect cells. The addition of tunicamycin reduces expression levels significantly, but the resulting polypeptide lacking sugar residues is soluble, and still binds to kainate receptor ligands. Using a combination of affinity and ion-exchange chromatography we are able to purify several milligrams of the GluR6 S1S2 protein per litre of culture medium. These results demonstrate that the ligand-binding site of GluR6 is completely contained within subunits and that oligomerisation is not necessary for binding.

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MOLECULAR DETERMINANTS FOR AMPA RECEPTOR DESENSITIZATION.

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Desensitization of the a-amino-3-hydroxy-5-methly-4-isoxazole-propionate (AMPA) subtype of ionotropic glutamate receptors is thought to shape synaptic responses and act as a neuroprotective mechanism at synapses in the central nervous system. Desensitization at the molecular level is poorly understood, however. In this study, we have analyzed the kinetic properties of chimeric receptors in which regions of the AMPA receptor subunit GluR3 are exchanged for the corresponding regions of the kainate receptor subunit GluR6. Glutamate receptors and chimeras were transiently expressed in HEK293 cells and desensitization was measured by application of 10 mM glutamate onto outside-out patches using a rapid solution exchange system.

Replacing the binding domain S1 of GluR3 with the corresponding S1 of GluR6 resulted in a fully active, but completely nondesensitizing receptor. Backsubstitutions of GluR3 sequences revealed three regions within S1 that modified desensitization properties. Mutations in a region near the first transmembrane domain resulted in partial block of desensitization. Another region in the N-terminal part of S1 reduced the rate of resensitization. Thirdly, a single amino acid exchange in vicinity of residues involved in glutamate binding, completely blocked the desensitization. Mutation at the same position had an identical effect on other AMPA receptors. Further mutations indicate that this site interacts with residues either outside S1 or with neighboring subunits. The close spatial arrangement of residues involved in binding and gating provides a structural link between these two events.
Supported by BSF (Y.S-B.) and a Helmholtz fellowship (C.R).

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The predicted structure of the ligand-binding domain of glutamate-receptor channels

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Receptors for neurotransmitters have long been recognized as major targets for drug action. Yet, in all cases, the drugs available today were developed on the basis of extensive structure-activity studies without any detailed knowledge of the three dimensional (3D) structure of their receptor targets. As these receptors harbour membrane-spanning segments in addition to their large hydrophilic ligand-binding and cytoplasmic domains, none of them has successfully been crystallized so far.

Nevertheless, over the last decade, information regarding the primary sequences of many proteins has been accumulated. Detailed analyses of this information with the aid of the advanced computational technology led to the recognition that large proteins and in particular integral membranous receptors may be composed of individual domains with autonomous protein folding pattern. In addition, the availability of an ever growing number of protein structures, resolved at the atomic level, have allowed the elucidation of the tertiary structures of protein domains by homology protein modelling. Attracted by this novel opportunity and by the subtle similarities in sequence patterns identified between glutamate receptors and bacterial periplasmic substrate-binding proteins (PBPs), we and other research groups have undertaken to identify residues involved in ligand binding to glutamate-receptor channels (ionotropic GluR; iGluRs). The latter being crucial actors in brain physiology and pathology.

We embarked on a study that included: (1) photoaffinity labelling experiments and (2) an interplay between computer-assisted molecular modelling and comprehensive site directed mutagenesis followed by ligand-binding measurements. Consequently, we identified subsets of amino acids accounting for the binding free energies and specificities of the chick kainate binding protein (cKBP, a member of the iGluR family) for two agonists, glutamate (Glu) and kainate (KA) and the specific competitive antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). We also identified an extracellular motif involved in the binding of GTP to cKBP and showed that GTP competitively antagonizes electrophysiological responses elicited in *Xenopus* oocytes expressing recombinant N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptors. A 3D structural model was elaborated based on the templates available from the X-ray co-ordinates of two PBPs, the lysine/arginine/ornithine- and phosphate-binding proteins. This model structure delineates the microarchitecture of the predicted KA/Glu-binding pocket that is engulfed by two PBP-like globular lobes. The model structure is coherent and in agreement with the experimental results. Moreover, the residues found to be located at the predicted ligand-binding pocket of the cKBP model structure and that were implicated in ligand binding based on the mutagenesis studies are homologous with residues implicated in ligand recognition by other iGluRs such as the NMDA receptor subunits NR1, NR2A and NR2B, the AMPA receptor subunit iGluR1, and the kainate receptor subunit iGluR6.

A hypothetical dynamic model that may correspond to the molecular mechanism that couples PBP-like movements of the ligand-binding lobes to channel activation and desensitization of iGluRs will be presented.

Instituto Juan March (Madrid)

Extracellular Domains of the AMPA Receptor

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Ionotropic glutamate receptors are oligomers of homologous subunits of 900-1500 amino acid residues. The N-terminal 550 residues and the 150-residue region between two membrane-associated segments, M3 and M4, are extracellularly located. The M3-M4 segment (known as S2) and the 150 N-terminal residues adjacent to M1 (S1) form a bipartite ligand-binding site, whereas the functional role of the N-terminal 400 residue segment ("X domain") is currently unclear. In order to gain insight into the respective functions of these domains, we have expressed and purified recombinant proteins representing the entire ectodomain (XS1S2), and the separate X domain and S1S2 ligand binding domain of the GluRD AMPA receptor. In filtration binding assay, both S1S2 and XS1S2 fusion proteins bind [^{3}H]AMPA with a high affinity and show very similar ligand-displacement pharmacology, indicating that the presence (or absence) of the X domain has either no or only minor effects on ligand binding. The separately produced X domain does not bind radiolabeled AMPA or L-glutamate, but may fold to a native-like structure as suggested by its binding to a conformation-specific monoclonal antibody. In gel filtration and density gradient centrifugation, S1S2 and X behave as monomers and XS1S2 as dimers. Our results are consistent with a modular structure for ionotropic glutamate receptor subunits, and should facilitate more detailed analysis of their structural domains.

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"Biochemical and Crystallographic Studies of Glutamate Receptor Ligand Binding Regions".

Eric Gouaux, Columbia University, New York.

"Ionotropic glutamate receptors are important mediators of synaptic transmission in the vertebrate nervous system and are grouped according to their response to the agonists AMPA, kainate and NMDA. Based on the analysis of the amino acid sequence of iGluRs, along with a large number of genetic, biophysical and molecular modelling studies, it has been demonstrated that the ligand binding region of the iGluRs is composed of two discontinuous segments of the receptor polypeptide, the S1 and S2 regions. In an effort to determine relationships between iGluR structure and function, we have undertaken biochemical and crystallographic studies. Here we report the structure of the ligand binding core of the GluR2 receptor in complex with the agonist kainate."

Instituto Juan March (Madrid)

Session 3: Potassium channels

Chair: Roderick MacKinnon

Instituto Juan March (Madrid)

Molecular Basis of K^+ conduction and Selectivity in Potassium Channels.

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Potassium channels are proteins that control the passive flow of K^+ ions across cell membranes. This K^+ flow is necessary for producing electrical signals in the nervous system, for governing the rate at which our hearts beat, and for the secretion of specific hormones such as insulin. There are many different kinds of K^+ channels but they are all built on a common theme; all have four identical subunits surrounding a central membrane-spanning pore. The pore is ion selective, ensuring that only K^+ (radius 1.33 Å) and not Na^+ (radius 0.95 Å) is permitted to enter and cross the cell membrane. The structure of a K^+ channel from the bacterium *Streptomyces lividans* has been determined using data to 3.2 Å resolution. The K^+ channel has the appearance of a basket of α-helices surrounding a central pore that is coincident with a molecular four-fold rotation axis. The central helices of the basket hold the selectivity filter near the extracellular surface of the cell membrane. This filter is 12 Å long, is comprised of main chain atoms, and contains two ions in it. We propose that selective ion coordination is similar to that occurring in valinomycin and nonactin, but that close spacing of two ions in the filter allows for mutual repulsion and therefore a high conductance. Below the selectivity filter, at the center of the membrane, the pore dilates to create a cavity large enough to hold 60 to 100 water molecules. The cavity, together with four helices oriented with their C-termini directed toward the cavity center, can overcome the dielectric barrier facing a cation at the membrane center. These findings help to further our understanding of ion conduction through K^+ channels.

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STRUCTURAL REARRANGEMENTS UNDERLYING ACTIVATION GATING IN THE *Streptomyces* K^+ CHANNEL

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The structure of the *Streptomyces lividans* K^+ channel is now known at atomic resolution (Doyle et al., Science 280:69). While careful analysis of this three-dimensional model has established the structural basis for permeation and selectivity in K^+ channels, the picture is less clear regarding the molecular basis of gating. We have pursued a systematic exploration of the structural properties of KcsA using site-directed spin labeling and EPR spectroscopy, looking to detect and characterize conformational changes associated with channel opening. Initial functional studies have shown that lowering the external pH promotes channel opening (Cuello et al, Biochemistry 37:3229), and that a large conformational change consistent with an opening of the internal vestibule of the channel can be detected at selected positions of the internal transmembrane helix TM2 (Perozo et al., Nature Struct. Biol. 5:457). Here, we extended these results by comparing the EPR signal from spin-labeled KcsA cysteine mutants obtained at neutral or acidic pH. Three separate portions of the channel were studied: TM1 (residues 26-49), TM2 (residues 90-120), and residues flanking the selectivity filter (external residues 81-83, internal residues 72-74). The mutants were labeled with an MTS spin label and X-band CW EPR spectra were obtained from liposome-reconstituted channels at room temperature, using a loop-gap resonator. Secondary structure information was obtained from Fourier-transform periodicity analysis of position-specific environmental properties: probe mobility (ΔHo^{-1}) and O_2 and NiEdda accessibility parameters (ΠO_2 and ΠNiEdda). For each spin-labeled mutant, probe mobility, solvent accessibility and inter-subunit spin-spin coupling were determined at pH 7 (closed state) and pH 3.5 (open state). Large conformational changes were detected on TM2 (particularly its C-terminal end) and to a lesser extent in TM1, while the pore regions show little change in its extracellular portion. Overall, these results are consistent with both rotational and translational movements in the transmembrane helices of the channel, and point to the cytoplasmic face of the channel as the site of the main open-closed conformation. (Supported by NIH grants GM54690, GM57846 and The McKnight Endowment Fund).

Instituto Juan March (Madrid)

Conformational Changes and Subunit interactions in ion channel gating.

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Voltage-gated ion channels and their relatives, such as cyclic-nucleotide activated channels, are functional tetramers of identical or similar subunits. Gating of these channels involves a high degree of cooperativity between the conformational changes in the subunits. We have investigated the nature of subunit cooperativity in gating in the calcium and voltage gated potassium channel slo1. Calcium binding and positive voltage changes both increase the probability of channel opening, with a complex interaction between calcium and voltage effects, when determined macroscopically. Higher calcium concentrations shift the voltage-dependence of opening to more negative voltages with little change in the slope of the open probability versus voltage relationships. More positive voltages increase both the apparent affinity and Hill coefficients of the open probability versus calcium relationships. The channels can be activated by membrane voltage in the absence of Ca-binding, indicating that channels contain an intrinsic voltage sensor. These relationships can be explained by an allosteric gating model where calcium binding and voltage-sensor activation independently affect the equilibrium between closed and open conformations. This model makes several predictions about the kinetics and voltage dependence of macroscopic ionic currents, single-channel currents and gating currents. We have tested these predictions quantitatively and have found such a model to provide an accurate description of gating. The model has allowed us to calculate the energetic effects of calcium binding and voltage sensor activation on channel opening. An allosteric model for voltage-dependent gating provides some unusual interpretations of gating behavoior. For example, the relationship between gating charge movement and channel opeing is not fixed, the charge versus voltage relationships are different for open and closed conformations. Whether a similar mechanism for gating of purely voltage-gated channels, such as Shaker potassium channels, remains to be determined.

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Tetrameric Assembly of Voltage-dependent K channels

S. Choe

The N-terminal, cytoplasmic tetramerization domain (T1) of voltage-gated K+ channels encodes molecular determinants for subfamily-specific assembly of channel subunits into functional tetrameric channels. Crystal structures of T1 tetramers from Shaw and Shaker subfamilies reveal a common four-layered scaffolding. Within layer 4 near the hypothetical membrane-facing side of the tetramer, the Shaw T1 tetramer contains four zinc ions near the T1 pore at the tetramer center. Each zinc is coordinated by a histidine and three cysteines, a HX5CX20CC sequence motif of which is highly conserved among all Shab, Shaw and Shal subfamily members. But it is never found in Shaker subfamily members. Zn and characteristic residues in the tetrameric subunit interface are crucial elements to govern the channel diversity through subfamily-specific tetramerization.

Instituto Juan March (Madrid)

Genetic Investigation of Potassium Channel Transmembrane Structure

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Inwardly rectifying potassium channels posses the simplest architecture of known potassium channels. These channels are comprised of tetramers wherein each subunit contains only two transmembrane domains linked by an extracellular region responsible for monovalent cation selectivity (the P-region). We have developed a system for investigating the amino acid tolerances of the transmembrane domains (designated 'M1' and 'M2') of the inwardly rectifying potassium channel IRK1. Yeast deficient for potassium transport (*Δtrk1,Δtrk2*) were used to screen for functional IRK1 sequences from libraries of IRK1 genes in which the transmembrane domains were subjected to extensive mutagenesis. A large number of functional sequences were isolated from these libraries. Analysis of the variation of allowed amino acid substitutions by Fourier methods suggests that M1 and M2 are indeed helical. Moreover, a clear segregation of allowed amino acid types occurs on the helical faces suggesting which regions of the helices are oriented towards protein-lipid, protein-protein and protein-water interfaces. We are currently using this genetic data in conjunction with computational modeling to derive a testable model of potassium channel pore structure.

Instituto Juan March (Madrid)

Session 4: Potassium and other ion channels

Chair: Senyon Choe

Instituto Juan March (Madrid)

The Structure of a Mechanosensitive Ion Channel Homologue (MscL) from Mycobacterium tuberculosis

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The ability to sense physical forces within our environment such as gravity, sound, or pressure is primarily mediated by a specialized class of membrane proteins known as mechanosensitive ion channels.

Although scant information is available on the molecules mediating mechanosensation in eukaryotes, the large-conductance mechanosensitive ion channel (MscL) from prokaryotes has been relatively well-characterized.

The MscL family is widely distributed among prokaryotes and may participate in the regulation of osmotic pressure changes within the cell.

In an effort to better understand the structural basis for the function of these channels, we have determined the structure of the MscL homologue from Mycobacterium tuberculosis by x-ray crystallography to 3.5 Å resolution. The channel is organized as a homopentamer with each subunit containing two transmembrane a-helices and a third cytoplasmic a-helix which assembles to form a helical bundle.

At the extracellular side, an ~18 Å diameter water-filled opening leads into a funnel-shaped pore lined with hydrophilic residues which narrows at the cytoplasmic side to an occluded hydrophobic apex that may act as the channel gate.

This structure may serve as a model for other mechanosensitive channels, as well as the broader class of pentameric ligand-gated ion channels exemplified by the nicotinic acetylcholine receptor.

Instituto Juan March (Madrid)

Structure/Function Studies on Polypeptide Ion Channels

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Polypeptides have been useful as model systems for studying the structure and functions of ion channels. This paper will discuss two particular systems examined in our lab, gramicidin and antiamoebin.

Gramicidin is a polypeptide antibiotic, consisting of 15 amino acids with alternating L- and D- amino acids, and is probably the best-studied ion channel to date in terms of its structure and function. It is a polymorphic molecule, forming several different types of dimeric structures, which have different functional properties. The helical dimer structures, known as the "channel" forms, have been defined by NMR spectroscopy, while the double helical structures, also known as "pores", have been characterized in several states (open, closed, blocked) by X-ray crystallography and NMR. The relationships between the different forms and how they interconvert have been well-characterized by CD spectroscopy. Comparisons between "mutant" molecules produced by peptide synthesis methods, have given insight into features important for its various functional properties. Most importantly, comparison of the crystal structure of the K^+/gramicidin complex with the recently solved crystal structure of the *S. lividans* K^+ channel, shows that despite the fact that gramicidin contains D-amino acids, both use the same type of motif for binding ions in their central pore: the carbonyl groups of their polypeptide backbones. Thus, this suggests that studies with this relatively simple model ion channel may provide useful information on ion channels in general.

Recently we have determined the crystal structure of a 16-residue peptaibol, antiamoebin I, which exhibits membrane-modifying activity, but does not haemolyze erthyrocytes. It is a helical molecule, which has a deep bend in the middle resulting from the presence of three imino acids. This bend is larger than has been found for any other pepaibol studied to date, and has important consequences for the dipole moment and length of the molecule. Functional studies have shown that antiamoebin can act as both a channel and carrier, perhaps the first instance of this dual functionality in a transport molecule. CD studies have shown that the crystal structure of this molecule is very similar to the structure it adopts in phospholipid bilayers and so has permitted us to propose model multimeric structures for both the channel and carrier forms.

In summary, ion channels with very different structures can be formed by polypeptides, and these have proved to be of considerable value in understanding the underlying principles of ion transport across membranes.

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Instituto Juan March (Madrid)

Three-Dimensional Structure of a Recombinant Cardiac Gap Junction Channel at 7.5Å Resolution by Electron Cryo-Crystallography

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Gap junction membrane channels directly connect the cytoplasms of adjacent cells and thereby mediate intercellular electrical and metabolic coupling. The principal gap junction protein in the heart is α_1 connexin (Cx43). Cardiac gap junctions play an important functional role by electrically coupling adjacent cells, thereby organizing the pattern of current flow to allow a coordinated contraction of the muscle. They are therefore intimately involved in the normal coordinated depolarization of heart muscle as well as cardiac arrhythmias causing sudden death.

Previous analyses of two-dimensional (2-D) crystals of gap junctions from endogenous sources were limited to 15-20Å resolution, presumably due to the existence of multiple connexins and different degrees of post-translational modification in gap junctions isolated from heart or liver tissue. To obviate these problems, a truncated form of the cardiac gap junction protein that lacks most of the large C-terminal domain (designated α_1Cx-263T) has been expressed in stably transfected BHK cells. Freeze-fracture and negative stain electron microscopy demonstrated that recombinant α_1Cx-263T assembles with the characteristic morphology of gap junctions and forms small 2-D crystals *in vivo*. The crystallinity was improved by extracting an enriched membrane fraction with Tween20 and DHPC. Image analysis of computed diffraction revealed that the best crystals were coherent over several hundred unit cells, and the hexagonal lattice manifested p6 plane group symmetry ($a = b = 79.4\pm0.3$Å with $\gamma = 119.8\pm0.2°$).

Electron cryo-microscopy and image analysis of frozen-hydrated 2D crystals yielded a projection map at 7Å resolution that displayed a ring of transmembrane α-helices that lines the aqueous pore and a second ring of α-helices in close contact with the membrane lipids. The distribution of densities allowed us to propose a model in which the two apposing connexons that form the channel are staggered by ~30°. Furthermore, apparent non-crystallographic twofold axes predicted that the two apposing connexons adopt identical conformations.

We have now recorded images of tilted, frozen-hydrated 2D crystals, and our current 3D map has an in plane resolution of ~7.5Å and a vertical resolution of ~25Å. To our knowledge this is the first example where structure analysis of a membrane protein has been accomplished using microgram amounts of recombinant starting material. The dodecameric channel is about 150Å in length with a diameter of ~65Å within the membranes and ~55Å in the extracellular gap. The intercellular channel is formed by the end-to-end docking of two hexameric connexons, each of which displays 24 rods of density in the membrane interior, consistent with an α-helical conformation for the four transmembrane domains of each subunit. The closely packed, transmembrane α-helices merge with a continuous ring and arcs of density in the extracellular gap. The extracellular vestibule is bounded by a continuous wall of protein that provides a conduit for intercellular communication and also forms a tight seal that is essential for excluding exchange of substances with the extracellular milieu. We anticipate that this molecular design will be a common folding motif for the family of gap junction channels.

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Instituto Juan March (Madrid)

THE VENUS FLYTRAP FUNCTION AND TETRAMERIC STRUCTURE OF AN AMPA RECEPTOR CHANNEL

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Studies carried out with the GluR1 subtype of AMPA receptor and on some of its bilobated ligand binding domain mutants suggest that the ligand-induced activation and desensitization of the receptor are processes tightly linked to the dynamics of the interlobe interactions. We have described these interactions metaphorically as a venus flytrap-like mechanism. I will briefly discuss this functional model as well as the data I. Mano and myself have obtained to propose that the quaternary structure of the AMPA receptor is tetrameric

Instituto Juan March (Madrid)

Session 5: Ligand gated ion channels II

Chair: Nigel Unwin

Instituto Juan March (Madrid)

ACETYLCHOLINE RECEPTOR: APPROACHING ATOMIC RESOLUTION.

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We are investigating the structure of the nicotinic acetylcholine receptor in the closed-channel form by electron microscopy of tubular crystals of Torpedo postsynaptic membranes embedded in amorphous ice. The analysis is being conducted on images recorded at 4°K with a 300 kV field emission source. The present resolution is about 4.6Å, making it possible now to see clearly the inter-chain periodicity of beta-sheet in some regions of the structure. We can also observe the main functional components of the ion channel with better definition than was previously possible at 9Å resolution. The results provide new insight into the molecular architecture of the receptor and physiological properties of the postsynaptic membrane.

Instituto Juan March (Madrid)

Molecular interactions in the gating of cyclic nucleotide-gated channels

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Cyclic nucleotide-gated (CNG) ion channels of retinal photoreceptors play a fundamental role in the response to visual stimuli by converting the chemical signal that the cyclic nucleotide concentration has changed into an electrical signal. The NH2-terminal domain and the COOH-terminal cyclic nucleotide binding domain of bovine rod CNG channels (BROD) have been shown to be involved in the allosteric transition, but the sequence of molecular events occurring during the allosteric transition is unknown. We have recorded single-channel currents from BROD channels in which mutations have been introduced in the binding domain at position 604 and/or the rat olfactory CNG channel NH2-terminal auto-excitatory domain has been substituted for the BROD NH2-terminal domain. Using a hidden Markov model (HMM) approach, we analyzed the kinetics of the allosteric transition in these channels and determined the effects on the energy profile for the allosteric transition for these mutant channels activated by cGMP, cIMP, and cAMP. We find that mutations at position 604 in the binding domain alter both rate constants for the allosteric transition, indicating that the interactions between the cyclic nucleotide and this amino acid are partially formed at the time of the transition state. In contrast, the NH2-terminal domain affects primarily the closing rates for the allosteric transition, suggesting that the state-dependent stabilizing interactions between NH2-and COOH-terminal domains form after the channel has switched to the activated conformation. We propose that the sequence of events that occurs during the allosteric transition involves the formation of stabilizing interactions between the purine ring of the cyclic nucleotide and the amino acid at position 604 in the binding domain followed by the formation of stabilizing interdomain interactions.

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A molecular switch for glycine-glutamate binding cooperativity in the NMDA receptor

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The NMDA ionophore-receptor complex has the unique property of requiring the simultaneous binding of two different agonists, glycine and glutamate, to open its intrinsic cationic channel. Although the binding sites for glycine and glutamate are segregated into the NR1 and NR2 subunits, respectively, there is a reciprocal influence on the apparent binding affinity of one ligand by the other agonist, indicating that both binding sites interact functionally. We have localized a region inserted into the proximal N-terminal S1 binding domain that plays a critical role in this allosteric coupling. Three dimensional molecular modelling indicates that this region is not involved in ligand binding and forms a solute accessible loop protruding out of the binding pocket, making it suitable for short-range interactions between adjacent subunits.

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Mutations within the pore affect the high-affinity zinc inhibition of NMDA NR1-NR2A receptors.

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We have recently shown (Paoletti et al., 1997, J. Neurosci. 17: 5711-5725) that Zn^{2+} ions specifically inhibit with a very high affinity recombinant NMDA receptors containing NR1 and NR2A subunits. This inhibition is voltage-independent and observed in the presence of saturating doses (100 μM) of the two coagonists glutamate and glycine. Zn^{2+} inhibiting effects therefore involve that they participate to a decrease of the efficacy with which agonist binding leads to NMDA channels gating, and identifying the molecular determinants of the Zn^{2+} inhibition should help understanding the molecular mechanism involved in the gating of ligand-activated channels. We have found that several mutations within the TM2 reentrant loop of the NR1 subunit (a structure assumed to participate to the selectivity filter in glutamate receptors) markedly affect Zn^{2+} inhibition of recombinant NR1/NR2A NMDA receptors. The Zn^{2+} inhibition is weakened by mutations at two positions (W590 and N598), whereas it is strengthened by mutations at two other positions (G594 and E603). These residues are presumably located either deep in the pore or in the inner vestibule of the channel, and it is very unlikely that they participate to the Zn^{2+} binding site. The phenotypes of the mutations rather point toward an involvement of the corresponding residues in the final steps of the gating process.

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THE KAINATE RECEPTOR: AN ION CHANNEL BECOMING A METABOTROPIC RECEPTOR

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Despite the identification of several subunits of the kainate receptors, these receptors have been rather elusive and their function remains enigmatic. To get an insight into the function of these receptors in brain physiology, we have carried out studies in cultured neurons and hippocampal slices. The onset of desensitization of native receptors is rapid while recovery is slow and agonist-dependent. In neurons, the concentrations for half activation and half inactivation differ by two orders of magnitude such that the maximum response to a maintained concentration of glutamate is small, and the dose response curve bell shaped. Under complete block of other glutamate receptors, kainate induces a reduction in the effectiveness of GABAergic synaptic inhibition. Kainate increased synaptic failures and reduced the frequency of miniature IPSC, indicating a presynaptic locus of action. *In vivo* experiments, using brain microdialysis, demonstrated that kainate reversibly abolished inhibition and induced an epileptic-like EEG activity. The results indicate that kainate receptor activation downregulates GABAergic inhibition by modulating the reliability of GABA synapses. We have found that regulation of GABA release by kainate receptors is independent of ion channel activity but involves the activation of Phospholipase C and Protein Kinase C through a Pertussis toxin-sensitive G-protein. In addition, it can be demonstrated that the two function modes of kainate receptors are independent. As ion channels, their activation depolarize the membrane, bringing the neurons to repetitively fire. This action remains after G-protein blockade by treating slices with Pertussis toxin (PTx). In contrast, the ability to modulate GABA release disappears in PTx-treated slices. These results provide strong support for the functional coupling of kainate receptors to a second messenger signaling cascade, in other words, for the metabotropic action of ionotropic glutamate receptors in the brain and indicate the existence of kainate receptors with different signaling mechanisms. One type would be located in the somato-dendritic compartment and act as ion channels. The other should localize to inhibitory presynaptic terminals and be coupled to a second messenger cascade. Both promote hyperactivity of hippocampal neurons and may be responsible for the strong epileptogenicity of kainate and related compounds.

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POSTERS

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Regulation by second messengers of human-*erg* K^+ channel gating after activation of phospholipase C-coupled receptors

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Modulation of K^+ channel function constitutes an important system for control of cell excitability. Regulation of an inwardly rectifying K^+ current constitutes a main control point of electrical activity in adenohypophysial cells (Barros et al. (1994) Pflügers Arch. 426, 221-230). Such a regulation is exerted by a phosphorylation mechanism specifically reverted by protein phosphatase 2A (Barros et al. (1993) FEBS Lett. 336, 433-439). Recent results from our laboratory indicate that the K^+ current is mediated by a human *ether-a-go-go*-related gene (HERG)-like K^+ channel (Barros et al. (1997) Pflügers Arch. 435, 119-129). By co-expressing HERG channels and phospholipase C-activating hormone receptors in *Xenopus* oocytes we found clear alterations in HERG activation and deactivation kinetics in response to receptor estimulation. Neither the inactivation nor the inactivation recovery rates were altered in the presence of receptor agonists. Acceleration of channel closing and slow down of activation at a given voltage is the dominant effect detected after hormonal treatment. These kinetic alterations are due to the operation of a protein kinase C-dependent phosphorylation pathway. However, opposite effects to those induced by protein kinase C activation were caused by increases in intracellular Ca^{2+} levels. It is concluded that stimulation of phospholipase C-coupled receptors can have a dual regulatory effect on HERG channel gating. This provides a mechanism for the physiological regulation of cardiac function and for modulation of adenohypophysial neurosecretion in response to this type of receptors.

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EXTRACELLULAR Ca^{2+} MODULATES THE αZ1 HOMOMERIC GLYCINE RECEPTOR FUNCTION.

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Calcium ions modulate the function of many cellular processes, including voltage-gated and receptor-operated ionic channels. In this study we investigated the effects of extracellular Ca^{2+} ($[Ca^{2+}]_o$) on the functional properties of the recently cloned αZ1 subunit of zebrafish glycine receptor (αZ1 GlyR) expressed in the human cell line BOSC23. Whole-cell recordings showed that the removal of $[Ca^{2+}]_o$ (i) inhibited glycine-activated Cl^- currents in a dose-dependent manner, reducing the apparent affinity for glycine 3-fold; (ii) depressed sensitivity of αZ1 GlyR to voltage. Increasing of intracellular Ca^{2+} buffer capacity (from 0.5 to 10 mM BAPTA) had no effect on the inhibition, suggesting that the cytoplasmic domain is not involved in this modulation.

Single channel analysis revealed that removal of $[Ca^{2+}]_o$ modulates gating kinetics inducing the appearance of multiple intraburst closures. The double mutation E191A-E192Q strongly diminished the effect of $[Ca^{2+}]_o$ on GlyR affinity, but did not interfere with the $[Ca^{2+}]_o$ modulation of voltage dependence. Single channel kinetics of mutated receptors in 2 mM $[Ca^{2+}]_o$ were similar to that of wild type in Ca^{2+}-free medium.

Our results suggest that extracellular calcium interacts with N-terminal domains of the αZ1 GlyR, through two functionally distinct sites: one involved in voltage-dependent modulation, the other coupled to ligand binding.

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Molecular determinants of ion permeation and agonist-gating in $GABA_A$ receptors.

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The $GABA_A$ receptor is an anion selective ligand-gated ion channel responsible for the majority of post-synaptic inhibitory currents in the central nervous system. It is a member of the Cys-loop superfamily, which includes the nicotinic acetylcholine receptor (AChR). There is good evidence that members of this superfamily are pentameric, with a central ion channel lined by the same face of M2 helices from each subunit. On this face of M2, polar hydroxylated residues at the 2' position (or "central ring") are significant determinants of conductance in AChRs and are thought to be in the narrowest region of the open channel.

To investigate the involvement of M2 polar sidechains in ion permeation through $GABA_A$ receptors, we have removed the polar groups by mutation of polar residues to smaller non-polar residues. In $GABA_A$ receptors composed of $\alpha 1$ and $\beta 1$ subunits, which lack polar residues at the 2' position, we found that the removal of all polar groups from the 6' position abolished ion channel activity, without affecting the level of assembled receptors in the plasma membrane. A polar sidechain at the 6' position of either subunit was sufficient for ion channel activity to be retained. In $\alpha 1\beta 1\gamma 2s$ $GABA_A$ receptors, however, the removal of polar groups from the 6' position in all subunits did not completely eliminate ion channel activity. The remaining channel activity was abolished by the further removal of a polar group at the 2' position, present only in the γ subunit. Ion channel activity, abolished by removal of all polar groups from the 6' position of $\alpha 1\beta 1$ $GABA_A$ receptors was restored by mutation of β(A2'S), adding a polar group at the 2' position. Taken together these data indicate that ion channel function requires polar sidechains at the 2' or 6' position, but a polar sidechain at either position and in any subunit type is sufficient to satisfy this requirement. The simplest explanation for this reqirement, consistent with earlier evidence that these residues line the channel, is that the polar sidechains interact directly with permeant ions and provide a vital step in the ion permeation pathway.

We have also carried out mutagenesis of conserved residues in M1 and the loop between M2 and M3. Several of these mutations eliminate agonist-activated channel activity without affecting agonist binding. These effects are strongly dependent on the subunit type mutated which, considering the location of these residues, is more consistent with a disruption of the coupling between agonist-binding and channel opening than a disruption of ion permeation directly. The major effect is caused by mutations in the α subunit, indicating that this region of the α subunit is particularly important in the coupling of agonist-binding to channel activation.

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BIOCHEMICAL CHARACTERIZATION OF NMDA RECEPTORS PRODUCED IN AN HETEROLOGOUS CELL SYSTEM USING VACCINIA VIRUS

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We have recently developed a highly efficient expression system based on infection/transfection of Human Embryonic Kidney 293 cells with vaccinia virus to produce functional N-Methyl-D-Aspartate (NMDA) receptors containing NR1 and NR2A subunits which sustain calcium influxes dependent on receptor agonists and inhibited by receptor antagonists. The entrance of calcium through these recombinant receptors induces delayed toxicity, demonstrated by approximately a three-fold increase in the number of dead cells obtained 12 hr after antagonist 2-amino-phosphopentanoic acid (DL-AP5) removal from the culture, and by more than 88% inhibition in the expression of a luciferase reporter gene observed 24 hr after DL-AP5 removal. Calcium toxicity can be completely abolished by specific antagonists of the NMDA receptor.

Subcellular fractionation and immunocytochemistry demonstrate that the recombinant receptors are properly arranged in membrane structures where NR1 and NR2A subunits colocalize. Physical interaction of the subunits has been demonstrated by immunoprecipitation of each subunit with antibodies specific for the other one. Pulse-chase experiments have shown a similar half-life of approximately 12 hours for both subunits, and a shift in the mobility of NR1 after *de novo* synthesis that is due to some modification in the carbohydrates of this protein. Preliminary results suggest that NR2A only interacts with the mature form of the NR1 subunit.

In experiments where NR1 or NR2A where expressed alone, we did not observed any difference in cellular localization, stability or maturation of the subunits compared to results obtained in cells transfected with genes coding for both proteins.

Treatment of cell extracts with N-glycosydase F showed that both receptor subunits are N-glycosylated. In our expression system, tunicamycin prevents calcium toxicity and protection is likely due to specific and very fast degradation of the NR1 subunit. We are currently characterizing other toxic and metabolic insults which, as tunicamycin, also perturb function of the endoplasmic reticulum (ER stress) to analyze if they similarly induce degradation of NR1. Data concerning the mechanism of NR1 proteolysis will be also presented.

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Design of a Synthetic, Sodium-Conducting Channel that Functions in a Phospholipid Bilayer Membrane

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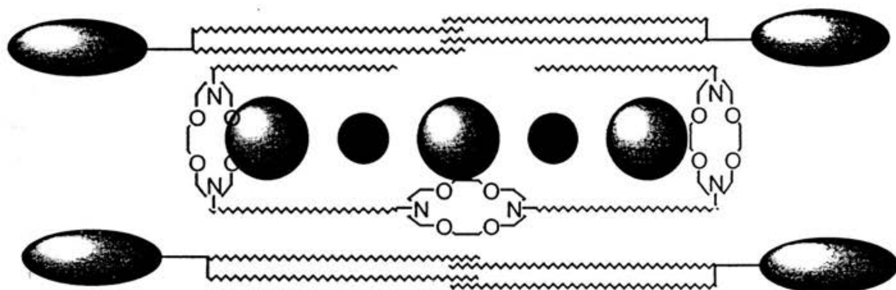
A family of tris(macrocycle)s was designed *de novo* to function as cation channels in phospholipid bilayers. Four structural features were judged at the outset to be critical and were incorporated into the design. First, crown ethers were chosen to function as headgroups and portals through which cations would enter and exit the channels and, in turn, the membrane. Second, a central "relay" unit was thought to be required as a polar "way-station" to accommodate the transient cation and water in the least polar segment of the bilayer. Third, the "portal" macrocycles were separated from the central relay by dodecyl chains. This made the effective portal-relay distance 12-14Å. The extended central macrocycle, parallel to the lipid axis and perpendicular to the distal macrocycles is ~6Å between nitrogen atoms making the overall extension 30-34Å. This distance corresponds well with the typical "hydrocarbon slab" thickness in phospholipid bilayers. Fourth, flexible sidearms were incorporated on the opposite "side" of the channel to provide "coverage" for the transient cation.

Cation flux was assessed by fluorescence measurements ($H^{\oplus}$), dynamic ^{23}Na-NMR ($Na^{\oplus}$), and by planar bilayer conductance (pbc) techniques ($Na^{\oplus}$, $K^{\oplus}$). Using the ^{23}Na-NMR method, sodium transport in a pc/pg lipid bilayer was found to be ~25-30% that observed for the peptide channel-former gramicidin. Numerous control experiments were conducted to eliminate the possibility of detergent effects, a simple carrier mechanism, and differential solubility. Fluorescence techniques were used to (1) establish headgroup position and (2) aggregation number. Blockage of the channel pore by hydrogen bond formation was also demonstrated. The overall structure as it is currently believed to exist is represented in the figure below.



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ROLE OF ATP IN EXPERIMENTAL HYPERTENSION

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One of the most extensively investigated examples of cotransmission has been the rodent vas deferens. In rat vas deferens, the electrical field stimulation (EFS) produces a biphasic contraction of the smooth muscle.The initial peak is mediated by ATP and the second, slower contraction, by noradrenaline (NA). In vas deferens, ATP and NA appear to activate separate transduction mechanisms to produce contraction. Activation of P_2-purinoceptors by ATP produces depolarisation of the muscle, manifested as an excitatory junction potential, or EJP. Trains of EJPs will summate to produce action potentials that result in an influx of calcium through voltage-sensitive channels, leading to the initial, rapid contraction. NA, however, does not appear to contribute significantly to the nerve-mediated membrane depolarisation; α_1-adrenoceptors stimulation mediates the slow phase of the neurogenic contraction by generation of second messengers, which are able to release calcium from intracellular stores. Without stimulation, it is possible to register spontaneous excitatory junction potentials (SEJP) produced by spontaneous release of ATP in the synapsis.It has been documented that spontaneously hypertensive rats (SHR) can be used as a pathophysiological animal model for human essential hypertension. These animals show an hyperreativity of the smooth muscle when that is assossiated with a general modification of the adrenergic mechanisms. In the present study, we re-investigate the purinergic contribution to hyperreatctivity by intracellular recording in the prostatic end from vas deferens of SHR i WKY animals.

The prostatic end of vas deferens from SHR and WKY animals was pinned in a 3 ml recording chamber perfused continuosly with a physiological solution. The tissue was excited by EFS and intracellular recording from smooth muscle cells were made by use of borosilicate filament microelectrodes coupled to an amplifier and displayed on a computer.

The resting membrane potential in SHR was significantly lower than WKY animals, and it was not affected by the addition in the medium of selective α_1-adrenocetor or P_{2x}-purinoceptors antagonists. In smooth muscle cells from SHR and WKY animals, SEJP were recorded in the absence of stimulation, and EJP after EFS. EJP, but not SEJPs, were abolished by terodotoxin 1 μM indicating that the first ones were mediated entirely by sympathetic nerves while SEJPs were independing from nerve stimulation. In general, the amplitude of EJPs and SEJPs was greater in SHR than WKY. SEJPs, in SHR animals, appeared more frequently with a width rank of amplitudes (Preliminary results).

It is concluded that the contribution by ATP to hypersensitivity in vas deferens exists although the effect of exogenous ATP is similar in both groups of animals. So, it should be kept in mind the possible role of calcium channels in the changes of responsiveness in smooth muscle of hypertensive animals.

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BINDING SITE DOCKING OF THE WILLARDIINES IN THE ACTIVATION AND DESENSITIZATION OF THE GLUR1 SUBTYPE OF AMPA RECEPTOR.

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To gain a better understanding of the process by which glutamatergic agonists bind to an ionotropic glutamate receptor channel and cause its activation and desensitization, we carried out a systematic study of the interactions of a set of desensitizing ligands with wild type GluR1 and with four mutants of its ligand binding domain E398Q, Y446F, L646A and S650V using the Xenopus oocyte expression system and two electrode voltage clamp measurements. The agonists selected were the willardiines since they differ chemically only by the nature of their 5' substituent H-, F-, Br-, I- while causing different extents of desensitization, and because they are the only glutamatergic agonists for which systematic structure-function relationships can be established.

To interpret our results in terms of Gibbs free energies of interactions, we calculated apparent ΔG values as $\Delta G_{app} = -RT\ln EC_{50}$ and the contribution to the ligand interactions of each mutated amino acid residue was defined as $\Delta\Delta G = \Delta G_{(WTGluR1)} - \Delta G_{(mutGluR1)}$ expressed in Kcal/mole.The specific contributions of a given amino acid of the binding domain to the receptor-ligand interactions were determined by using the double mutant cycle method. In the latter, pairwise interactions are studied by "removing", one at a time, one of the components of the interaction. In the case of the willardiines, the interactions of the ligand binding residues are studied by mutating, one at a time, each of the amino acid binding residues while the effects of substituting within the willardiine structure the 5' moiety, F to H, Br to H and I to H, are measured. The amplitude and sign of the coupling energies provide information on the existence of favorable or unfavorable interactions between the two interacting components under investigation.

Our results suggest that positional rearrangements of the ligand binding residues within the binding domain occur in order to accomodate the various willardiines. Furthermore, each willardiine has an unique mode of interaction that differs in the active conformation of the receptor from that prevailing in its desensitized conformation..This interpretation might account for the fact that the various willardiines desensitize the GluR1 channel complex to different extents . Inspection of the values and sign of the coupling energies allows also to propose a possible mode of docking of the willardiines within the binding domain.

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Conversion of the ion selectivity of the the 5-HT$_3$ receptor from cationic to anionic: a conserved feature of the ligand-gated ion channel family?

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The 5-HT$_3$ receptor is a ligand-gated ion channel which together with the nicotinic acetylcholine (nACh) , GABA and glycine receptors forms an ion channel superfamily. These receptors are pentameric assemblies of subunits arranged around an axial channel. Each subunit is thought to consist of four transmembrane domains (M1-M4) with M2 lining the wall of the channel . Galzi and co-workers (*Nature*, 359, 500; 1992) identified regions of the channel that on mutation converted the ion selectivity of the α7-nACh receptor from cationic to anionic. To investigate whether the 5-HT$_3$ receptor possesses similar determinants of ion selectivity, we generated equivalent mutations (see figure) and assessed changes of the properties of the mutant receptors using whole cell patch clamp.

```
WT 5-HT3 R     DSG ERVSFKITLLLGYSVFLIIVSDTLP
MUTANT         ---PA-------------T----------
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Primary sequence of M2 and flanking regions of the 5-HT$_3$ receptor. Dashes represent unchanged amino acids.

The coding sequence of the 5-HT$_3$ receptor was subcloned into the expression vector pRc/CMV. Mutants were generated by site-directed mutagenesis, stably expressed in HEK293 cells and characterised using whole cell patch clamp. For ion selectivity experiments cells were voltage clamped over the range -80 to +40mV and the peak current evoked by 10mM 5-HT determined in the presence of normal extracellular solution (EC; 140mM NaCl, 2.8mM KCl, 2.0mM $MgCl_2$, 1.0mM $CaCl_2$, 30mM glucose, 10mM HEPES, pH 7.2.) , 50% NaCl-mannitol (0.5NaCl; as EC except 65mM NaCl, 130mM mannitol) and NaCl-Isethionate solutions (Na-Ise; as EC except 15mM NaCl, 115mM Na Isethionate). Current voltage plots were constucted and the reversal potential for the wild type (WT) and mutant receptors determined in each solution.

The mutant receptors responded to 5-HT, although with a higher affinity (EC_{50} = 0.24 $\pm$ 0.03μM, n=5) than WT receptors (EC_{50}= 2.18 $\pm$ 0.13μM, n=6), and most of the desensitization in response to agonist was abolished. The reversal potential for the WT receptor was similar in both EC and Na-Ise solutions (E_{rev} was -4.75 $\pm$ 1.61mV and -5.98 $\pm$ 1.33mV respectively, n=5) but was shifted to a more negative potential by the removal of 50% of the NaCl (E_{rev} = -22.40 $\pm$ 2.03mV, n=5) indicating that WT receptors are predominantly cation-selective. In contrast equivalent experiments for mutant receptors indicated that their reversal potential in EC solution (4.09 $\pm$ 1.81mV) shifted to more postive values in both 0.5NaCl and Na-Ise solution (E_{rev} respectively 18.49 $\pm$ 0.94 and 28.01 $\pm$ 2.88 mV, n=5) indicating that this mutant receptor had appreciable chloride permeablity.

The results indicate that the changes in amino acid in this mutant were sufficient to switch the ion selectivity of the 5-HT$_3$ receptor. As the α7nACh and 5-HT$_3$ receptors appear to share common determinants of ion selectivity, this may be a conserved feature of the ligand-gated ion channel family.

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A Synthetic Cation-transporting Calix[4]arene Derivative Active in Phospholipid Bilayers

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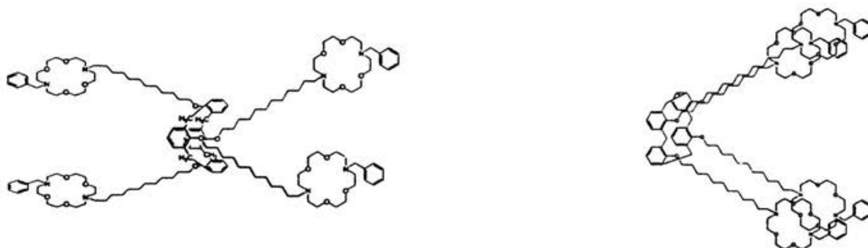
A novel, completely synthetic cation channel based on the calix[4]arene platform has been designed, synthesized, and studied. The calixarene is thought to serve primarily as a non-polar structural element from which hydrophobic chains extend in both directions. The structural design of the channel is aided by the 1,3-alternate conformation of the calixarene. This orients alternate chains in opposite directions so that two -C_{12}crownC_{12} chains reside in each bilayer leaflet. The corresponding channel whose central calix has a cone conformation orients all of the -C_{12}crownC_{12} chains in the same direction. According to the design criteria, the 1,3-alternate form was expected to conduct cations but the cone analog was not. The former has chains capable of traversing the full bilayer. All four chains of cone isomer extend in the same direction and the molecule does not possess sufficient overall length to transcend the membrane.

Cation transport was assessed using the planar bilayer conductance (pbc) technique. As anticipated, cation flux was observed for the 1,3-alternate channel but not for the cone isomer. The two isomeric structures (1,3 alternate left and cone right) are illustrated be-



low along with the pbc conductance results (conditions: 500 mM NaCl, pH = 6.7, top +100 mV; bottom -100 mV, vertical scale, 100 pS, time scale, 10 sec)).

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MOLECULAR DETERMINANTS OF THE GLYCINE RECEPTOR CHANNEL CONDUCTANCE: STUDIES USING CYSTEINE MUTAGENESIS

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Ligand-gated ion channels mediate fast synaptic transmission in the mammalian central and peripheral nervous system. They are pentameric proteins whose ion channel is formed by the second transmembrane segment (M2) in each of the five sub-units. This pore-forming M2 segment is flanked by charged amino acids. In the human glycine receptor (GlyR), mutations to the positively charged arginine 271 (R271), at the external end of the M2 region, are responsible for the genetic disorder, hyperekplexia, and reduce both the efficiency of glycine gating and the amplitude of single channel conductance states (Rajendra *et al.*, 1995). To further investigate the role of an added positive charge at this residue we have been using standard patch clamping techniques to examine the effects of methanethiosulfonate (MTS) reagents on wild type and R271C mutants transiently expressed in HEK-293 cells. The positively charged water soluble MTS derivative, MTSET, increased the amplitude of glycine activated whole cell currents in R271C mutants without affecting the response of WT receptors. Excised outside-out patches held at -60 mV revealed that the WT GlyRs show up to 6 subconductance levels with the most common being about 80 and 90 pS. R271C GlyRs also have multiple conductance levels with the most common being between 30 and 50 pS. Acute perfusion of MTSET, or pre-incubation with MTSET, had no effect on WT GlyR single channel measurements. In contrast MTSET irreversibly and markedly increased both channel open probability and single channel conductance. In the presence of MTSET the dominant conductance states of R271C GlyRs was increased to between 60 and 80 pS. These results confirm the importance of the positive charge on arginine 271 in determining the single channel conductance of the human GlyR.

Rajendra, S., Lynch, J.W., Pierce, K.D., French, C.R., Barry, P.H. and Schofield, P.R. (1995), Neuron 14, 169-175.

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Instituto Juan March (Madrid)

Activation of hIK channels by 1-ethyl-2-benzimidazolone is obligatory Ca-dependent.

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The human intermediate-conductance, Ca2+-activated K+ channel, hIK, was identified by searching the expressed sequence tag database. HIK was found to be identical to two recently cloned K+ channels, hSK4 and hIK. RNA dot blot analysis showed a widespread tissue expression with the highest levels in salivary gland, placenta, trachea, and lung. Using fluorescent in situ hybridization and radiation hybrid mapping, hIK mapped to chromosome 19q13.2 in the same region as the disease Diamond-Blackfan Anemia. Stable expression of hIK in HEK cells, revealed single Ca2+-activated K+ channels exhibiting weak inward rectification (30 and 11 pS at -/+ 100 mV, respectively). Whole-cell recordings showed a non-inactivating, inward rectifying K+ conductance. Ionic selectivity estimated from bi-ionic reversal potentials gave the permeability sequence (PK /PX): K+=Rb+(1.0)>Cs+(10.4)>>Na+,Li+,NMDG+(>51). NH4+ blocked the channel completely. The classical inhibitors of the Gardo's channel, charybdotoxin (IC50=28 nM) and clotrimazole (IC50=153 nM) as well as nitrendipine (IC50=27 nM), stichodactyla-toxin (IC50=291 nM), margatoxin (IC50=459 nM), miconazole (IC50=785 nM), econazole (IC50=2.4 µM), and cetiedil (IC50=79 (M) blocked hIK. 1-ethyl-2-benzimidazolinone (EBIO), an opener of Ca^{2+}-activated K^{+} channels in T84 cells, activated hIK with an EC50 of 74 µM. The effect of EBIO was voltage-independent, but was strictly dependent on the presence of internal Ca^{2+}. EBIO (50 µM) hyperpolarized the hIK expressing cells to E_K within seconds. In conclusion, we have cloned the human IK channel, characterized its chromosomal localization, tissue expression, sensitivity to K^{+} channel blockers, and have identified an IK channel opener.

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High-level Expression of Neuronal Voltage-Sensitive K' Channels in the Methylotropic Yeast *Pichia pastoris*

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Neuronal voltage-dependent K^+ channels of the voltage-sensitive, delayed rectifier type are large complexes containing 4 pore-forming α-subunits and 4 auxiliary β-subunits [1]. Isolation of these proteins from natural sources [2] [3] is made difficult by their relative scarcity and is further complicated by the existence of multiple heterooligomeric sub-types [4]. In order, therefore, to obtain sufficient amounts of material for structural determination we have expressed singly, several delayed-rectifier α-subunits, Kv1.1 Kv1.2 and Kv1.4 , in the methylotrophic yeast *Pichia pastoris*. Using the binding of ^{125}I-α-dendrotoxin (^{125}I-α-DTX), a potent and specific blocker of Kv1.2 channels, and hydrodynamic measurements we show that Kv1.2 is produced in *P. pastoris* as an active tetrameric complex. Furthermore, site-directed mutagenesis indicates that the phosphorylation status of the protein greatly affects its mobility on SDS-PAGE as found for Kv1.1 channels expressed in *Xenopus* oocytes [5]. Calculations based on the binding data indicate expression levels in the order of 80mg of K^+ channel protein per litre of fermentation culture. Addition of a cleavable N-terminal polyhistidine tag has allowed the routine purifcation of 5-10mg of K' channel protein with full retention of ^{125}I-α-DTX binding activity. We are now embarking upon 2- and 3 dimensional crystallisation attempts with the view to obtaining an atomic or near-atomic structure of the delayed rectifier-type K^+ channels. This information should complement and extend the information gleaned from the recently solved structure of a simple prokaryotic K^+ channel [6].

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Instituto Juan March (Madrid)

CALCIUM SIGNALING IN PLANTS.

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Plants use calcium as a second messenger in a wide variety of responses. The intracellular changes of this ion as a consequence of different biotic and abiotic stimuli has been well documented. It is known that the kinetics of calcium fluxes in the plant cell is different in response to cold, pathogen or hormone stimuli. However information on the molecular level of calcium channels, channel regulators or associated proteins is still missing in plants. Therefore we are interested in developing different approaches in order to isolate genes involved in the regulation of calcium signaling.

1.- T-DNA insertional mutagenesis of tobacco protoplasts. Wild type tobacco protoplasts are unable to grow and develop to microcalli in culture medium without addition of calcium. We have mutagenized (through Agrobacterium T-DNA transfer) 57,000 protoplasts, and obtained 18 clones that were able to regenerate in selection medium. Currently we are characterizing the phenotype of these mutants, which carry the calcium indicator protein *aequorin.* Segregation analysis, and measurements on calcium changes of the putative clones in response to different stimuli will be discussed on the poster.

2.- Complementation of two calcium sensitive yeast mutants with an Arabidopsis cDNA library. Two mutant strains which are affected in the control of calcium homeostasis in yeast have been described: the calcium-sensitive growth mutants *csg1* and *csg2* (provided by Dr. Dunn). These mutants are affected by an increase in a calcium pool distinct from the vacuolar calcium pool, where most of the calcium in wild type cells is stored. These strains are unable to grow on yeast media containing 100mM calcium at 37°C. From this screening we obtained four novel *Arabidopsis thaliana* genes which show no homology to known proteins. At the moment we are investigating the regulation and function of these genes in the plant.

3.- Knock out of an L-type calcium channel of *S. cerevisiae.* The YGR217w open reading frame listed in the *S. cerevisiae* genomic database as CCH1, encodes a protein of 2,039 aminoacids with a significant sequence similarity to the α1-subunit of the dihydropyridine-sensitive family of voltage dependent calcium channels. To perform a knock out of this gene, a 1,508 bp fragment containing the N-terminal part (-4 bp tp +1,504 bp) of the CCH1 gene, and an 829 bp fragment (+2,222 to +3,051) were amplyfied by PCR and introduced to the left and right side respectively of a kanamycin resistance cassette. This construct was linearized and used to transform the wild type strain DBY689. Putative knock out strains were isolated on G-418 plates. The southern analysis of three of these colonies revealed that, in fact, the CCH1 gene had integrated the kanamycin cassette by homologous recombination. This strain is being used to complement a cDNA library of *A. thaliana*. Analysis of complemented clones is ongoing.

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3D MAPPING OF RYANODINE RECEPTOR LIGANDS

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Structural determination of large membrane bound macromolecular complexes by diffraction methods is limited by difficulties in their crystallization. Cryo-electron microscopy together with single-particle image processing methods have been used as a successful alternative to these methods in determining the structure of the ryanodine receptor (RyR), a 2.3 Md tetrameric intracellular Ca^{2+} channel. In skeletal muscle, the RyR, under the control of the dihydropyridine receptor (DHPR), releases Ca^{2+} from the sarcoplasmic reticulum and thereby initiates muscle contraction. The DHPR interacts directly with the RyR through the intracellular II-III loop of its α-subunit with a currently unknown site on the RyR.

Other molecules, such as calmodulin (CaM) further modulate the RyR. At submicromolar Ca^{2+} concentrations, up to 16 CaM molecules bind to the RyR with an activating effect. We are utilizing cryo-electron microscopy and single particle image processing to study the location of bound CaM under these conditions. The 3D difference map shows that CaM binds to 4 equivalent areas in the cytoplasmic assembly, distant from the transmembrane region of the receptor. The RyR with bound CaM in these conditions seems to undergo a conformational change in other regions of the channel, perhaps the activating effect of that modulator.

Similar studies are in progress to determine the site of interaction with the DHPR intracellular II-III loop. Our preliminary results suggest that the interaction could occur in 4 equivalent areas, located in the so-called “clamp” domains.

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THE POTASSIUM CHANNEL KCSA OF *STREPTOMYCES LIVIDANS*

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Ion channels are found in eucaryotic cells and are involved in many physiological processes, such as secretion, regulation of membrane potential, signal transduction and osmoregulation. Within the Gram-positive bacterium *Streptomyces lividans* we discovered the first procaryotic potassium channel gene *kcsA*. It encodes a predicted 17.6 kDa protein with two potential membrane-spanning helices linked by a central domain which shares a high degree of similarity with the H5-segment conserved among eucaryotic ion channels. Multiple alignments of deduced amino acids suggest that the novel channel has the closest kinship to the S5-, H5- and S6-regions of voltage-gated K^+-channel families, mainly to the subfamily represented by the *Shaker* protein from *Drosophila melanogaster*.

S. lividans protoplasts were fused with liposomes and, within the resulting vesicles, K^+-channel activity was recorded by patch clamp analysis. KcsA was purified in larger quantities and functionally reconstituted in a bilayer system (1). The data show that KcsA is so far the best model system. The recent elucidation of the crystal structure of the pore region of KcsA allows detailed conclusions about the principle underlying selective K^+-conduction (2). It is expected that additional KcsA studies will provide further insights into the functioning of K^+-channels.

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2) Doyle et al. 1998. Science 280, 69-77

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Structural and biochemical analysis of soluble nicotinic acetylcholine receptor extracellular domains.

Dr M. L. Tierney.

Crystallographic studies of water soluble domains of neurotransmitter-gated ion channels will provide a powerful system for generating structural information, ideal for the structural modelling of the intact receptor. This important class of receptors is responsible for chemical signalling in the brain and at the neuromuscular junction. Insight into their mechanism of action at the molecular level is fundamental to our understanding of normal and dysfunctional brain and muscle activity. The nicotinic acetylcholine receptor (nAChR) is the prototypic neurotransmitter ion channel in a family which includes the excitatory 5-hydroxytryptamine$_3$ (5-HT$_3$) receptor and the inhibitory GABA$_A$ and glycine receptors. Viewed under the electron microscope, negative stained samples of purified neurotransmitter receptors have a distinct 'donut-like' appearance created by the arrangement of their five homologous subunits in a pseudo symmetric ring with the N-terminal extracellular half of each subunit forming the water-filled vestibule through which ions are funnelled towards the transmembrane pore. The receptors architecture incorporates the binding sites for agonists and antagonists in the hydrophilic extracellular domains, distinct from the more hydrophobic C-terminal region which folds to form the pore through the lipid bilayer. The independent functionality of these two domains has been demonstrated experimentally using chimeric neuronal nicotinic $\alpha 7$ and 5-HT$_3$ receptors; the binding of the $\alpha 7$ ligand to it's extracellular domain opened the conduction pore of the fused 5-HT$_3$ C-terminal domain (1). Specific assembly signals for pentamer formation and mature ACh binding sites have also been localised to the extracellular domains (2,3). Given the domain structure of neurotransmitter receptors recombinant expression of N-terminal soluble domains offers a potential strategy for determining the 3 dimensional structure.

Recombinant expression of individual and various subunit combinations of the EC domains of the nAChR ($\alpha 2\beta\gamma\delta$) has been achieved using the multi-promoter based baculovirus insect cell system. Subunit specific polyclonal antibodies confirm that each of the four extracellular (EC) domains are synthesised in the insect cells. Furthermore, the binding of ^{125}I-α-bungarotoxin and the conformationally sensitive monoclonal antibody, mAb35, to the $\underline{\alpha}$ EC domain, expressed alone or in combination, indicates subunit folding and /or maturation occurs in the insect cells. Purification strategies have focused on isolating the soluble ligand binding domain of the $\underline{\alpha}$ subunit as well as complexes formed from the co-expression of all four subunit EC domains.

The incorporation of a hexahis tag on the $\underline{\alpha}$ EC domain enables its subsequent purification on nickel nitrilotriacetic acid (NiNTA) resin from both the Hi5 cell membrane fraction (endoplasmic reticulum) and the media, ie secreted. The secreted form of the $\underline{\alpha}$ EC domain corresponds to the higher molecular weight glycosylated product while its isolation from the cell also includes the lower molecular weight, non-glycosylated form.

To form a functional complex composed of just the soluble EC domains of the nAChR a major difficulty is that the four polypeptides have to assemble together in a stoichiometry of $\alpha 2\beta\gamma\delta$. Under the electron microscope assembled complexes of the EC domains are likely to resemble the donut-like images characteristic of the full length receptor given that it is the arrangement and folding of the EC domains which creates the vestibule. Co-expression studies have used a recombinant virus carrying the four nAChR EC domains in which one of the domains, the $\underline{\delta}$ EC, contains a hexahis tag. Small quantities of material have been partially purified on the NiNTA resin from insect Hi5 cells and the media following secretion. Under the electron microscope images of negative stained samples reveals 'donut-like' complexes. Single particle averaging of these complexes shows 5-fold pseudo symmetry. Such complexes are absent when the α EC domain is expressed alone.

Characterisation of the soluble EC complexes identified under the electron microscope is required to confirm their make up and domain arrangement. Potentially, the baculovirus system, with its ability to co-express multiple proteins, may prove to be a viable source of soluble neurotransmitter EC domains for structural studies.

1) Eisele, J.L. *et al.*, 1993, Nature 366: 479-483.
2) Yu, X-M. & Hall, Z.W. 1991 Nature 352: 64-67.
3) Verall, S. & Hall, Z.W. 1992 Cell 68: 23-31.

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Abstract: Expression and Tetramer formation of Kch, a putative K+-Channel of Escherichia coli

Thomas Urbig, Marie Johansson, and Gunnar von Heijne

The gene encoding Kch has been amplified from E. coli genomic DNA by PCR and cloned into expression vectors. The expression was then studied in a variety of host strains and vectors. Under control of IPTG-inducible T7 promotors the Kch protein expresses quite well. A significant amount of the full-length protein is overexpressed upon induction, but the majority accumulates as a degradation product. Interestingly, a fairly large amount of a high-molecular weight species is seen after separation of E. coli protein extracts with SDS-PAGE. According to the molecular weight this high-molecular band represents most likely a tetramer of Kch. Surprisingly, this species is stable under the rather harsh conditions of denaturing SDS gel electrophoresis. The stability of this complex allowed us to study the oligomerization by insertion mutagenesis to determine if and which transmembrane helices are critical for tetramerization. So far, only the so-called P-loop has been found to be critical while insertions of amino acids into transmembrane helices do not affect the oligomer formation. A number of N- and C-terminal truncation mutants are being made to further map the domains which facilitate the protein-protein interactions. This might also allow conclusions about the influence of the extramembraneous domain in the oligomerisation.
Protein-protein cross-linking in vitro was performed to confirm the subunit composition by biochemical means.
Currently, we are working on the purification of the recombinant protein. Expression of Kch with fusion tags should allow us easy purification of the recombinant protein. Once this is achieved we aim for the crystallization of the protein to gain more information about the structure/function relationship of potassium channels and of the structures of membrane proteins in general.

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