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Jan Egebjerg, Søren-P. Olesen and Povl Krogsgaard-Larsen

Abstracts - MONDAY, August 14, 2000

Kainate Receptor-Deficient Mice
Stephen Heinemann, Molecular Neurobiology Laboratory, Salk Institute, La Jolla, Ca, U.S.A
Abstract not received

Importance of AMPA Receptors in synaptic plasticity
Sprengel R., Borchardt T., Mack V., Jensen V., Ovind H.[1], Feldmeyer D., Burnashev N.[2]& P.H. Seeburg
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We have adopted genetic strategies to determine the function of AMPA receptors in the formation of synaptic plasticity i.e long term potentiation (LTP) at CA3/CA1 synapses in the hippocampus of adult and juvenile mice. Using a variety of different mouse lines with perturbations in AMPA receptor expression, the involvement of the AMPA receptor subunits GluR-A and GluR-B in long term LTP was dissected.
The GluR-B subunit was found to have its major function during development of a mouse brain. Up to postnatal day 15 to 25 the GluR-B subunit has to maintain distinct AMPA receptor channel properties, such as Ca^{2+}-impermeability and linear current/voltage relationship. Mice with disturbed AMPA receptor mediated Ca^{2+}-flux in principal neurones develop neurological phenotypes which can result in epileptic attacks and premature death of the carriers. At all ages examined LTP was present at CA3/CA1 connections indicating that GluR-B is not essential for synaptic plasticity. In contrast, abolished GluR-A subunit expression had minor effects on mouse development but on LTP formation, which was diminished completely in adult mice. In the absence of the GluR-A subunit the total amount of functional AMPA receptors was strongly reduced in CA1 pyramidal cells, and most of the remaining receptors were localised in synapses. In wild-type mice the majority of AMPA receptors are in extra-synaptic sites, which might indicate that the presence of extra-synaptic receptors is important for the formation of LTP. This is supported by the presence of CA3-CA1 LTP in young GluR-$A^{-/-}$ mice. At this age the ratio of extra-synaptic versus synaptic receptor is more in favour of extra-synaptic receptors.
In summary, our mouse models might indicate that the AMPA-receptor level in the postsynaptic neuron is more important for LTP than the presence of individual AMPA receptor subunits.

Regulation of Glutamate Receptor Function and Synaptic Plasticity
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Neurotransmitter receptors mediate signal transduction at the postsynaptic membrane of synaptic connections between neurons in both the central and peripheral nervous systems. We have been studying the molecular mechanisms in the regulation of neurotransmitter receptor function. Recently we have focused on glutamate receptors, the major excitatory receptors in the brain. Glutamate receptors can be divided into two major classes: non- NMDA and NMDA receptors. Non-NMDA receptors mediate rapid excitatory synaptic transmission while NMDA receptors play important roles in neuronal plasticity and development. Studies in our laboratory have found that both non-NMDA and NMDA receptors are multiply phosphorylated by a variety of protein kinases. Phosphorylation regulates several functional properties of these receptors including conductance and membrane targeting. For example, phosphorylation of the GluR1 subunit of non-NMDA receptors by multiple kinases including PKA, PKC and CaM kinase II regulates its ion channel function. Recent studies have demonstrated that the phosphorylation of AMPA receptors is regulated during cellular models of learning and memory such as long term potentiation (LTP) and long term depression (LTD). We have also been examining the mechanisms of the subcellular targeting and clustering of glutamate receptors at synapses. We have recently identified a variety of proteins that directly or indirectly interact with non-NMDA and NMDA receptors. We have found a novel family of proteins that we call GRIPs (Glutamate Receptor Interacting Proteins) that directly bind to the C-termini of the GluR2/3 subunits of non-NMDA receptors. GRIPs contain seven PDZ domains, protein-protein interaction motifs, which appear to crosslink non-NMDA receptors or link them to other proteins. In addition, we have recently found that the C-termini of GluR2 also interacts with the PDZ domain of

PICK1, a protein kinase C-binding protein that is found at excitatory synapses. Finally, the GluR2 subunit also interacts with the NSF protein, a protein involved in the regulation of membrane fusion events. These non-NMDA receptor interacting proteins appear to be involved in the proper subcellular targeting and synaptic clustering of these receptors. In summary, we have examined the molecular mechanisms underlying the regulation of glutamate receptor function. These studies have suggested that regulation of glutamate receptor function may be a major mechanism for the regulation of synaptic plasticity in the nervous system.

Kainate Receptors: New Functions, New Mechanisms

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Kainate administration in experimental animals induces seizures and patterns of neuronal damage closely resembling those observed in epileptics and has been widely used as a chemical model for human temporal lobe epilepsy. For this and other reasons, it has become important to understand the physiology of these receptors in brain function. The discovery of a specific AMPA receptor antagonist, GYKI53655, has made such studies feasible. Consistent with a role in epilepsy, kainate has been found to depress GABA inhibitory transmission in the rat hippocampus. Remarkably enough, this effect is dependent on G-protein activation and disappears by blocking PKC activity. In addition, a small part of the excitatory input to CA1 inhibitory interneurons seems to be driven by kainate receptors. Therefore, bath application of kainate or ATPA, the postulated specific agonist of GluR5-containing receptors, depolarizes interneurons and increases their firing rate, a phenomenon that has been suggested to produce the overinhibition of pyramidal cells. We have carried out experiments aimed at clarifying this aspect of kainate receptor functioning. we found that glutamate, when applied at low concentrations (10 μ M) was able to significantly inhibit the evoked IPSC, without increasing the frequency of sIPSC, indicating that at low concentrations, glutamate acts weakly on kainate receptors that depolarize interneurons. An inverse situation was found for ATPA. At 1 μ M, this compound depolarized interneurons, increasing their firing rate and the spontaneous IPSC in pyramidal cells. However, at this concentration, ATPA reduces the amplitude of evoked IPSC only slightly. *In vivo* experiments revealed that kainate and ATPA have different potency to reduce GABA inhibition and to induce epileptic activity. Therefore, both effects of kainate, reduction of the inhibitory drive and increase in interneuron firing rate, can be dissociated and must be mediated by two different populations of kainate receptors.

Action of Agonists and Antagonists on the GLuR2 Ligand Binding Core Defined by X-ray Crystallography

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Ionotropic glutamate receptors (iGluRs) define a family of ligand-gated ion channels that are essential for the development and function of the mammalian nervous system and are implicated in processes that include memory and learning, and diseases such as schizophrenia. Within the iGluR family of receptors, pharmacologically distinct subtypes include the AMPA, kainate and NMDA receptors. The region of iGluRs that defines receptor pharmacology is the ligand binding core or S1S2 region. My laboratory has developed methods for the over-expression and folding of the GluR2 (AMPA) S1S2 and has crystallized it alone and with a series of ligands. Here I report the structures of the bilobed GluR2 S1S2 core in an apo form and bound to the agonists AMPA, glutamate, kainate and the antagonist DNQX. Agonists and antagonists bind between domains 1 and 2, bring the domains closer together, and reduce the volume of the interdomain cleft. By constrast, in the apo and the antagonist-bound state, the domains are significantly farther apart and the active site cleft is expanded. Weakly activating and partially desensitizing agonists such as kainate produce a degree of domain separation that is intermediate between the glutamate- or AMPA-bound state and the apo form. Additional high resolution studies involving a series of 5-substituted Willardiine compounds (hydro, fluoro, bromo and iodo will also be discussed.

Molecular Pharmacology of the Recombinant Glutamate Receptors

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The native ionotropic glutamate receptors are based on pharmacological criteria divided in NMDA, AMPA and kainate receptors. Identification of the genes encoding the glutamate receptor subtypes showed that the receptors are formed of at least 16 subunits. GluR1-GluR4 form the AMPA receptors while the kainate receptors are formed of the subunits GluR5-GluR7 and KA1-KA2. Studies on the recombinant receptors have revealed that neither AMPA or kainate exhibit particular high selectivity between the recptors. We have studied a collection of AMPA derivatives (synthesized in Krogsgaard-Larsens laboratory). Changes at the 5-position of the isoxazolyl ring strongly influences the potency of the derivatives but also the selectivity. The site for interaction on the receptor has been referred to as the hydrophobic pocket. We have by mutagenesis tried to identify the residues responsible for the selectivity. Changes within the isoxazolyl ring influences the steady state current evoked by the compounds. These data are combined with mutagenesis data suggest that the degree of closure of the binding pocket influences the ratio between the peak current and the steady state current.

Each subunit form a three transmembran (TM) spanning elements and a pore forming reentry loop between the first and the second TM element. The agonist binding site is located between two lobes formed of a segment N-terminal to TM1 and a segment C terminal of the second TM, respectively.
Heteromer expression of the subunits often changes the phamacological properties significantly, we have introduced artificial Zn^{2+} binding sites in order to distinguish between a binding site formed between lobes from one subunit (intrasubunit) vs. a binding sites formed from lobes on adjacent subunits (intersubunit).

Block of Glutamate Receptor Channels by Polyamines
Mark Mayer, LCMN, NICHD, NIH, Bethesda, MD 20892 USA.
When expressed in neurons, HEK cells and oocytes the genomically encoded forms of AMPA and kainate receptor channels exhibit profound biphasic rectification. Current flow between -10 and +50 mV is almost completely blocked. Such rectification is lost when membrane patches are removed from cells, and is abolished when RNA editing converts a codon encoding a Gln to Arg in the pore loop of the GluR2, 5 and 6 subunits. In membrane patches rectification is restored by intracellular polyamines which behave as permeable ion channel blockers. Polyamines such as PhTX, which have large aromatic substituents at one end of the polyamine chain, also block from both sides of the membrane but show greatly reduced permeation. Studies with polyamines of different size show a linear correlation of binding energy with the number but not spacing of CH2 groups within the polyamine chain. The apparent valence of block differs little for spermine and spermidine which have 4 and 3 NH2 respectively. For diamines with a fixed separation of NH2 groups addition of external alkyl groups increases the valence of block most likely due to coupling of block with the movement of permeant ions. Kinetic analysis of voltage and concentration jump experiments revealed that the interaction of polyamines with GluR channels is complex and involves both permeation and molecular sieving effects as well as allosteric stabilization of closed states. A conserved Asp/Glu which is likely to be located near the cytoplasmic entrance to the channel plays a key role in establishing polyamine block. Introduction of Trp at the Q/R site produces a nM affinity binding site while other residues attenuate block.

Poster NO. 1
Quantitative single cell RT-PCR of AMPA mRNA following induction of ischemic tolerance or moderate ischemia
Alsbo C, Laboratory of Neuropathology, University of Copenhagen, Copenhagen, Denmark.
Introduction: Global cerebral ischemia can induce a delayed loss of hippocampal CA1 pyramidal neurons. The AMPA receptor is implicated in the selective vulnerability of these neurons. Previous results from our laboratory have shown that ischemia induces a general downregulation of the AMPA receptor subunits, and that a short tolerance-inducing period of ischemia leads to a general upregulation of the GluR2 subunit. To further differentiate the regulation level of the GluR1-4 subunits, quantitative single cells PCR were used to analyse hippocampal CA1 neurons from rats after moderate, damage-inducing ischemia or tolerance-inducing ischemia.
Methods. Three groups - each with 8 rats - were used. Ischemic animals: subjected to 7 minutes of global cerebral ischemia and reperfused for 24 hours. Tolerant animals: subjected to 3 minutes of ischemia and reperfused for 48 hours. Control animals: non-handled. Whole cells were picked up from acutely dissociated hippocampal CA1 neurons. The mRNA and the a RNA standard were reverse transcribed to cDNA. By the use of quantitative PCR GluR1-4 were amplified with common primers and the products were digested with subunit-specific enzymes.
Results: GluR1, GluR2 and GluR3 mRNAs in the ischemic animals were decreased to 59%, 67% and 66% of levels in control animals. The GluR4 subunit was not detectable in the ischemic animals. In addition, the balance between the levels of the GluR1-4 mRNA was unchanged compared to control animals. In tolerant animals GluR1-4 were increased to 151%, 401%, 120% and 226% , respectively. **Conclusion:** The ischemic animal shoed no correlation between cell loss in the hippocampal CA1 region and a selective downregulation of the GluR2 subunit. The upregulation of especially the GluR2 subunit in tolerant animals could alter the receptor complex characteristics, possibly making the cell more resistant to a subsequent ischemic insult.

Poster NO. 2
Crystal Structure of GluR2 S1S2 in the DNQX- and AMPA-bound states
Neali Armstrong and Eric Gouaux, Columbia University, New York, USA.
The bilobed AMPA receptor ligand binding core, GluR2 S1S2, contains a binding site located in the cleft between the two domains. The crystal structures of GluR2 S1S2 bound to the competitive antagonist, DNQX, and the full agonist, AMPA, have been determined at 1.8 Å and 1.7 Å resolution, respectively. We find that in the antagonist bound state the binding cleft is expanded and almost all interdomain contacts are severed. DNQX binds in the open cleft by stacking directly under Tyr-450 and forming hydrogen bonds with residues in domain 1 which are essential for agonist binding. Stabilization of the expanded conformation may occur through the interaction of a DNQX nitroxyl group with Thr-686 at the base of helix H in domain 2. The GluR2 S1S2-DNQX model illustrates the structural basis for quinoxalinedione antagonism and provides a picture of the ligand-binding core in the closed-channel state.

The GluR2 S1S2-AMPA structure reveals a notable difference in the mode of AMPA binding as compared to glutamate. The AMPA hydroxylate anion has been proposed as bioisosteric with the glutamate γ -carboxyl group. However, superposition of glutamate and AMPA structures shows that these anions do not occupy equivalent positions in the binding site. Instead, the AMPA oxyanion superimposes with O2 from the glutamate γ -carboxyl and is tightly tethered to a water molecule which is located in a position that superimposes with O1. Difference Fouriers calculated from the AMPA crystal form, which is isomorphous with the glutamate crystal form, clearly depict a striking peptide flip involving Asp-651 and Ser-652. As a result of this backbone conformational change, two additional hydrogen bonds are formed between domain 1 and domain 2 in the AMPA-bound, closed cleft state.

Poster NO. 3

Identification of Amino Acid Residues in the GluR1$_o$ AMPA-R Responsible for Desensitization

T.G. Banke, A. Schousboe and D.S. Pickering, Royal Danish School of Pharmacy, Dept. of Pharmacology, Copenhagen, Denmark.

Even though GluR1$_o$, -3$_o$ and -4$_o$ are highly homologous at the amino acid level (65 - 70 %), they show different desensitization patterns; i.e. GluR4$_o$ and GluR3$_o$ desensitize approximately four-fold faster than GluR1$_o$ (TAU = (ms ± S.E.M): 3.83 ± 0.12, n = 13; 1.49 ± 0.06, n = 16 and 1.18 ± 0.09, n = 8 for GluR1$_o$, GluR3$_o$, and GluR4$_o$, respectively). By creating chimeras of GluR1$_o$ and GluR3$_o$, we identified important S2-segments of the subunits that are involved in controlling this desensitization mechanism. We then proceeded to examine point mutations in the S2-region by changing non-conserved amino acids in the S2-region of GluR1$_o$ to their GluR3$_o$ counterparts and three amino acids were identified to be critical for GluR1$_o$ desensitization. We hereby confirm the importance of the R/G RNA editing site in desensitization, but this single point mutation [(R757G)GluR1$_o$: TAU = 2.55 ± 0.10 ms, n = 19] was not sufficient for complete reversal of the desensitization rate of GluR1$_o$ to that of GluR3$_o$. In addition, we located two amino acids in GluR1$_o$, Y716 and Y714, which are also involved in desensitization. Moreover, by creating the double point mutant, (Y716F, R757G)GluR1$_o$, we could completely exchange the desensitization rate of GluR1$_o$ to that of GluR3$_o$ [(Y716F, R757G)GluR1$_o$: TAU = 1.75 ± 0.07 ms, n = 21). However, we could not obtain the complementary exchange of desensitization rates from either a (F728Y, G769R)GluR3$_o$ mutant (TAU = 1.84 ± 0.10 ms, n = 10) or from a (F724Y,G765R)GluR4$_o$ mutant (TAU = 1.64 ± 0.06 ms, n = 4), indicating that the desensitization mechanism for GluR1$_o$ could be different than for either GluR3$_o$ or GluR4$_o$.

Poster NO. 4

Allosteric modulation of AMPA receptors

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The diversity of the AMPA subtype of the glutamate receptors is a result of the assembly of both homomeric and heteromeric complexes of AMPA receptor subunits (GluR 1-4). Each of the four subunits exists as two splice variants known as the flip/flop isoforms, that differ in a 38 aa module in the extracellular loop between M3 and M4. Certain drugs, such as the allosteric modulator cyclothiazide (CTZ) has been shown to suppress receptor desensitization. CTZ is known to distinguish between the flip and flop isoforms, the flip variants showing the highest affinity and considered the most sensitive isoforms. In the present study, CTZ and the CTZ analog NS 1376 have been used to characterize differences between AMPA receptor subtypes. Using the patch clamp technique, the effects of the compounds on whole-cell currents were studied on either mouse neocortical neurons, on HEK-293 cells transfected to give heteromeric combinations of GluR, or on *Xenopus* oocytes expressing homomeric receptors. Incubation with either CTZ or NS 1376 caused a reversible potentiation of the current. Potentiation by CTZ and NS 1376 both exhibited various effect on cortical neurons which correlated with the ratio between the peak and steady state levels of the response. Studies on recombinant receptors using kainate (1 mM) or AMPA (100 μM) as agonist, showed that heteromeric complexes containing GluR2 were not potentiated to the same degree as complexes lacking this subunit by either CTZ or NS 1376. Surprisingly, CTZ and NS 1376 displayed opposite selectivity on homomeric channels; NS 1376 (100 μM) was more effective on the flop isoform and, in contrast to CTZ, caused a right shift in the concentration-response curves for AMPA and Glutamate (from 4.6 to 168.2 μM for GluR4o). However with kainate as agonist the largest effect of NS 1376 was on GluR1 i/o (4-6 times potentiation).

Poster NO. 5

Structural studies on GluR2 ligand binding domain S1S2 in complex with AMPA analogs

A. Hogner[a], R. Jin[c], J.S. Kastrup[a], L. Brehm[a], T. Liljefors[a], J. Egebjerg[b], I.K. Larsen[a] and E. Gouaux[c], [a]Royal Danish School of Pharmacy, Dept. of Medicinal Chemistry, Copenhagen, Denmark. [b]University of Aarhus, Dept. of Mol. and Struct. Biol., Aarhus, Denmark. [c]Columbia University, Dept. of Biochem. and Mol. Biophysic, New York 10032, USA.

Detailed structural studies of AMPA receptors are necessary for a better understanding of ligand-receptor interactions. X-ray structure determinations of the ligand-binding domain complexed with different agonists and antagonists will

hopefully clarify the relationships between the activity and pharmacological profile of the ligand and the conformation of the receptor. Furthermore, structural studies will allow us to identify key functional sites for ligand recognition and thus facilitate the design of new ligands. The development of the GluR2-S1S2 construct ASDE1 by the group of E. Gouaux has made it possible to obtain detailed information of the binding site of GluR2. In the present study we have focused on a range of AMPA receptor agonists and one antagonist. The agonists are: ACPA ((S)-2-Amino-3-(3-carboxy-5-metyl-4-isoxazolyl) propionic acid), BAN ((S)-2-Amino-3-[3-hydroxy-5-(2-methyl-2H-tetrazol-5-yl)isoxazol-4-yl] propionic acid) and Br-Hibo ((S)-Amino-4-bromo-3-hydroxy-5-isoxazole propionic acid). The antagonist used is ATPO ((S)-2-Amino-3-[5-tert-butyl-3-(phosphonomethoxy)-4-isoxazolyl] propionic acid) [4]. We have obtained crystals from all four ligands in complex with ASDE1. X-ray data to 1.9Å resolution have been collected on ASDE1 complexed with BAN and ACPA, respectively. Binding affinity experiments show that ASDE1 is correctly folded and IC_{50} data for each ligand are similar to values given in the literature.

Poster NO. 6

Functional Concatemeric Glutamate Receptors Sussest a Tetrameric Receptor Complex Stroichiometry *(Oral presentation Tuesday 13:30-14:00)*

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Ionotropic glutamate receptors (GluRs) are multi-subunit complexes. Their stoichiometry has caused considerable debate as evidence for both pentameric and tetrameric assemblies has been reported. To obtain unequivocal, direct evidence for complex formation we set out to construct concatemeric glutamate receptors. As the N-terminus of GluRs is in the extracellular compartment while the C-terminus is intracellular, construction of dimeric receptors requires the introduction of an additional membrane-spanning domain between the C-terminus of subunit 1 and the N-terminus of subunit 2. We found the signal peptide of GluR1, the transmembrane domain B of GluR1, and the fourth transmembrane domain of the bovine $GABA_A$ receptor β -2 subunit to each allow the construction of functional dimeric GluR1 molecules. Dimeric GluR1 was expressed in *Xenopus* oocytes and gave small but reproducible currents. To exclude receptor formation by merely the first (or last) subunit of each dimer, we constructed dimers of two functionally different subunits. Such dimers had an edited GluR1(R) in the N-terminal position, while the C-terminal position was occupied by an unedited GluR1(Q), or vice versa. It is well known that GluR1(Q) has a rectifying current-voltage relationship (I/V) and considerable calcium permeability, while.GluR1(R) has a linear I/V, tiny currents, and very little calcium permeability. Heteromeric complexes of these two subunits behave much like the edited GluR1(R) except for larger currents. Our heterodimeric concatemers gave responses with properties indistinguishable from heteromeric receptors, irrespective which of the two subunits was placed N-terminally, thereby proving incorporation of both subunits into the complex. These findings suggest a tetrameric rather than a pentameric subunit stoichiometry.

Poster NO. 7

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Fourier transform infrared spectroscopy was used to investigate ligand-protein interactions in the GluR4 glutamate receptor subunit. Specifically, the asymmetric carboxylate vibrations of the ligands, the SH stretching mode of the single non-disulfide-bonded cysteine residue, and the amide vibrations of the protein were used to study the structural changes induced by ligand binding in the ligand-binding domain of the GluR4 subunit. These studies indicate that glutamate binding induces more extensive secondary structural changes in the ligand-binding domain than does kainate binding. Glutamate also alters the hydrogen-bonding strength of the single free cysteine side chain in the domain; while kainate does not. On the other hand, the interaction of a binding site arginine residue with kainate appears stronger than that with glutamate. These results, for the first time, identify chemical and structural differences that may explain the different functional characteristics of the two agonists acting on ionotropic glutamate receptors. In doing so, they complement and extend recent crystallographic structures of the ligand-binding domain.

Poster NO. 8

Molecular Evidence for an Intersubunit Agonist Binding in GluR1

Jensen, HS & Egebjerg J, Dept. of Molecular and Structural Biology, University of Aarhus, Denmark, Aarhus, Denmark.

The ligand-binding domain of the multioligomeric ionotropic glutamate receptors is composed of two extracellular segments (S1 and S2) which are contained within each subunit. The motions of S1 and S2 are believed to transmit a signal to the pore inducing channel opening and receptor desensitization. The subunit-origin of the two seg-ments composing the ligandbinding domain remains enigmatic. An *intersubunit*-binding situation simi-lar to the nACh and the $GABA_A$ receptors is supported by several observations on heteromeric receptors disclosing functional properties not present in the respective homomers. On the other hand, an engineer-ed S1-S2 linked construct has been crystallized as monomers and shows a pharmacological profile re-sembling the wildtype receptors suggesting an *intrasubunit*-binding situation. We approached this issue by constructing a range of mutant receptors with putative

metal ion binding sites spanning the ligand-binding gorge. Such receptors with engineered sites were foreseen to produce differential effects on the observed steady-state potencies and maximal steady-state currents in the presence of zinc compared to wildtype receptors when examined electrophysiologically in *Xenopus* oocytes. We have successfully characterized a GluR1 double mutant containing a bidentate zinc-binding site composed of two histidines from each side of the ligandbinding gorge. The coordination of zinc between $S1^H$ and $S2^H$ was dis-closed by an 8-fold increase in KA potency at 1 mM $ZnCl_2$. Coexpression of the complementary GluR1-($S1^H$) and GluR2($S2^H$) gave rise to heteromer receptors responding with a 9-fold decrease in KA poten-cy at 3 mM $ZnCl_2$. The heteromeric GluR1+GluR2($S1^H$, $S2^H$) receptors were insensitive to high zinc concentrations, implying that no coordination of zinc occurs between the two segments of GluR2. Fur-thermore, the observed reduction in KA potency by addition 3 mM zinc on heteromer GluR1($S1^H$, $S2^H$) +GluR2 receptors appear to be an additive effect of the two heteromers GluR1($S1^H$)+GluR2 and GluR1-($S2^H$)+GluR2 receptors. In conclusion, our results suggest that the binding of ligands in the ionotropic glutamate receptors take place on the interface of two neighboring subunits in the receptor complex.

Poster NO. 9

Resolution, Absolute Stereochemistry, and Enantiopharmacology of a 1,2,5-Thiadiazole containing Analogue of Glutamic Acid showing unexpected Stereoselectivity

Tommy N. Johansen, Yves Janin, Birgitte Nielsen, Karla Frydenvang, Hans Bräuner-Osborne, Tine B. Stensbøl, Stine B. Vogensen, Ulf Madsen, and Povl Krogsgaard-Larsen. Department of Medicinal Chemistry, Royal Danish School of Pharmacy, Copenhagen, Denmark.

In order to identify new subtype-selective excitatory amino acid (EAA) receptor agonists as useful pharmacological tools we have prepared the stereoisomers of the 4-hydroxy-1,2,5-thiadiazole analogue of glutamic acid (TDPA), a compound that in the racemic form previously has been preliminary pharmacologically characterized as an EAA receptor agonist (1). (*R*)- and (*S*)-TDPA were obtained using chiral HPLC with enantiomeric excesses of 99.9%. The absolute configuration of (*R*)-TDPA was established based on an X-ray analysis of the zwitterion. At cloned metabotropic EAA receptors, (*S*)-TDPA in a stereoselective manner activated class I receptors, whereas both enantiomers showed no activity at class II or III receptors. At native ionotropic EAA receptors, TDPA was also characterized to be a subtype selective agonist only acting at AMPA receptors. In [^{3}H]AMPA binding (*S*)-TDPA had almost the same affinity as compared to that of (*S*)-AMPA. Unexpectedly, TDPA showed a very low stereoselectivity, having an eudismic ratio of less than 5. For comparison, AMPA had an eudismic ratio of more than 3800 in the same binding assay. In electrophysiological studies using rat cortical neurons both (*R*)- and (*S*)-TDPA were AMPA receptor agonists. However, (*R*)-TDPA, being only approximately 3 times less active than (*S*)-AMPA, turned out to be the eutomer in this test system most likely due to a re-uptake of (*S*)-TDPA.

(1) Lunn et al. Book of Abstract, XIIth International Symposium on Medicinal Chemistry, Basel, 1992.

Poster NO. 10

Joergensen, R & Egebjerg J, Dept. og Molecular and Structural Biology, University of Aarhus, Aarhus, Denmark.

The N-terminal extracellular domain of glutamate receptors may interact with synaptic structural proteins or proteins that have a modulating effect on receptor kinetics. With the aim of identifying such interaction partners, we have expressed the N-terminal extracellular domain of GluR6 in an insect cell system. The construct has an N-terminal Heart Muscle Kinase (HMK) site and a C-terminal 6xHis-tag. We have used it in affinity assays with synaptosomal protein fractions from rat brain, where we have identified two potential interaction partners of 35 and 45 kDa.

Purified recombinant proteins were incubated with solubilised synaptosom fractions from rat brain and bound to NiNTA. When examining the eluat on silver stained SDS gel, a 45 kDa protein band occurs which is not seen in the control. Furthermore, when the recombinant N-terminal extracellular domain of GluR6 was radioactively labeled in the enginered HMK-site and used in a far western assay on protein fractions from rat brain, we identified a 35 kDa potential interaction partner which is not seen in the affinity assay.

Further studies to identify the interacting proteins are in progress.

Poster NO. 11

Glutamate-induced currents reveal three functionally distinct NMDA receptor populations in superficial dorsal horn - effect of peripheral nerve injury and inflammation

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NMDA receptor mediated whole cell currents were studied in acutely dissociated superficial spinal cord dorsal horn neurons from control rats and rats following peripheral nerve injury or joint inflammation as models of different pain states. Some of the neurons from the control and the peripheral nerve injury groups were harvested for subsequent analysis of NMDA subunit mRNA expression. The neurons were shown by single cell RT-PCR to express all four NR2 subunits and both splice variants of NR1 that were assayed for. A majority of the neurons expressed mRNA for more than one NR2 subunit, some neurons expressed all four NR2 subunits and both NR1 splice variants, indicating that a single neurons may express more than one type of NMDA receptor. The NR2B subunit was expressed with the

highest frequency, while NR2C was expressed in a limited number of neurons. Following nerve injury changes occurred in the mRNA expression pattern in the respect that statistically significantly fewer neurons expressed NR2A in the nerve injury population compared to the control population. This reduction was accompanied by a statistically significant difference in the glutamate concentration response relationship between the control and nerve injury populations. No such difference was found between the control and the inflammation groups. The concentration response relation ship for all neurons taken together was best described by a three component fit, indicating the existence of three major discrete populations of NMDA receptors in the superficial dorsal horn. In conclusion the results suggest that the type of peripheral nerve injury used in this study is accompanied by a decrease in the number of superficial spinal cord dorsal horn neurons expressing NR2A mRNA, as well as an altered glutamate sensitivity mainly affecting one of the three NMDA receptor populations.

Poster NO. 12

Spatio-temporal Gene Manipulation of NR2B C-terminus in Mouse Brain

Emese G. Kicsi, Noboru H. Komiyama, Rolf Sprengel, Seth G. N. Grant, Centre for Genome Research, The University of Edinburgh, Edinburgh, UK.

NMDA receptors belong to the group of ionotropic glutamate gated ion channels and are essential for some forms of synaptic plasticity, learning and memory formation. According to the age of expression, the different subunits, NR1 and NR2B may play an important role in brain development and therefore it is not surprising that mice lacking these subunits die shortly after birth. Mice expressing C-terminal truncated forms of the NR2A, NR2B and NR2C subunits showed similar phenotypes as mice lacking the respective subunits (Sprengel et al., 1998). This suggests the importance of the C-terminal domains in the signaling pathways. The NR2B subunit contains a carboxyl terminal valine residue in the cytoplasmic domain. The E-S/T-X-V motif of the NR2 C-terminus binds to the 1st and 2nd PDZ domain of PSD-95. PSD-95 mice show disrupted NMDA receptor signaling (Migaud et al., 1998). PSD-95 further interacts with other signaling molecules such as SynGAP and nNOS.

To examine the coupling beween NR2B and PSD-95 we are constructing mice containing a deletion of the carboxyl terminal valine of NR2B. Crossing the mutant mice obtained in this way with mice bearing the C-terminal truncated form of NR2A subunit will result in the disruption of the signaling pathway mediated by the PSD-95 family proteins.

Since mice expressing the C-terminal truncated form of the NR2B subunit of the NMDA receptor die shortly after birth, we are carrying out conditional deletion of the C-terminus, which is encoded by a single large exon, using the Cre/*lox* system. According to the strategy the targeted exon is flanked by two *loxP* sites. By crossing these mice with Cre-recombinase expressing transgenic strains, tissue-specific and time-point dependent disruption of the NR2B C-terminus can be achieved.

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Poster NO. 13

Neuronal Death Elicited by Glutamate and Secretory Phospholipase A_2 Synergy. Experimental Neuropathology

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Secretory phospholipase A_2 binding sites on neurons in culture potentiate glutamate-induced cell death (Kolko M et al., J Biol Chem 271:32722-32728, 1996). Secretory phospholipase A_2, OS_2 and OS_1, purified from the venom of the taipan snake *Oxyuranus scutellatus scutellatus*, and the excitatory amino acid glutamate (glu) were injected into the right striatum of male Wistar rat brains. OS_2 was given in three doses 10,20 and 50 pmol. Injection of 50 pmol OS_2 caused neuronal death. The brains of these rats were histologically characterized by well demarcated infarcts in the right hemisphere. 10 and 20 pmol OS_2 did not cause tissue damage. OS_1 showed no sign of neurotoxicity. Glu was given at doses of 2,5 µmol and 5,0 µmol. Neuropathologically the injection of 5,0 µmol glu caused a well demarcated infarct in part of the striatum. Injection of 2,5 µmol glu caused no infarct. When the nontoxic concentrations of 20 pmol OS_2 and 2,5 µmol glu were co-injected, a synergistic neurotoxicity was observed. Histologically the structure was missing in the intire right hemisphere, and in half the rats the infarct comprised also part of the left hemisphere as well. The NMDA receptor antagonist MK-801 was given i.p. in normothermic rats to abolish the toxicity elicited by 50 pmol OS_2 and the synergistic neurotoxicity caused by injection of both OS_2 and glu. The combination of administred MK-801 and injection of 50 pmol OS_2 resulted in immediate death of 7 out of 8 rats and we were not able to evaluate a possible neuroprotection. MK-801 did not show any neuroprotection in the animals which were co-injected with the toxic combination of 20 pmol OS_2 and 2,5 µmol glu. The toxicity of the $sPLA_2$, OS_2 is probably due to an initial specific binding to a neuronal receptor and it is tempting to suggest a role for this enzyme in the modulation of glutamatergic synaptic function and of excitotoxicity *in vivo*.

Poster NO. 14

Expression of AMPA and Kainate Receptor Subunits In Cultured Cortical and Spinal Cord Neurones: Correlation With Pharmacological and Electrophysiological Properties

John D.C. Lambert, Jan Egebjerg, Bjarke Ebert, Kenneth V. Christensen and Wei-Min Dai, Department of Physiology, University of Aarhus, Aarhus, Denmark.

Non-NMDA glutamate receptors are composed of AMPA (assembled from subunits GluR1-4) and kainic acid (KA - assembled from GluR5-7 and KA1-2) receptors. This diversity is enhanced by alternative splicing (flip (i) and flop (o) variants) and post-transcriptional editing. Cultured cortical (Cx) and spinal cord (SC) neurones show characteristic differences in their responses to the prototypical agonists, AMPA and KA. To investigate the molecular basis of this diversity, we have performed quantitative single-cell RT-PCR on these neurones. $GluR2_i$ (with a strong contribution from $GluR1_i$) was shown to be the major constituent of Cx AMPA receptors, while SC receptors are mainly composed of $GluR4_o$. The kinetics of responses of SC neurones to AMPA are twice as rapid as Cx neurones, and the response is enhanced to a significantly greater extent on blocking desensitization with cyclothiazide (CTZ). NBQX competitively antagonised responses of both neurone types to AMPA, and also reduced desensitization of AMPA receptors on SC neurones.

KA receptors are predominantly composed of GluR6+KA2 on Cx neurones and GluR5+KA2 on SC neurones. KA evoked non-desensitizing responses on both types of neurone. While much of action is mediated by AMPA receptors, CTZ only potentiated responses of Cx neurones. The action at KA receptors was disclosed using the AMPA selective antagonist, GYKI 53655. This revealed that SC neurones have a 7-fold higher affinity for KA than Cx neurones, while the overall kinetics of the desensitizing responses were slower. Con A potentiated responses of Cx, but not SC, neurones to AMPA, showing that heteromeric GluR6+KA2 can be activated to a significant degree by this agonist.

Poster NO. 15

Molecular Determinants of agonist discrimination in the nr2b subunit of the excitatory NMDA receptor *(Oral presentaion Tuesday 14:00-14:30)*

Laube, B., Schemm, R., Polzer, S., Denzel, A. and Heinrich Betz, Dept. of Neurochemistry, Max-Planck-Institute for Brain Research, Frankfurt, Germany.

The *N*-methyl-d-aspartate (NMDA) receptor, a member of the ionotropic glutamate receptor family, is thought to be a tetrameric protein complex composed of homologous membrane-spanning NR1 and NR2 subunits and requires both l-glutamate and the co-agonist glycine for channel activation. The glycine and the glutamate binding sites of the NMDA receptor are formed by distinct regions of the NR1 and NR2 subunits, respectively, which display sequence similarity to bacterial amino acid binding proteins (Kuryatov et al., 1994; Laube et al., 1997).

To investigate the structural requirements for the specificity of the glutamate binding site, we substituted residues of the NR2B subunit by site-directed mutagenesis within the binding pocket formed by the N-terminal domain and the loop region between membrane segments M3 and M4. Again, all mutants significantly reduced the efficacy of glutamate, but not glycine, in channel gating. After heterologous expression of the mutated subunits in the *Xenopus* oocyte system, we tested the affinity and efficacy of the glutamatergic agonists NMDA, aspartate and ACBD. Most mutations similarly reduced the affinities of all agonists. However, in some cases differences in maximal inducible currents and in relative affinities of NMDA and aspartate were observed. Inhibition of channel activation by the glutamate antagonists D-AP5 and (2R)-CPP was also affected. Homology-based molecular modeling of the glutamate binding region based on the known structures of the AMPA-selective GluR2 subunit (Armstrong et al., 1998) and bacterial binding proteins suggests that in the NR2B subunit some residues selectively interact with specific ligands.

Poster NO. 16

Purification and Characterization of the Ligand Binding site of GluR6

Marie-Louise Lunn[1,2], Jette Kastrup[1], Jan Egebjerg[2], [1]Dept. of Medicinal Chemistry, The Royal Danish School of Pharmacy, Copenhagen, Denmark and [2]Inst. of Molecular and Structural Biology, University of Aarhus, Aarhus, Denmark.

One of the three major subfamilies of the ionotropic glutamate receptors is the kainate (KA) receptor family. This receptor family is well characterized pharmacologically and functionally by studies of recombinant heteromeric receptor complexes and homomeric receptors. Interestingly, recombinant homomeric KA receptors of GluR6 subunits constitute a functional receptor with a distinct pharmacological profile from both the AMPA receptor subunits and from within the KA receptor family itself by being unable to bind AMPA and AMPA analogue compounds. A crystal structure of the KA receptor will therefore provide valuable information for understanding the receptor function, binding of selective agonists and thus the diversity even within the subfamily. Recently, the crystal structure of the ligand binding site of the AMPA receptor subunit GluR2 was determined. Structural data on the KA receptor is not yet available but different experiments have demonstrated that the ligand binding site can be expressed as a soluble protein. In the present work, we have employed a similar strategy for GluR6. A number of C-terminal His-tagged fusion proteins of the two extracellular ligand binding segments S1 and S2 of GluR6 have been constructed and combined by peptide linkers of various lengths. After expression in *E.coli*, the protein was purified from inclusion

bodies and further folded yielding a solution of the protein in a conformation that displayed ligand binding activity (KA, K_D = 25nM). The folded protein showed resistance to proteolysis when characterized by various proteases in the absence and presence of the endogenous ligand. Crystallization studies will be initiated in order to determine the 3D structure of the GluR6 ligand binding domain.

Poster NO. 17

Structure and Function of Glutamate Receptor Ion Channels

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Ionotropic glutamate receptors (GluR) are the predominant mediators of excitatory synaptic signalling in the CNS. The GluR ligand-binding domain (S1S2) is homologous to the bacterial glutamine-binding protein, with agonist bound in a cleft between two lobes of the protein. Despite the availability of a crystal structure of S1S2[1], however, little is known about the detailed processes that accounts for the coupling of agonist binding to channel gating and desensitization.

We have taken a dual approach to this issue. The first involves a biochemical characterization of agonist binding by S1S2 from GluRD. Using stopped-flow techniques and an intrinsic fluorescence signal, we have followed the kinetics of agonist binding to S1S2. Association and dissociation rates are comparable to those of the periplasmic binding proteins. Initial docking of ligand to the binding site is followed by protein isomerization. Solution X-ray scattering indicate, however, that high-affinity binding is achieved without dramatic motion of the lobes[2]. The agonist-binding kinetics of a panel of site-directed mutants have permitted us to identify side chains that are important for the conformational coupling between binding and subsequent gating and/or desensitization steps.

A second approach involves quaternary structure analysis of intact GluR. The molecule studied is a homomer composed of rat GluRB subunits (protomeric mass of 106 kD) that is expressed in insect cells, solubilized in Triton X-100 and purified by affinity chromatography. A single-particle reconstruction from negatively stained electron microscopic images reveals the molecular envelope of this ion channel at approximately 25 Å resolution. The molecule appears as an elongated rectangular volume lacking a central symmetry axis comparable to those seen for the AChR or K^+-channel structures. An extended central cavity is observed running parallel to the longest direction of the molecule.

Poster NO. 18

A Molecular Model for the Binding of an AMPA-R Subtype-Selective Agonist

D.S. Pickering, J.K. Christensen, T. Coquelle, T.G. Banke, T.Liljefors* and A. Schousboe, The Royal Danish School of Pharmacy, Dept. of Pharmacology and Dept. of Medicinal Chemistry*,Copenhagen, Denmark.

The pharmacological toolbox of compounds available for studying the non-NMDA ionotropic glutamate receptors has grown to include both selective agonists and antagonists of AMPA vs. kainate receptors as well as a GluR5 selective agonist, ATPA. Here, we report the pharmacological character-ization of an AMPA receptor subtype-selective agonist, (*S*)-bromohomoibotenic acid (BrHIBO).

BrHIBO exhibited a 37-fold selectivity among recombinant, homomeric AMPA-R expressed in *Xenopus* oocytes (EC_{50} (μM): $GluR2_o$(Q), 5.4; $GluR1_o$, 14; $GluR4_o$, 39; $GluR3_o$, 202). At heteromeric channels containing GluR2(R), the potency of BrHIBO matched that of the homomeric channels lacking GluR2, suggesting that agonist potency is independent of the presence of GluR2 in the complex.

To uncover the basis of this agonist's selectivity, a series of single point mutants between $GluR1_o$ and $GluR3_o$ were examined. Non-conserved residues in the S2 domain of $GluR1_o$ were changed to the corresponding $GluR3_o$ residue. Thereby, we have identified that Y716 in $GluR1_o$ is responsible for this selectivity of BrHIBO. The corresponding residue in $GluR3_o$ is F728 and interchange of these residues exchanges BrHIBO's selectivity in both functional (EC_{50}) experiments and [^{3}H]AMPA radioligand binding (K_i) experiments. Computer-assisted modelling of the agonist binding site of $GluR1_o$ and $GluR3_o$, based on the known X-ray crystal structure of GluR2, indicated that the isoxazole ring of BrHIBO is likely rotated 180° and twisted, with respect to AMPA. This allows an H-bonding of the 5'-hydroxyl anion of BrHIBO with T665. The residue Y716 is also in the binding pocket but does not directly interact with ligands. We propose that the mutation Y716F disrupts the local H-bonding matrix of water molecules in the binding site and consequently decreases the affinity of BrHIBO for $GluR1_o$.

Poster NO. 19

Resolution of ATPA and Thio-ATPA, two potent and selective GluR5 agonists

Stensbøl, T.B. [1]; Nielsen, B. [1]; Jensen, H.S. [2]; Frydenvand, K.[1]; Borre, L.[2]; Egebjerg, J. [2]; Johansen, T. [1]; Krogsgaard-Larsen., P. [11]Department of Medicinal Chemistry, The Royal Danish School of Pharmacy, Copenhagen, Denmark.[2]Department for Molecular and Structural Biology, University of Aarhus, Aarhus, Denmark.

The ionotropic glutamate receptors are divided into NMDA, AMPA and kainic acid receptors. The pharmacological distinction between AMPA and kainic acid receptors has, so far, been difficult, due to the lack of ligands that specifically interact with kainic acid receptor subtypes. Whereas AMPA and ACPA are highly selective AMPA receptor agonists, ATPA has recently been shown selectively to interact withGluR5 receptor subunits. Thio-ATPA,

has previously been characterize as a relatively weak AMPA receptor agonist. However, in oocytes expressing GluR5 receptor subunits Thio-ATPA was found to be an agonist three fold more potent (EC_{50} 0.23 μM) than ATPA and more selective at GluR5 receptors as compared to ATPA. Using chiral HPLC we have separated ATPA and Thio-ATPA into enantiomers in high yield and with high enantiomeric purity (>98.5% ee). The absolute configuration was, in both cases, assigned unequivocally. The molecular pharmacology at cloned Glu receptors revealed that the agonist activity of ATPA at GluR5 receptors resides exclusively in the (*S*)-enantiomer, whereas the (*R*)-enantiomer was shown to be a weak antagonist at AMPA receptor subunits (Ki 33–75 μM at GluR1–4). The pharmacology of the enantiomers of Thio-ATPA will also be presented.

[1] Stensbøl, T.B.; Borre, L.; Johansen, T.N.; Egebjerg, J.; Madsen, U.; Ebert, B.; Krogsgaard-Larsen, P. *Eur. J. Pharmacol.* **380**, 153–162, 1999.

Poster NO. 20

Localization of Domains and Amino Acids Involved in GluR6 and GLuR7 Ion Channel Function

N.Strutz, C.Villmann, A.Thalhammer and M. Hollmann. Glutamate Receptor Laboratory, Max-Planck-Institute for Experimental Medicine, Göttingen.

The kainate receptor subunits GluR6 and GluR7 belong to the same receptor subfamily and are 86% identical at the amino acid level. GluR6 gives large currents in oocytes, whereas GluR7 does only respond to unphysiologically high concentrations of agonist, and even then responses are only detectable in transfected mammalian cells but not in *Xenopus* oocytes. We set out to investigate if a domain, region, or even a single amino acid determines the functional differences between GluR6 and GluR7. We constructed several chimeras between GluR6 and GluR7 by replacing parts (N-terminal domain, pore forming region, C-terminal domain, S2 domain) of one receptor by homologous parts of the other. All chimeras were capable of forming functional ion channels which could be activated by physiological concentrations of Glu and KA. Interestingly, chimeras containing the region between transmembrane domains (TMDs) B and C (= L3 domain) of GluR7 in a GluR6 background gave very reduced currents whereas the current amplitudes of the reverse construct were comparable with GluR6 wild type. Thus, the functional differences between GluR6 and GluR7 appear to be linked to the L3 domain. We generated several GluR7 point mutants which are able to conduct currents that can be measured in *Xenopus* oocytes. All these point mutations were located in the L3 domain. It has been shown recently by coexpession studies that wild type GluR7 can have a reducing effect on wild type GluR6 current amplitudes. Using the same domain transplantation strategy we could localize the region in GluR7 which is responsible for this reducing effect to the L3 domain. For GluR6 the ratio of glutamate- to kainate-evoked currents after treatment with concanavalin A is one. All functional mutant GluR7 clones, however, showed an increased glutamate- to kainate-evoked response ratio. The domain in GluR7 which determines this ratio could also be localized to the L3 domain.

Poster NO. 21

Differentially Expressed Genes Following Induction of Ischemic Tolerance as Studied by RFDD-PCR, a New Differential Display Technique

Maria L. Wrang, Carsten W. Alsbo, Flemming Møller Jensen, Nils H. Diemer, Lab of Neuropathology, University of Copenhagen, Danmark.

Previous work has shown that ischemic preconditioning (3 min) induces a state of tolerance to subsequent ischemic insults (7 min) in the CA1 region of the hippocampus in rat brain. The tolerance has been found to be maximal three days after the tolerance inducing ischemia. The mechanisms whereby ischemic tolerance is induced are basically unknown. Activation of the NMDA receptors has been proposed to play as central role, but others find that tolerance can be induced in rats treated with MK-801 (an NMDA antagonist). A specific subunit of the other major glutamate receptor family, the AMPA subunit GluR2-flop, have recently been found to increase following induction of ischemic tolerance, but AMPA-antagonists do not affect tolerance induction.

Transcriptional regulations are believed to be a prerequisite for development of tolerance to ischemia. To detect these transcriptional regulations we have applied a new differential display technique called RFDD-PCR to mRNA purified from rat brains subjected to a tolerance inducing ischemia, with survival times of 1, 2, or 3 days. Naïve animals were used as controls.

We found 70 genes, whose expression is changed significantly by induction of ischemic tolerance. When grouping the genes according to their function we find that they fall in seven major groups, of which extracellular matrix proteins and receptors constitute a majority. By applying a new molecular biology technique to ischemic tolerance, we have thus detected the possible involvement of a group of genes hitherto unrelated to ischemic tolerance, namely the extracellular matrix proteins, and established the involvement of several genes likewise not related to ischemic tolerance previously.

BENZON SYMPOSIUM No. 47

MOLECULAR PHARMACOLOGY OF ION CHANNELS

AUGUST 13-17, 2000, COPENHAGEN, DENMARK

Organizing committee:
Jan Egebjerg, Søren-P. Olesen and Povl Krogsgaard-Larsen

Abstracts - TUESDAY, August 15, 2000

Structure and Function in P2X Receptors
North RA, Institute of Molecular Physiology, University of Sheffield, Sheffield, UK.
Currently known forms of P2X receptor channels form as homomultimers (P2X1-P2X5, P2X7) or heteromultimers (P2X1/5, P2X2/3, P2X4/6). Functional and biochemical evidence suggests that the channel might form from three subunits. We recorded currents in response to activation of heteromeric P2X1/5 and P2X2/3 receptors expressed in HEK293 cells to characterize further their functional properties. At the P2X1/5 receptor ATP concentration-response curve had a very low threshold concentration (1 nM), and a Hill slope of one. TNP-ATP was a weak partial agonist, and a non-competitive antagonist which inhibited maximal ATP currents by 60%. Increasing or decreasing pH from 7.3 shifted the ATP concentration-response curves to the right by 5-fold and decreased the maximum current by 40%. Calcium permeability was lower than that observed for other P2X receptors (PCa/PNa ratio = 1.1), and no change in cation permeability with time during prolonged ATP application was observed. Similar studies at the P2X2/3 receptor, in combination with substituted cysteine mutagenesis, showed that this heteromer also has properties fundamentally different from those known for any of the homomeric channels. The results will be discussed with relation to the properties of P2X receptors expressed by native cells and tissues.

Inhibitory and excitatory glycine receptors: Structures, functions and synaptic localization
H. Betz, B. Wittekind, A. Denzel, K. Hirzel and B. Laube, Max-Planck-Institut für Hirnforschung, Abt. Neurochemie, 60528 Frankfurt, FRG.
The strychnine-sensitive glycine receptor (GlyR) is a pentameric chloride channel protein that exists in developmentally and regionally regulated isoforms in the CNS. These result from the differential expression of four genes encoding different variants (α 1-α 4) of the ligand-binding subunit of the GlyR. Their assembly with the structural β subunit is governed by "assembly motifs" within the extracellular domains of these proteins and creates chloride channels of characteristic conductance properties. GlyR gating is potentiated by Zn2+, a metal ion coreleased with different neurotransmitters. In the adult organism, GlyR activation produces a potent inhibition of neuronal firing. Consequently, mutations in GlyR subunit genes result in motor disorders characterized by hypersensitivity to sensory stimuli, convulsions and muscle stiffness. The *spastic* and *spasmodic* phenotypes in mouse as well as human hereditary startle disease will be discussed.
During development, glycine receptors mediate excitation that results in Ca2+ influx and neurotransmitter release. Ca2+ influx triggered by the activation of embryonic GlyRs is required for the synaptic localization of the GlyR and its anchoring protein gepyhrin. The latter forms a cytoskeleton-attached scaffold at developing postsynaptic sites. Gene inactivation studies have documented an essential role of gephyrin in the synaptic localization of both GlyRs and $GABA_A$ receptors.

GABAA-receptors functions in vivo revealed by targeted point mutations
Möhler H, Crestani F, Fritschy J.-M, Benke D & Rudolph, U, Institute of Pharmacology, ETH and University of Zürich, Zürich, Switzerland.
GABAergic transmission represents the major inhibitory control in the brain. The drug-induced enhancement of GABAA-receptor function, in particular by drugs acting at the benzodiazepine (BZ) site, elicits a broad therapeutic spectrum. The classical benzodiazepine drugs interact with all GABAA-receptor subtypes with comparable affinity. In order to generate drugs with a more selective profile and fewer side effects an attempt was made to attribute a pharmacological repertoire to particular GABAA-receptor subtypes. Earlier studies on recombinant receptors (1) had identified a conserved amino acid in the drug binding site of all BZ-sensitive GABAA-receptor subtypes as a molecular switch to abolish ligand affinity. A corresponding point mutation was therefore introduced into distinct GABAA-receptor subtypes in mice in order to render the respective receptors diazepam-insensitive. The deficit in the pharmacological profile of diazepam in these mutant mice can be attributed to the respective silenced GABAA-subtype receptor. Thus, the pharmacological profile of ligands of the benzodiazepine site can be genetically dissected

(2). Using this approach, the 1 type of GABAA-receptor was found to mediate the drug-induced sedation and amnesia. The anxiolytic activity was attributed to the receptors 2, 3 and/or 5 which are presently subdivided by further genetic analysis. These results demonstrate that specific GABAA-receptor subtypes are promising targets for the development of selective anxiolytic and hypnotic drugs. In addition, therapeutic indications beyond those of the classical benzodiazepine drugs my be envisaged.

1) Wieland, H.A. et al. J. Biol.chem. 267, 1426-1429 (1992)
2) Rudolph, U. et al. Nature 401, 796-800 (1999)

$GABA_A$ Receptors: Determinants of Agonist Activity

Bjarke Ebert, Martin Mortensen, Povl Krogsgaard-Larsen, Sally A. Thomson and Keith A. Wafford, Department of Pharmacology, The Royal Danish School of Pharmacy, Copenhagen, Denmark

The binding site for the GABA molecule of the $GABA_A$ receptor is located at the interface between the α and the β subunit. The amino acid residues contributing to the binding site are conserved across the different subunits, thus restricting the possibility for the development of affinity based subtype selective compounds. However, characterization of a series of GABAergic ligands have shown that the functional consequences of binding to the $GABA_A$ receptors are highly dependent on the subunit composition. Compounds like piperidine-4-sulphonic acid (P4S) and analogues acts as full agonists at some receptor subunit combinations and as partial agonists or antagonists at other combinations. It is therefore possible to obtain a functional subtype selectivity of the GABA ligands irrespective of the affinity.

Co-expression of α 1 and α 6 subunits in GABA receptors found in the cortical neurones adds a further layer of complexity to the understanding of the functional consequences of subunit composition. Thus, whereas P4S is a low efficacy partial agonist at both α 1 and α 6 containing receptors, co-expression of α 1 and α 6 in the same oocyte results in a pharmacological profile, where P4S is acting as a high-efficacy partial agonist. However, when same approach is used with α 1 (P4S, low efficacy) and α 3 (P4S, high efficacy) or α 1 and α 5 (P4S, high efficacy) in oocytes the functional consequence is for P4S a low efficacy and a significantly reduced potency.

Taken together, our data clearly indicate that current knowledge on the determinants of potency and efficacy still is sparse and further studies are needed.

Nicotinic Cholinergic Mechanisms in the Central Nervous System

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Neurons that use acetylcholine as their neurotransmitter drive or modulate a wide variety of behaviors. Cholinergic neurons usually make broad, diffuse, sparse projections that innervate nearly every neural area. By initially acting on nicotinic acetylcholine receptors (nAChRs) within these cholinergic systems, nicotine can increase arousal, enhance attention, influence cardiovascular properties, and influence a number of cognitive functions. The results of synaptic studies from my lab will serve as a summary of potential nicotinic mechanisms that underlie those higher level consequences.

Nicotinic nAChRs form a family of ligand-gated channels that are composed of five polypeptide subunits surrounding a central, water-filled pore. The broad, sparse cholinergic projections ensure nicotinic influences on multiple neurotransmitter systems and enable nAChRs to modulate neuronal activity on a wide scale. Thus, fast excitatory nicotinic transmission in the CNS reaches wide areas, but it is usually provides only a small component of the overall excitatory inputs into an area. Presynaptic and preterminal nAChRs can have global effects by influencing many neurotransmitter systems. Presynaptic nAChRs can initiate a raise in calcium in the presynaptic terminal that enhances the release of various neurotransmitters, including GABA and glutamate. In addition, nAChRs can serve other modulatory roles. For example, excitation of GABAergic interneurons via nAChRs can induce either inhibition or disinhibition of hippocampal pyramidal neurons. Thus, nAChRs are capable of modulating circuits by exciting interneurons and, subsequently, inhibiting or disinhibiting pyramidal neurons. In the midbrain, nicotine, as obtained from tobacco, can activate nAChRs and excite dopaminergic neurons in the mesotelencephalic system (Pidoplicko et al., 1997, Nature, 390:401-404). Our results suggest that many factors contribute to the multiple effects on the CNS produced by nicotine.

Gating and Electrostatics in the Acetylcholine Receptor Channel

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The cation-conducting channel of the nicotinic acetylcholine receptor is lined by the first (M1) and second (M2) membrane-spanning segments of each of its five subunits ($\alpha_{2\beta\gamma\delta}$). The narrowest part of the channel is formed by a barrel of five-residue stretches, one from each subunit, starting in the predicted cytoplasmic loop between M1 and M2 and extending into the predicted cytoplasmic end of M2. In the mouse-muscle α subunit, these residues are $G_{240}EKMT_{244}$. In the open state of the channel G, E, K, and T, when substituted by C, are accessible to a small, positively charged, SH-specific, methanethiosulfonate reagent from both the extracellular and the intracellular sides of the membrane. In the closed state, G is accessible only from the intracellular side, T is accessible only from the extracellular side, and E and K are inaccessible. Hence, the activation gate lies between G and T in the vicinity of E

and K. Previous work by others showed that T and the aligned residues in the other subunits are crucial for selectivity among cations and that E and its aligned residues (E in β and δ and Q in γ) are crucial for cation conductance. The ring of four E and one Q also gives rise to a large, negative, intrinsic electrostatic potential (~ -200 mV) in the narrow part of the open channel. This was estimated by comparing the rate constants of reaction of a positively charged reagent and a polar but neutral reagent with $T_{244}C$. As each E was mutated to an uncharged Q, the potential was shifted about 50 mV toward zero, extrapolating to zero potential when all four E were neutralized. Also, each of the subunits contributes a K to this region; yet, surprisingly, mutation of these K had little effect either on the intrinsic electrostatic potential or, as shown by others, on cation conductance. In the open state of the channel, the ε $-NH_3^+$ group of the K (unlike the SH of the substituted C) must be oriented away from the channel lumen. Thus, a short, narrow part of the channel at its cytoplasmic end contains the gate and the selectivity filter. The nature of the gate, however, whether the block is steric or electrostatic or both, is not yet known.

Poster NO. 22

Rat $P2X_1$ and $P2X_2$ isoforms assemble after heterologous expression in *xenopus* oocytes

Aschrafi A, Rettinger J & Schmalzing G, Institute for Pharmacology, University of Frankfurt, Frankfurt, Germany.

P2X receptors represent a family of transmitter-gated ion channels activated by extracellular ATP. To date, seven different P2X isoforms have been discovered through cDNA cloning.

P2X receptors function as homo-multimeric cation-permeable ion channels and, in some cases, as heteromeric channels consisting of two different P2X receptor subtypes. the P2X receptor subtypes, $P2X_2$ and $P2X_3$ function as a heteromeric channel in rat nodose ganglion neurons where it exhibits distinct pharmalogical and electrophysiological properties.

To investigate a possible heteromeric assembly of $P2X_1$ and $P2X_2$ subtypes, we utilized a co-immunoprecipitation assay in which the hexa-histidyl tagged rat $P2X_1$ were expressed together with non-tagged $P2X_2$ receptor subunit in *xenopus* oocytes. Subsequent Blue-Native PAGE analysis revealed a multimeric complex with a mass intermediating homomeric $P2X_1$ and $P2X_2$ receptors. Treating of the natively isolated probes with the reducing agent DTT revealed two further bands on the Blue-Native polyacryamide gel, indicating that also the heteromeric P2X1-P2X2 possibly consist of a trimeric structure.

Poster NO. 23

Biochemical and electrophysiological characterisation of $P2X_1$ concatamers in *Xenopus* oocytes. What makes the current?

Nicke, A., Rettinger, J., and Schmalzing, G., Dept. of Pharmacology, J. W. Goethe-University, Biocenter Niederursel, 60439 Frankfurt, Germany.

Determination of the subunit composition and stoichiometry of ion channel subtypes is an essential but still very challenging problem in ion channel research. Although the electrophysiological characteri-sation of concatenated ion channel subunits has been successfully applied to determine quaternary structures and functional properties of a variety of ion channels, ambiguous results have been reported.

Here we present the first detailed biochemical analysis of the synthesis, assembly and surface expression of concatamers using the $P2X_1$ receptor as a model. Full-length $P2X_1$ concatamers consisting of 2-6 subunits were synthesised with the expected masses and assumed the correct membrane topology as inferred from their carbohydrate content. All constructs gave rise to ATP-triggered ion channels upon expression in *Xenopus* oocytes. Surprisingly, blue native PAGE analysis of the plasma membrane form of all concatamers exclusively revealed complexes equivalent to three P2X subunits. These complexes were mainly composed of either three monomers or one monomer plus a concatenated dimer. Only small amounts of concatenated trimers reached the plasma membrane. No significant assembly of full-length concatamers resulting in complexes with more than three subunits occurred. Deletion of codons that might form internal translation initiation sites in and before the linking sequence had no influence on the formation of lower order side products. Likewise, analysis of concatamers containing nonfunctional mutant $P2X_1$ subunits indicated that the formation of lower order concatamers and monomers was not due to a false translation initiation. Taken together, the results demonstrate that the construction of concatamers does not guarantee the appearance of defined ion channel complexes in the plasma membrane and that a thorough biochemical analysis might be required. Furthermore, they strongly support a trimeric structure of the P2X receptor (Nicke *et al., EMBO J.* **17**, 3016-3028).

Poster NO. 24

Distinct properties of $P2X_{cilia}$ - an airway P2X receptor regulated by Na^+

Silberberg, SD, Ben GurionUniversity of the Negev, Beer Sheva, Israel.

Airway ciliated cells express an extracellular ATP -gated channel ($P2X_{cilia}$ channel) that is competitively inhibited by extracellular Na^+, (Ma, *et al. Nature* **400**:894; 1999) and strongley attenuated by extracellular divalent cations. Recombinant $P2X_7$ receptors have also been shown to have similar properties (Michel *et al. Naunyn Schmiedebergs Arch Pharmacol.* 359:102; 1999), raising the possibility that $P2X_{cilia}$ is $P2X_7$. In this study we investigated whether

P2X$_{cilia}$ channels are distinct form P2X$_7$ channels. To address this question, we characterized the binding site for ATP, the binding site for Na^+, and the pore properties of *P2X*$_{cilia}$ channels. As for the cloned P2X$_7$ receptor, the agonist potency order for eliciting inward currents through *P2X*$_{cilia}$ channels was 3'-O-(4-benzoyl)benzoyl ATP (BzATP) > ATP >> ADP. The relative permeability of *P2X*$_{cilia}$ channels to various cations including Na^+ was estimated with the GHK equation by measuring the zero-current (reversal) potential of the net current activated by ATP_o. Whole-cell patch-clamp recordings were made from freshly dissociated rabbit airway ciliated cells. The reversal potential was measured using an intracellular solution containing Cs^+ as the chief cation and an extracellular solution containing the tested cation. Unlike P2X$_7$ channels, *P2X*$_{cilia}$ channels did not exhibit a time-dependent change in ion selectivity during long exposures to ATP_o. The permeability (relative to Cs^+) of NH_4^+ : Li^+ : K^+ : Rb^+ : $NMDG^+$ were estimated to be 2.9 : 1.6 : 1.2 : 1.1 : 0.2, respectively. The permeability to Na^+ (relative to Cs^+) was estimated to be 1.27, by comparing the reversal potentials measured in extracellular Cs^+ solutions containing different concentration of Na^+ to the reversal potentials predicted by the GHK equation. Using a similar approach, Ca^{2+} was estimated to be 9-fold more permeant than Cs^+. Thus, *P2X*$_{cilia}$ channels have both common and unique properties in comparison to the cloned P2X$_7$ channels, suggesting that *P2X*$_{cilia}$ may be a P2X$_7$ variant.

Poster NO. 25

The mechanism of action of an inhibiting neurosteroid, pregnenolone sulfate, on recombinant α 1β 2γ 2 GABA$_A$ receptors

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The functional properties of GABA$_A$ receptors can be modified by a variety of pharmacological agents including benzodiazepines, barbiturates and neurosteroids. We have examined the mechanism of action of an inhibiting neurosteroid, pregnenolone sulfate (PS) naturally present in the mammalian central nervous system, using cell-attached single-channel patch clamp technique. Our results show that when the receptors are activated by high ($\geq$50 μ M) concentrations of GABA, the receptor activity takes place in isolated clusters arising from openings and closings of a single receptor-channel. The intracluster activity can be characterized by the sum of three exponentials for channel openings, and by the sum of three exponentials for channel closed periods. Co-application of GABA and progressive concentrations of PS does not affect the open or closed time distributions but results in a voltage-independent shortening of single-channel clusters. The IC$_{50}$ for the PS-caused reduction in cluster durations is at ~1 μ M. The magnitude of PS effect is similar when receptors are activated by 50 μ M or 1 mM GABA suggesting that the number of bound ligands does not affect inhibition. Activation of the receptors by a low efficacy agonist piperidine-4-sulfonic acid does not affect inhibition by PS. Addition of a potentiating neurosteroid, ACN, or a GABA$_A$ receptor potentiator, pentobarbital does not prevent PS-caused inhibition. Site-directed mutagenesis studies demonstrate that mutation of the 2' (RDL) residue in M2 segment to serine in the α - (V256S) but not β -subunit (A252S) prevents inhibition by PS. We propose that PS acts as an allosteric, state-independent inhibitor of the GABA$_A$ receptor whose binding site is distinct from those for barbiturates and potentiating neurosteroids. *GA is a McDonnell Center for Molecular and Cellular Neurobiology fellow; supported by P01 GM47969 to JHS.*

Poster NO. 26

Glycine receptor channels: rapid potentiation byintracellular calcium

Bregestovski P, Fucile S, De Saint Jan D & Prado De Carvalho L, InstitutePasteur, Paris, France. Glycine receptors (GlyRs) belong to the family ofligand-gated ion channels which includes receptors for γ-aminobutyric acid (GABA), serotonin and acetylcholine. GlyR provideinhibitory neurotransmission mainly in spinal cord and brainstem synapsesof vertebrates. Its function is known to be regulated by protein phosphorylation and several other pathways (Betz et al., Ann NY Acad Sci.**868**: 667, 1999). We describe here anew regulatory mechanism: fast potentiation of GlyR channels byintracellular Ca2+.

Using apatch-clamp and imaging techniques we demonstrated that in spinal cordneurons and the HEK cells expressing homomeric GlyRs: (i) Ca2+ influx through receptor-operated or voltage-gated Ca2+-permeable channels causes rapid and transientaugmentation the amplitude of GlyR currents; (ii) the minimal intervalnecessary for GlyR channel potentiation is less than 100 ms; (iii) phosphorylationand G-protein pathways do not underlie this phenomenon; (iv) elevation ofintracellular Ca2+ results in prolongation of single channel burst kinetics; (v) ininside-out patches, exposure of the cytoplasmic side of the membrane toCa2+ had no effect on activity of GlyRchannels; (vi) Ca2+ potentiates GlyR by increasing its apparent affinity toglycine. Our results suggests thatCa2+ ions trigger a powerful and rapidmodulation of neurotransmission at glycinergic synapses controlling a gating of GlyR channels througha diffusible Ca2+-sensitive cytoplasmicintermediate.

Poster NO. 27

The Metabotropic $GABA_B$ Receptor Directly Interacts with the Activating Transcription Factor 4 (ATF-4)

R.Nehring[1], H. Horikawa[2], O. El Far[2], M. Kneussel[2], S. Stamm[3], E. Wischmeyer[1], H. Betz[2] and A. Karschin[1], [1]Dept. Molecular Neurobiology of Signal Transduction, MPI Biophysical Chemistry, Göttingen; [2]Dept. Neurochemistry, MPI Brain Research, Frankfurt; [3]Research Group Neuron-Specific Splicing, MPI Neurobiology, Martinsried, Germany.

G protein-coupled receptors regulate gene expression by cellular signaling cascades that target transcription factors and their recognition by specific DNA sequences. In the central nervous system heteromeric metabotropic $GABA_B$ receptors through adenylyl cyclase regulate cAMP levels which may control transcription factor binding to the cAMP response element (CRE). Using yeast-two hybrid screens of rat brain libraries we now demonstrate that $GABA_B$ receptors are engaged in a direct and specific interaction with the activating transcription factor 4 (ATF-4), a member of the CREB/ATF family. As confirmed by pull-down assays, ATF-4 associates via its conserved basic leucine-zipper domain with the C-termini of both $GABA_B$R1 and $GABA_B$R2 at a site which serves to assemble these receptor subunits in heterodimeric complexes. Confocal fluorescence microscopy shows that $GABA_B$R and ATF-4 are strongly coclustered at the dendritic membrane surface of cultured hippocampal neurons. In oocyte coexpression assays short-term signaling of $GABA_B$Rs via G proteins was only marginally affected by the presence of the transcription factor, but ATF-4 was moderately stimulated in response to receptor activation in *in vivo* reporter assays. Thus inhibitory metabotropic $GABA_B$Rs may regulate activity-dependent gene expression via a direct interaction with ATF-4.

Poster NO. 28

Gating of nicotinic receptors by tubocurarine and acetylcholine

Heckmann & Dudel, Institut für Physiologie der Technischen Universität München, Germany.

In experiments with 1 μM tubocurarine (TC), quantal end-plate current amplitudes from up to 3 days old (fetal channels) and also from adult mice were reduced to less than half. In addition current rise times were increased and their decay time constants reduced. The effects were larger after inhibition of ACh hydrolysis. The classical explanation for an effect of competitive blockers on the time course of synaptic currents is that TC reduces ACh binding to post-synaptic receptors and thereby allows ACh to be cleared more rapidly from the synaptic cleft (Katz and Miledi, 1973 *J. Physiol.* 231:549-574). On the other hand TC is known to be a weak partial agonist (see Steinbach and Chen, 1995 *J. Neurosci.* 15:230-240). To test for a direct effect of TC on the kinetics of the postsynaptic receptors we applied 100 μM ACh in 30 ms pulses to outside-out patches from mouse myotubes. Preincubation of patches with 0.03-1 μM TC reduced the amplitude of the response and in addition clearly changed the current time course relative to the controls. The current rise was biphasic with 0.1-0.5 μM TC. The initial phase was similar to the rise in control recordings and thus probably the response of unblocked channels. The second slowly rising current component was somewhat masked by the response of unblocked channels at low TC concentrations but almost pure with 1 μM TC. At this TC concentration the current reached 1/5 of the control amplitude with a rise time of 11.2 ± 2.5 ms compared to 0.46 ± 0.08 ms in controls (n=6). In view of the different affinities of TC for the two ligand binding sites of nicotinic receptors (Sine and Taylor, 1980 *J. Biol. Chem.* 255:10144-10156) we propose that the slow current component is the response of nicotinic receptors with one TC and one ACh bound.

Poster NO. 29

Ca^{2+} permeability of heterologously expressed human neuronal nicotinic L248Tα 7 receptors

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We expressed a cDNA coding for the human neuronal nicotinic α 7 receptor subunit mutated from Leu-248 into threonine in *Xenopus* oocytes. When activated by nicotine the human receptors expressed generated currents showing low desensitization, linear current-voltage relation and high apparent affinity for the ligand. These characteristics are similar to those described for the chick α 7 mutant. These properties were maintained when the human L248Tα 7 mutant was expressed in human Bosc 23 cells. Whole-cell clamp and fluorescence measurements, using the Ca^{2+} indicator dye fura-2, showed that nicotine induced a Ca^{2+} influx in standard medium. The average fractional Ca^{2+} current flowing through L248T α 7 nicotinic acetylcholine receptors (nAChRs) was ca. 7 %, which is much larger than that flowing through muscle nicotinic ε -nAChRs (c.a. 4 %). Determinations of the relative Ca^{2+} permeability in oocytes, made in the absence of Cl^- by measuring the shift in reversal potential due to the increase of the external Ca^{2+} concentration from 1 to 10 mM, showed that the human wild type α 7 nAChR was more Ca^{2+} conductive than the L248Tα 7 mutant. We conclude that the Ca^{2+} permeability of the *homomeric* α 7 nAChR is larger than that of the *heteromeric* neuronal nicotinic receptors studied to date.

This work is supported by Telethon (grant n. E0912 to F.E.)

Poster NO. 30

Kinetic analysis of alpha7 nAChR fast desensitization in acutely dissociated hypothal- amic neurons: implications for therapeutics *(Oral presentation Tuesday 14:30-15:00)*

Roger L. Papke, Edwin Meyer, and Vladimir V. Uteshev. University of Florida, Department of Pharmacology and Therapeutics.

The alpha-bungarotoxin sensitive alpha7-type receptors of the brain have been identified as potentially important therapeutic targets for cognitive and neurodegenerative disorders. We have developed the use of acutely dissociated hypothalamic neurons for the study of this unique receptor subtype. The hypothalamus shows strong expression of the alpha7 nicotinic receptor gene product and neurons acutely dissociated from the tuberomammillary (TM) nucleus of the posterior hypothalamus have robust and stable nicotinic receptor-mediated current responses that can be associated with alpha-bungarotoxin sensitive alpha7-type receptors exclusively. We have studied the nicotinic receptor-mediated responses of these cells to the rapid application of ACh and the alpha7-selective agonist 4OH-GTS-21. The rapid application of high agonist concentrations leads to a brief period of maximal receptor-activation followed by profound desensitization. Rise rates, decay rates, and the degree to which current was desensitized were all agonist and concentration-dependent. Our kinetic analyses of alpha7 receptor activation and desensitization have permitted us to develop and test models for alpha7receptor function. We have found that most of the salient features of the data can be explained by a fractional occupancy model, which includes both fast and slow desensitization processes. Fast desensitization is fundamentally concentration-dependent and can beexplained by associating stable closed states to a higher level of agonist occupancy than what is effective for opening the ion channel. Recovery from this fast desensitization appears to occur as rapidly as agonist dissociates. In addition to this concentration-dependent fast desensitization, channels also convert more slowly to another form of desensitization, which can be described with conventional first-order kinetics of onset and recovery. We observe that there can be a dynamic equilibrium between activatable and desensitization that supports significant levels of persistent current with agonist concentrations several orders of magnitude lower than those used to evoke maximal peak currents. This suggests a role for alpha7 receptors in regulating calcium homeostasis within a narrow range when alpha7-selective agonists are applied at low concentrations. In separate experiments, we have shown such low concentration applications of alpha7 agonists to be cytoprotective in models of both apoptotic and necrotic cell death. These observations are likely to be of special significance for the potential therapeutic targeting of alpha7 receptors in neurodegenerative conditions such as Alzheimer's disease.

Poster NO. 31

Co-Agonist Effects og Choline on α 4β 2 and α 4β 4 nAChRs

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Since the discovery that choline is a low affinity, full agonist on α 7 subunit-containing nAChRs, choline is considered to be an important signalling molecule in the CNS. On heteromeric neuronal and endplate nAChRs choline acts as a very weak, partial agonist. Weak partial nAChR agonists and competitive nAChR antagonists, e.g. d-tubocurarine and atropine, may have unusual co-agonist effects on nAChRs. These drugs potentiate ion currents evoked by low concentrations of ACh on heteromeric nAChRs. Potentiation occurs when one of the agonist binding sites of the nAChR is occupied by an agonist molecule and the other by d-TC or atropine. We have investigated whether the weak partial agonist choline has similar effects on heteromeric neuronal α 4β 2 and α 4β 4 nAChRs expressed in *Xenopus* oocytes using the two-microelectrode voltage clamp technique.

We found a bell-shaped concentration-effect relation for choline when ion currents were evoked by low concentrations of ACh. Low concentrations ($\geq$10 μ M) of choline strongly potentiated, whereas high concentrations of choline (>1 mM) inhibited ACh evoked ion currents. The potentiating effect of choline at low ACh concentrations is surmounted at elevated concentrations of ACh and only inhibition by choline is observed when ion currents are evoked by high concentrations of ACh. Fitting the data to a two-site equilibrium receptor occupation model resulted in a good approximation of the effects of choline on α 4β 4 nAChRs, but a discrepancy between the predictions of the model and the experimental data for α 4β 2 nAChRs was detected. This discrepancy is attributed to heterogeneity of α 4β 2 nAChRs.

It is concluded that the weak partial agonist choline is a potent endogenous co-agonist of heteromeric neuronal nAChRs. Since co-agonist effects are observed at physiologically relevant concentrations of choline, these findings may have important implications for nicotinic neurotransmission in the CNS.

Poster NO. 32

The 5-HT$_3$ receptor: relating structure to function

Lummis S.C.R., Spier, A.D. and Gunthorpe, M.J. Division of Neurobiology, LMB and Dept of Biochemistry, University of Cambridge, Cambridge, CB2 1AG, UK.

The 5-HT3 receptor belongs to the family of ligand-gated channels that includes nicotinic acetylcholine (nACh), glycine and GABAA receptors (Maricq et al. 1991). In common with other members of this family, the 5-HT3

receptor is a pentameric assembly of subunits (Boess et al. 1995). Two 5-HT3 receptor subunits, 5-HT3A (Maricq et al., 1991) and 5-HT3B (Davies et al., 1999) have been identified so far, and heterologously expressed receptors function with distinctive biophysical properties as either homo-oligomeric 5-HT3A or hetero-oligomeric 5-HT3A and 5-HT3B subunit complexes (Davies et al., 1999). We have been using molecular biology, immunocytochemistry and electrophysiology to explore the role of specific amino acids in the structure and function of homo-oligomeric 5-HT3A receptors, and have recently examined the role of tryptophan (Trp) residues in the ligand binding domain. Our data suggest that Trp90, Trp183 and Trp195 are intimately involved in ligand binding, whilst Trp95, Trp102, Trp121 and Trp214 have a critical role in receptor structure or assembly (Spier & Lummis, 2000). We have also explored the proposed pore-lining region (the second transmembrane domain M2) and show that changing three amino acids results in a change in selectivity of the channel from cationic to anionic; similar studies have previously been performed on the nACh α 7 receptor (Eisele et al.,1993). Thus our data exemplify the high degree of structural and functional homology between the receptors in the cys-loop ligand-gated ion channel family, and provide insights towards the subtle differences which may be responsible for 5-HT3 receptor-specific characteristics.

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Poster NO. 33

Probing Potassium Channel Dynamics With a Unique Intrinsic Fluorescence *(Oral presentation Tuesday 15:00-15:30)*

Bruce E. Cohen, Yuh Nung Jan, and Lily Yeh Jan, Howard Hughes Medical Institute and Departments of Biochemistry and Physiology, UCSF, San Francisco, CA 94143-0725 USA

Tryptophan residues give proteins an intrinsic fluorescence which has found widespread use in studies of protein structure, folding, dynamics, and protein-ligand interactions. Tryptophan's environment sensitivity might make it a valuable probe for characterizing the molecular motions that underlie K^+ channel activity, but its utility is limited by its complex photophysical characteristics and by the difficulty of distinguishing between the many tryptophans within the membrane. To overcome these limitations in studying K^+ channel dynamics, we have synthesized a novel fluorescent amino acid (β-(*N*,*N*-dimethyl-aminonaphthoyl)alanine, Aladan) which shows acute environment sensitivity and may be selectively excited within proteins. We have incorporated Aladan site-specifically at multiple positions within the T1 domain of Shaker and within the transmembrane domains of IRK1 by nonsense suppression in *Xenopus* oocytes. Aladan fluorescence was shown to vary dramatically in bulk solvent, with the emission maximum of *N*-Ac-aladanamide shifting from 409 nm in cyclohexane to 542 nm in water. To characterize its behavior within proteins, Aladan was incorporated at buried, interface, and solvent-exposed positions in GB1, a well-characterized soluble protein, and showed a distinct fluorescence at each site. Minor changes introduced at neighboring residues produced readily observable changes in fluorescence. These results suggest that Aladan should be useful for detecting even small structural changes caused by channel gating, inactivation, ligand binding, or subunit interactions.

Poster NO. 34

Modulation of 5-HT_{1A} receptor activation by its interaction with wild-type and mutant $G\alpha_{i3}$ proteins

Delphine S. Dupuis, Thierry Wurch, Stéphanie Tardif, Francis C. Colpaert & Petrus J. Pauwels. Constitutive and agonist-dependent activation of the recombinant human 5-HT_{1A} receptor (RC: 2.1.5HT.01A) was investigated by co-expression with a rat $G_{\alpha i3}$ protein in Cos-7 cells. The interaction between the 5-HT_{1A} receptor and rat $G_{\alpha i3}$ protein was modulated by substitution of the $G\alpha_{i3}$ protein site for pertussis toxin-catalysed ADP-ribosylation (cysteine^{351}) by each of the natural amino acids. Enhanced basal [^{35}S]GTPγ S binding responses (+24 to +189 %) were observed with the mutant $G\alpha_{i3}$ proteins containing at position 351 either a histidine, glutamine, serine, tyrosine or a nonpolar amino acid with the exception of a proline. With each of these mutant $G\alpha_{i3}$ proteins, spiperone (10 μM), but not WAY 100635 (10 μM), reduced (-22 to -60 %, $p < 0.05$) the enhanced basal [^{35}S]GTPγ S binding response. 5-HT (10 μM)-mediated [^{35}S]GTPγ S binding responses attained for some of the mutant $G\alpha_{i3}$Cys^{351} proteins (Phe, Met, Val and Ala) more than 300 % of that obtained with the wt $G\alpha_{i3}$ protein. Similar results were also obtained with the prototypical 5-HT_{1A} agonist 8-OH-DPAT and the partial agonist (-)-pindolol. Fusion proteins assembled from the 5-HT_{1A} receptor and either the wt $G\alpha_{i3}$Cys^{351}, mutant $G\alpha_{i3}$Cys^{351}Gly or $G\alpha_{i3}$Cys^{351}Ile protein displayed similar observations for each of the ligands as obtained by co-expression of the 5-HT_{1A} receptor with these $G_{\alpha i3}$ proteins. Both the degree of 5-HT_{1A} receptor activation by 8-OH-DPAT and (-)-pindolol, and its inhibition by spiperone, strongly correlate (r^2: 0.78 to 0.81) with the octanol/water partition coefficients of the mutated amino acid at position 351 of the $G_{\alpha i3}$ protein. The

present data also suggest the wt $G_{\alpha i3}$ protein does not result in maximal activation of the 5-HT_{1A} receptor by the agonists being investigated.

Poster NO. 35

Two-dimensional analysis suggests non-sequential kinetic gating of mechano-sensitive channels in *Xenopus* oocytes

Ziv Gil,* Karl L. Magleby,[#] and Shai D. Silberberg*, *Department of Life Sciences and the Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer Sheva, Israel and [#]Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida, USA.

Xenopus oocytes express mechanosensitive (MS_{XO}) channels that can be studied in patches of membrane with the patch clamp technique. This study examines the steady-state kinetic gating properties of the MS_{XO} channels using detailed single-channel analysis. The open and closed one-dimensional (1-D) dwell-time distributions were described by the sums of 2-3 open and 5-7 closed exponential components, respectively, indicating that the channels enter at least 2-3 open and 5-7 closed kinetic states during gating. Dependency plots revealed that the durations of adjacent open and closed intervals were correlated, indicating two or more independent transition pathways connecting the open and closed states. Maximum likelihood fitting of 2-D dwell-time distributions to both generic and specific models was used to examine gating mechanism and rank models. A kinetic scheme with five closed and five open states, in which each closed state could make a direct transition to an open state (two-tiered model) could account for the major features of the single-channel data. Thus, the gating mechanism of MS_{XO} channels differs from the sequential (linear) gating mechanisms considered for MS channels in bacteria, chick skeletal muscle, and *Necturus* proximal tubule. These differences in gating might reflect differences in structure and/or in the mechanism of activation.

Poster NO. 36

NeuroPatch - the fully automated patch clamp system

R. Schrøder[1,2], P. Christophersen[1*], M. Bech[1*]. S-P Olesen[1], [1]NeuroSearch A/S, DK-2750 Ballerup, Denmark, [2]Aalborg University, department of control engineering, DK-9220 Aalborg, Denmark. [*] Inventors of the system.

NeuroPatch is a fully automated patch clamp system, which has been developed for the purpose of pharmacological screening on any ion channel such as voltage gated, ligand gated and intracellular gated. All manual maneuverings of doing patch clamp experiments have been computerised, with the perspective that NeuroPatch will be a system of improved speed for industrial search for possible drug candidates on ion channels.

Based on the conventional patch clamp technique the NeuroPatch system controls all the distinctive parts of an experiment. Thus, NeuroPatch is able to automatically get and replace a pipette, to recognize the pipette tip, to identify a proper cell for patching, to connect the pipette to the cell, to establish the desired patch clamp configuration, to monitor the experiments and apply test compounds to the test chamber.

The identification of the pipette tip and the selection of a cell for patching is performed via a visualization system including a microscope, a video camera, a frame-grabber, a computer and new software methods for automatic focusing and image recognition. The exchange of pipettes and cells is made possible by the use of a computer controlled micromanipulator and a computer controlled specimen stage equipped with a pipette rack containing 18 pipettes and a carousel containing 8 micro perfused chambers. Furthermore, a special pipette holder has been developed to allow a common connection to the exchangeable pipettes.To establish a high resistance seal and the patch clamp configuration suction in the pipette is provided by a computer controlled suction pump. The amplifier used with NeuroPatch is the EPC-9 from HEKA. It is controlled by the PULSE program from HEKA, which has been expanded with a communication interface that allows the NeuroPatch program to control it during experiments. The application of test compounds is performed by a HPLC auto-sampler controlled via an accompanying program.

Besides controlling electrophysiological recordings, as well as monitoring an ongoing experiment, error conditions are also handled by the NeuroPatch. Here, we introduce and discuss the distinctive parts of the NeuroPatch system.

Poster NO. 37

Evidence for competition of gadolinium and fluoxetine at plasmalemma-integrated porin / VDAC channels

Thinnes FP, Hellmann KP & Hilschmann N, Max-Planck-Institut für Experimentelle Medizin, Abteilung Immunchemie, D-37075 Göttingen, Germany.

We recently documented by light scattering measurements that gadolinium, applied as gadolinium chloride in micromolar amounts, induces cell swelling on human healthy or CF B-lymphocyte cell lines in isotonic Ringer solution. In high potassium Ringer solution additional swelling was observed. The agonist induced excessive swelling of the cell lines in hypotonic Ringer solutions, containing 70 mM NaCl or 135 mM taurine, respectively. The gadolinium effect disappeared whenever NaCl was replaced by Na-gluconate. Furthermore, by video camera monitoring we showed that HeLa cells react corres-pondingly under gadolinium. The effect of the agonist was dose-dependent and it was always blocked by the extracellular application of anti-human type-1 porin antibodies. Additionally, in artificial lipid bilayer measurements gadolinium decreased the voltage dependence of the channel. As

a mechanism we proposed that ionic gadolinium opens up plasmalemma-integrated porin channels, chloride or taurine then following their concentration gradients into the cell. However, the results indicate the involvement of porin in the swelling behaviour of different cells and, furthermore, argue for a single pathway for inorganic and organic osmolytes during regulatory volume decrease after cell swelling (Thinnes et al., Mol. Gen. Metab. 69, in press). Using same approaches, we now elaborated that fluoxetine abolishes the gadolinium effects at the plasma membrane of B lymphocytes and HeLa cells in a way corresponding to anti-porin antibodies. The data may help to further establish the involvement of porin channels in cell volume regulation as part of the ORCC (Thinnes & Reymann, Naturwissenschaften 84, 480-498 (1997).

BENZON SYMPOSIUM No. 47

MOLECULAR PHARMACOLOGY OF ION CHANNELS

AUGUST 13-17, 2000, COPENHAGEN, DENMARK

Organizing committee:
Jan Egebjerg, Søren-P. Olesen and Povl Krogsgaard-Larsen

Abstracts - WEDNESDAY, August 16, 2000

Gβ γ Regulated Ion Channels In Heart And Brain

David Clapham, HHMI Children's Hospital, Harvard Medical School, Boston, USA

At least eight transmitters activate a class of inward rectifier K^+ channels via an apparently identical GTP binding protein- (G protein) linked signal transduction mechanism. The G protein-linked receptor subtypes that activate these channels include muscarinic (m2), γ -aminobutyric acid ($GABA_B$), serotonin (5HT1A), adenosine (P1), somatostatin, enkephalin (μ, κ , δ), α 2-–adrenergic, and dopamine (D2) receptors. G protein-linked receptors couple to a heterotrimeric protein complex of Gα and Gβ γ subunits. After these receptors catalyze the transfer of GTP to replace GDP in the Gα subunit, the freed Gβ γ subunit directly binds and activates the GIRK channel**.** These G protein-linked Inwardly Rectifying K^+ (GIRK) channels play a role predominantly in the pacing range of cardiac cells and in the regenerative firing of neuronal cells where they oppose the slow depolarization of such currents as I_f in heart or I_h in neurons (HCN channel class) and can compensate for inactivation of the M current (neurons).

The GIRK channel class has four members, GIRK1, GIRK2, GIRK3, and GIRK4. GIRK1 is unique among the four in having a long carboxy terminal tail, while GIRK2, 3, and 4 are quite similar. GIRK1 combines with GIRK2,3,or 4 to make tetrameric K^+ channels. GIRK1/GIRK4 comprise the cardiac I_{KACh} channel while GIRK1/GIRK2 and GIRK2/GIRK3 channels are predominantly in the central nervous system.

I_{KACh} channel activity is also *modulated* by levels of intracellular Na, ATP, and phosphatidylinositol bisphosphate (PIP_2). In this presentation I will discuss how phophorylation regulates the activation of theGβ γ activated K^+ channel.

Tetrameric Assembly of Voltage-gated K^+ Channels

Senyon Choe, The Salk Institute, La Jolla, CA – USA.

The T1 domain is a highly conserved N-terminal cytoplasmic portion of the voltage-dependent K+ channel (Kv) α subunit that encodes the subfamily specific tetramerization properties of Kv channels. We report the identification of a set of mutations made in the central water-filled cavity of the T1 tetramer that alter the gating properties of the Kv channel. Two of those mutants produce a leftward shift in the activation curve and slow the channel closing rate while a third mutation produces a rightward shift in the activation curve and speeds the channel closing rate. The crystal structures for these T1 domain mutants containing each of these mutations show intriguing alteration in the part of the T1 structure. The leftward-shifting mutations produce a similar conformational change in the putative membrane-facing surface of the T1 domain, whereas the rightward-shifting mutation produces no significant alteration as compared to the wild-type conformation. These results suggest that the conformational states of the T1 domain, perhaps more in this region, is structurally tightly coupled to varying degrees of channel¹s global conformations involved in gating. We will highlight the role of the cytoplasmic domain as a docking station for various protein-protein interaction with cytoplasmic components to modulate ion conductance occurring in the transmembrane part of the channel.

Reference:

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Cushman, S.J., Nanao, M.H., Kunjilwaar, K., Jahng, A.W., DeRubeis, D., Choe, S., Pfaffinger, P.J. (2000) Voltage-dependent activation of potassium channels is coupled to T1 domain structure. ***Nature Struc. Biol.,*** May 2000 issue.

Structure-driven Investigations of Voltage-gated K^+ Channels

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The high-resolution structure of the prokaryotic KcsA K^+ channel provides a wealth of novel information about the ion permeation pathway and the chemical mechanism of K^+ selectivity in all K^+ channels. But it says very little about the overall molecular architecture of eukaryotic K_v channels. Using the *Shaker* channel, we are beginning to develop a picture of the physical locations of the transmembrane segments S1-S4 outside of the pore-region made by the S5-P-S6 segments, as well as the disposition of the cytoplasmic "T1" domain. Tryptophan-scanning mutagenesis is used to

show that S1, S2, and S3 are mostly helical, and to identify the parts of these transmembrane segments that are exposed to bilayer lipid. Distances to selected residues on the extracellular ends of the S1-S4 helices are measured by a tethered blocker strategy, which is also employed to trace the trajectories of extracellular "loops." These studies alone do not provide enough constraints on a unique model for helix packing in K_v channels, but they do lead to plausible guesses that may be tested by cysteine-crosslinking methods. One surprising conclusion of the mutagenesis work is that S5 may not be completely surrounded by protein, but may be directly exposed to a lipid environment along a narrow surface. The location and functional purposes of the T1 domain are currently in dispute. It is clear that this domain does not contribute to ion permeation behavior, but the role this sequence plays in cytoplasmic access to the pore is unknown. We are attempting to assess whether T1 in fact takes on a homotetrameric structure in the mature channel, and, if so, to ascertain whether it is attached to the membrane-embedded parts of the channel in a "dangling gondola" configuration

Gating of the K_{ATP} Channel and Its Modulation by Mg-Nucleotides

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ATP-sensitive K-channels (K_{ATP} channels) couple the metabolic state of the cell to its electrical activity, and thereby play important roles in processes such as insulin secretion, vascular smooth muscle tone, and ischaemic preconditioning. The K_{ATP} channel consists of 4 pore-forming Kir6.2 subunits and 4 regulatory sulphonylurea receptor subunits (SUR1 in pancreatic B-cells, SUR2A in heart, and SUR2B in smooth muscle). Metabolic regulation involves both Kir6.2 and SUR subunits: thus, ATP and ADP bind to an intracellular site on Kir6.2 and induce channel closure, whereas a range of Mg-nucleotides (including ATP, ADP, GTP, GDP, and UDP) interact with the nucleotide-binding domains (NBDs) of SUR and thereby increase channel activity. Channel activity is determined by the balance between these inhibitory and stimulatory effects. Mutations in SUR1 that result in loss of Mg-nucleotide regulation produce unregulated insulin secretion and thereby congenital hypoglycaemia of infancy.

We examined the regulation of cloned K_{ATP} channels by Mg-nucleotides in giant inside-out membrane patches excised from *Xenopus* oocytes heterologously expressing wild-type or mutant K_{ATP} channel. We found that Kir6.2/SUR1, Kir6.2/SUR2A and Kir6.2/SUR2B channels were activated by MgADP to similar extents. In contrast, Kir6.2/SUR1 and Kir6.2/SUR2B, but not Kir6.2/SUR2A, were activated by MgATP. The latter finding may help explain the different sensitivities of Kir6.2/SUR2A and Kir6.2/SUR2B to K-channel openers. This talk will focus on the molecular mechanisms involved in Mg-nucleotide activation of the K_{ATP} channel, and its physiological implications. It will also show how most channel regulators, including ATP and Mg-nucleotides, influence the slow gating of the channel, demonstrate that the fast and slow gates are independent, and consider where they are located within the channel.

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Structure, Function, and Physiology of Small and Conductance Calcium-activated Potassium Channels

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Small-conductance calcium-activated potassium channels (SK channels) are fundamental regulators of neuronal excitability. Structure-function studies examined the molecular mechanisms underlying calcium-gating for SK channels. The results revealed that functional SK channels are heteromeric complexes of the pore forming alpha subunits and the ubiquitous calcium sensor, calmodulin. Calcium binding to calmodulin induces conformational changes in calmodulin, and consequently in the alpha subunits, that open the channels. The association into SK channels is different than the interactions of calmodulin with other proteins, being constitutive, and the lower affinity E-F hands 1 and 2 on calmodulin are necessary and sufficient for calcium-gating. Calmodulin associates with a highly conserved domain at the proximal end of the intracellular C-terminus. Structural studies of the protein-protein interaction will be discussed. Three highly homologous SK channel subunits have been cloned. They are expressed in distinct but overlapping patterns in the brain. Hippocampal CA1 pyramidal neurons show both a medium and a slow AHP with different pharmacologies and kinetics. The roles of the SK channels in the medium and slow AHPs in CA1 neurons will be discussed. To examine the individual roles of the three SK subunits, a transgenic strategy based upon homologous recombination has been developed. Insertion of a novel regulatory cassette permits acute regulation of gene expression in vivo, while retaining cell type and developmental specificity. Mice harboring the regulatory cassette in the SK3 gene will be described..

Molecular Pharmacology of Calcium-Activated K^+ channels

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Ca^{2+}-activated K^+ channels have been implicated in a number of physiological functions, which suggest they may be effective drug targets. The pharmacology of subtypes of Ca^{2+}-activated K^+ channels has been addressed in this study by patch clamp measurements on SK1, SK2, SK3, and IK channels stably expressed in HEK cells.

All SK channels are sensitive to apamin, albeit with different IC_{50} values of 3.3 nM, 0.083 nM and 0.6 nM for SK1, SK2 and SK3, respectively. Apamin bears some of the same pharmacophore as UCL 1684, which, however, shows no differences in selectivity between the three SK channel subtypes. IC_{50} values of UCL 1684 were 0.76 nM on SK1 and 0.36 nM on SK2. Thus, for a blocker to discriminate between the SK subtypes it is not sufficient to have an 'apamin-like' structure and to be potent.

IK channels are highly sensitive to the internal pH, with the highest activity at pH_i 7.2 and no activity at pH_i 6.0. IK is activated by imidazolone derivatives such as 1-ethyl-2-benzimidazolone. The activation is obligatory dependent on Ca^{2+}_i above 10 nM. IK channels are potently inhibited by charybdotoxin (ChTx), clotrimazole (CLT) and nitrendipine (Nit), whereas highly selective blockers are unknown. Activation of human T-cells and subsequent release of interferon-γ is inhibited by ChTx, CLT and Nit, an effect not shared by close analogues being devoid of IK channel blocking effect.

In conclusion, the pharmacology of Ca^{2+}-activated K^+ channels is of great value in delineating the function of these channels at a molecular level.

2P-Domain K^+ Channels: Structure, Physiological Functions, Pharmacology and Therapeutic Implications

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We have recently cloned and expressed a new family of K^+ channels comprising 4 transmembrane domains and 2P regions. These channels are non voltage-dependent background channels and are expressed in different tissues but are usually very abundant in the brain. Two types of channels have a particular interest with respect to CNS function. The first one is TASK, a channel type that strictly follows the Goldman equation and which is highly regulated by external pH (active at a physiological pH of 7.3, inactive at pH < 6.9-7). Changes of this channel activity probably play an important role in situations such as epileptic seizures and ischemia. The second type is TREK-1, a K^+ channel that is activated by arachidonic acid and polyunsaturated fatty acids. This channel is also highly mechano-sensitive and inhibited by intracellular increases of cAMP and by PKC. The molecular mechanisms by which the regulations take place will be discussed. This class of channels is (i) the target of volatile anaesthetics and (ii) of drugs that provide potent neuroprotection. TRAAK is another mechano-sensitive channel also activated by polyunsaturated fatty acids, but it has a different regulation and a different pharmacology. Both TREK-1 and TRAAK are potently activated by lysophospholipids and platelet activating factor. TREK-1, but not TRAAK, is heat activated and looks as an ideal candidate as a thermoreceptor in peripheral sensory neurons and central hypothalamic neurons. Polyunsaturated fatty acids are potent blockers of glutamatergic transmission and potent neuroprotectors against ischemia and epilepsy and these properties seem to be related to channels of the TREK-1/TRAAK family.

Poster NO. 38

Modulation of the cardiac I_{KS} channel gating: the same IsK residues slow activation and prevent channel inactivation *(Oral presentation Thursday 14:00-14:30)*

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The cardiac I_{KS} potassium channel complex was recently shown to consist of the heteromeric assembly of two structurally distinct α and β subunits called KvLQT1 and IsK, respectively. Tail current analysis suggests that channels formed by KvLQT1 populate multiple open states, while those formed by I_{KS} segregate into a single open state or partition into kinetically indistinguishable open states. We show that I_{KS} channel activators, such as stilbenes and fenamates accelerate KvLQT1 activation kinetics, slow deactivation, reduce inactivation and produce a leftward shift in the voltage-dependence of activation. Importantly, stilbenes and fenamates restore normal I_{KS} channel gating in otherwise inactive IsK C-terminal mutants, including the naturally occurring LQT5 mutant, D76N. Deletion of residues 39-43 of human IsK, at the N-terminal boundary of its single transmembrane segment, renders I_{KS} insensitive to stilbenes and fenamates and leads the mutated I_{KS} channels to repopulate multiple open states. The same IsK domain appears to be involved in slowing activation gating and in preventing I_{KS} from inactivation. Our data support a model in which allosteric interactions exist between the extracellular and intracellular boundaries of the IsK transmembrane segment as well as between domains of the α and β subunits. Owing to such allosteric interactions, stilbenes and fenamates can rescue the dominant-negative suppression of I_{KS} produced by IsK mutations and thus, may have important therapeutic relevance for LQT syndrome.

Poster NO. 39

Molecular and functional properties of human SK2 Ca^{2+}-activated K^+ channels in leukemic Jurkat T cells: mapping domains important for channel function

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Previous electrophysiological studies have demonstrated the presence of apamin-sensitive, small-conductance Ca^{2+}-activated K^+ channels (SK) in human leukemic Jurkat T cells. Using a combined cDNA and RT-PCR cloning strategy, we have isolated from Jurkat T cells a 2.5 kb cDNA, hSK2, encoding the human isoform of SK2 channel α subunit. Northern blot analysis reveals the presence of a 2.5 kb hSK2 transcript in Jurkat T cells, however, no hSK2 mRNA could be detected in resting and activated normal human T cells. The protein encoded by hSK2 is 579 aminoacid long and exhibits 97% identity with its rat protein counterpart rSK2. Similar to the native Jurkat SK currents, hSK2 produces in transfected mammalian cells a time- and voltage-independent K^+ current which is inhibited by apamin and d-tubocurarine. Overexpression of the Src-family tyrosine kinase $p56^{lck}$ up-regulates the Jurkat SK channel activity. The calmodulin antagonist W7 inhibits the SK currents in Jurkat cells and in hSK2-transfected CHO cells. Combined cross-linking and immunoprecipitation experiments performed in Jurkat T cells and in hSK2-transfected cells indicate that [^{125}I] apamin binds to high (57 kDa) and low (31 kDa) molecular weight polypeptides, suggesting that native SK2 channels consist of hetero-oligomeric complexes comprising α and β subunits, respectively. Current mutagenesis experiments identify novel domains important for SK channel function.

Poster NO. 40

Haloperidol increases the rate of inactivation of Kv4.2 current while the rate of recovery from inactivation remains unchanged

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We have shown recently that Kv4.2 subunits are major constituents of somatodendritic A-type current in four types of central neurons (J. Neurosci. 20:579-88). Here we demonstrate that haloperidol, a known antipsychotic, can dramatically (from ~50% to >500%) accelerate A-type current inactivation in all four types of neurons, namely in striatal medium spiny, basal forebrain and pallidal neurons and striatal cholinergic interneurons. The effect usually reached steady state within 5 sec. The inactivation rate in the presence of haloperidol had very little voltage dependence between –75 mV and –10 mV. Surprisingly, the recovery from inactivation was not affected even at the potentials close to activation threshold of the A-type current (-75 mV). Similarly, voltage dependence of inactivation was not altered. The increase of inactivation rate was significantly reduced in the presence of high concentration (50 μ M) of sigma receptor antagonist 3-PPP HCl.

Several mechanisms could account for the observed results. Haloperidol can block several types of voltage gated channels and activate or interfere with several types of receptors, including dopamine, sigma and serotonin. Experiments to distinguish between receptor mediated mechanisms, open channel block and allosteric modulation will be performed.

Poster NO. 41

TASK, a background K^+ channel is expressed and regulated by angiotensin II in glomerulosa cells

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As a consequence of their high resting potassium conductance, adrenal glomerulosa cells possess a very negative membrane potential which is reduced by extracellular [K^+] elevation and by angiotensin II (AII). While electrophysiological studies indicate the presence of several different K^+ channels in this tissue, only background K^+ channels are likely to be operational under resting conditions.

We showed by Western blot and RT-PCR experiments that TASK, a member of the two-pore (2P) domain K^+ channel family, is expressed abundantly in adrenal zona glomerulosa cells. When mRNA, prepared from glomerulosa tissue, was injected into *Xenopus Laevis* oocytes the development of the increased potassium conductance (as measured by the two-electrode voltage clamp method) was prevented by coinjecting TASK antisense oligonucleotide. The sense failed to have any inhibitory effect.

When in vitro synthesized AT1 angiotensin receptor and TASK cRNA were coexpressed in the oocytes, application of AII inhibited the expressed channel activity. The inhibition could be mimicked by intracellular application of GTPγS. The effect of AII was maintained even when the Ca^{2+} signal was abolished either by depletion of the intracellular Ca^{2+} stores by thapsigargin (administered prior the application of AII) or by intracellular application of 10 mM EGTA.

Our results indicate that the TASK channel in the adrenal glomerulosa cells is a target of AII action. This inhibition which is independent of the Ca^{2+} signal may depolarize the cell and thus contibute to the stimulatory effect of AII via activation of voltage sensitive Ca^{2+} influx mechanisms.

Poster NO. 42

G_{i2} Proteins Couple Somatostatin Receptors to K^+-Channel Activity and Exocytosis in Glucagon-Secreting Rat A-Cells

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Aim: To explore the types of G proteins that mediate the inhibitory effects of somatostatin on K^+-channel activity and Ca^{2+}-dependent exocytosis in single rat pancreatic A-cells. **Methods**: Patch-clamp techniques were used to record ion channel activity and changes in cell capacitance (reflecting exocytosis) in single rat pancreatic A-cells. **Results**: Somatostatin inhibited spontaneous electrical activity in the absence of glucose and repolarised the cell by >15 mV. Pretreatment with pertussis toxin abolished the inhibitory response to somatostatin, indicating the involvement of an inhibitory G-protein. These effects on electrical activity were associated with an increase in the whole-cell K^+ conductance by 3.8±1.0 nS from a control level of 1.0± 0.2 nS (n=4) at 25.6 mM $[K^+]_o$. In support for a direct interaction between G-proteins and the somatostatin-activated K^+-channel, intracellular application of GTPγS led to activation of the current. This effect was mimicked by G-protein β γ -subunits. Activation of the K^+ conductance by somatostatin was inhibited in cells treated with antisense oligonucleotides against G-proteins of the subtype G_{i2} but not G_{i1}, G_{i3} or G_o. Somatostatin reversibly inhibited depolarisation-induced increases in cell capacitance by >85% without affecting the whole-cell Ca^{2+}-current. This action was abolished in cells pretreated with pertussis toxin and the serine/threonine protein phosphatase calcineurin inhibitors deltamethrin and cyclosporin A. Finally, the effect of somatostatin on exocytosis was also prevented by antisense oligonucleotide treatment against G_{i2} but not G_{i1}, G_{i3} or G_o proteins. These data suggest that somatostatin inhibits glucagon release by G_{i2}-protein-dependent interaction with both proximal and distal inhibitory steps in the stimulus-secretion coupling of the rat A-cell.

Poster NO. 43

Localization of big conductance Ca^{2+}-activated K^+ channels in distal colon epithelium.

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Water and salt homeostasis in mammalians is largely maintained by transepithelial transport in specialized epithelial cells, e.g. in the distal colon. Big conductance Ca^{2+}-activated K^+ channels (BK channels) may play an important role in the overall regulation of this transport, but little is known about the expression level of BK channels in epithelia.

The aim of the present study was to quantify and localize the BK channels in rabbit epithelia by iberiotoxin (IbTX) binding using the radiolabelled double mutant ^{125}I-IbTX-D19Y/Y36F, autoradiography and immunohistochemical studies.

These experiments demonstrated a distinct distribution of BK channels in the colon epithelium. The surface cells responsible for Na^+ absorption contained a high number of BK channels (78 fmol/mg protein) whereas the expression level of the channels was approx. 10 times lower in the Cl^- secreting crypt cells. This expression pattern could be confirmed by autoradiography and immunohistochemical studies. Surprisingly, the immunohistochemical studies apparently showed expression of BK channels in apical as well as basolateral membranes of the surface cells. This may reflect expression of different BK channel splice variants in these cells.

In conclusion, the significant and distinct expression of BK channels in epithelia, combined with their strict regulation, indicate that the these channels may play an important role in the overall regulation of salt and water absorption.

Poster NO. 44

KCNQ4 channels expressed in mammalian cells: Functional characteristics and pharmacology

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KCNQ1-4 channels constitute a family of voltage-gated potassium channels with about 40% identity at the amino acid level. Besides the homology at the protein level, these K^+ channels share biophysical characteristics, i.e. they activate and deactivate slowly, albeit with subtype differences. Further, they all conduct a significant non-inactivating current at negative membrane potentials. KCNQ4 is expressed in the outer haircells of the cochlea, and mutations in the channel is known to underlie a form of non-syndromic dominant deafness (Kubisch et al., 1999; Coucke et al., 1999). KCNQ4 channels share some of the characteristics of KCNQ2+3 and of the native M-current, but when expressed in *Xenopus* oocytes there may also be incongruities such as too low sensitivity to M-current blockers and a too positive activation threshold (Kubisch et al., 1999).

We have expressed KCNQ4 channels stably expressed in HEK 293 cells and characterized with respect to function and pharmacology. Patch-clamp measurements showed that the KCNQ4 channels conducted slowly activating currents at potentials more positive than –60 mV. From the Boltzmann function fitted to the activation curve, a half-activation potential of –29 mV, and an equivalent gating charge of 1.4 elementary charges was determined. The instantaneous current-voltage relationship revealed strong inward rectification. KCNQ4 channels were blocked in a

voltage-independent manner by the memory-enhancing M-current blockers XE991 and linopirdine with IC_{50} values of 5.5 μM and 14 μM, respectively, whereas the anti-arrhytmic KCNQ1 channel blocker bepridil inhibited KCNQ4 with an IC_{50} value 9.4 μM. The KCNQ4-expressing cells exhibited an average resting membrane potentials of –56 mV in contrast to –12 mV recorded in the non-transfected cells. Pharmacological inhibition of the KCNQ4 channels depolarized the membrane of the transfected cells. In conclusion, the activation and pharmacology of KCNQ4 channels resemble that of M-currents, and it is likely that the function of the KCNQ4 channel is to regulate the sub-threshold electrical activity of excitable cells.

Poster No. 45

Pharmacological characterization of SK3 potassium channels stably expressed in HEK293 cells

Thomas Jespersen[1], K. A. Pedersen[1], D. Klaerke[1], S.-P. Olesen[1,2], B. S. Jensen[1,2], M. Grunnet[1,] 1) The Panum Institute, Department of Medical Physiology, Institute of Cellular and Molecular physiology, University of Copenhagen, Copenhagen, Denmark. 2) Division of Ion Channel Physiology, NeuroSearch A/S, Ballerup, Denmark.

Small-conductance, calcium-activated K^+ channels (SK channels) are gated solely by intracellular calcium ions and their activity is responsible for part of the afterhyperpolarization (AHP) that follows an action potential in many excitable cells. Presently three subtypes of SK channels have been described at a molecular level within the last few years. The rat small-conductance, calcium-activated K^+ channel subtype 3, rSK3, has previously been expressed in Xenopus laevis oocytes, an expression system that is well-suited for electrophysiological studies, but not for pharmacological characterisation.

To characterise rSK3 channels with respect to their basic pharmacology, we choose to stably express rSK3 channels in HEK293 cells. Expression of rSK3 was confirmed by an ^{125}I-apamin binding assay on a membrane preparation. Apamin was found to block the potassium channel by an IC_{50} value of 630 pM (n=5). With an intracellular Ringer buffered at 100 nM free Ca^{2+} the rSK3 whole-cell current is activated by 1-ethylbenzimidiazolone (EBIO) in concentrations above 1 μM. The effect of EBIO is blocked by 100 nM apamin. The EBIO activation of rSK3 channels is strictly calcium-dependent. 100 μM EBIO in whole-cell recordings with 0 Ca^{2+} in the pipette gave no effect. 4-aminopyridine (4-AP) is a potent inhibitor of SK3 channels with an IC_{50} value of 512 μM (n=4).

Data from pharmacological analyses of both openers and blockers will be presented.

Poster No. 46

The small- and intermediate-conductance, Ca^{2+}-activated K^+ channels: Regulation by changes in pH

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Minor changes in pH in the physiological range have been shown to modulate potassium channels. In the present study we investigated the effect of changes in intra- and extracellular pH (pH_i and pH_o) on the activity of the cloned human intermediate conductance, Ca^{2+}-activated K^+ channel (hIK) and the rat small conductance, Ca^{2+}-activated K^+ channel (rSK3) using the patch-clamp technique.

Multi-channel inside-out recordings on patches from HEK-293 cells stably expressing hIK-channels, revealed that the channel activity was modulated by changes in pH_i. Changes in pH_o in the range from pH 6.0 to 8.2 did not affect the hIK whole-cell current. Intracellular acidification gradually decreased the open state probability of the hIK channel, approaching zero activity at pH_i 6.0. This proton-induced inhibition of the channel was reversible. The hIK channels exhibit weak inward rectification with a corde conductance of 29 and 11 pS at +/- 100 mV, respectively. Decreasing pH_i altered neither the conductance nor the inward rectification of hIK channels. The proton-induced inhibition of the multi-channel hIK patch current could not be counteracted by increasing the cytosolic Ca^{2+} concentration to 30 μM. The results obtained from the hIK channel will be compared to similar experiments performed on the rSK3 channel and the molecular sensory mechanism underlying the proton-induced modulation will be investigated.

Poster NO. 47

Bioactive Peptides from the Venom of Cone Shells as Probes for Ion Channels and Receptors

Silke Kauferstein (1), J. Tytgat (2), A. J. Menez (3), F. B. Sotto (4), D. Mebs (1), (1)Zentrum der Rechtsmedizin der University Frankfurt, Frankfurt; (2)Laboratory of Toxicology, University of Leuven, Leuven, Begium; (3)Department d` Ingenierie ed d`Etudes des Proteines, C.E Saclay, Gif-sur-Yvette, France; (4)University of San Carlos, Marine Biology Section, Cebu, Philippines.

Cone shells produce a complex venom which contains a large number of biologically active peptides. For several ion channels and receptors, they are the most specific ligands known. We have used conotoxins as tools for characterizing ion channels, in particular potassium channels. Venom of several Conidae species was fractionated by HPLC. These fractions and the further purified toxins were tested on various ion channels expressed in *Xenopus* oocytes. Amino acid sequencing and mass spectrometric analysis were performed. Our studies indicate that venom from the cone shells tested contain highly specific toxins acting on vertebrate potassium channels. Considering the diversity of potassium channels and the high subtype selectivity as a general feature of conotoxins it seems reasonable to conclude

that the venom of cone shells may provide novel highly specific ligands, targets of various potassium channel subtypes.

Poster NO. 48

Regulation of cloned Ca^{2+}-activated K^+ channels by volume changes

Klaerke DA, Boxenbaum N, Jensen BS, Olesen, SP & Grunnet M. Dept. of Medical Physiology, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark.

Ca^{2+}-activated K^+ channels of big conductance (BK channels), intermediate conductance (IK channels) or small conductance (SK channels) were co-expressed with aquaporin 1 in *Xenopus laevis* oocytes. BK channels were activated by depolarization, whereas IK and SK channels were activated by direct injection of Ca^{2+} or Cd^{2+} into the oocyte cytoplasm, before the oocytes were subject to hyperosmolar or hyposmolar (± 50 mOsm mannitol) challenges. In all cases, the oocytes rapidly responded to the osmotic changes with shrinkage or swelling, and the effects on the K^+ currents were measured. The IK and SK currents were extremely sensitive to volume changes; for both channel types the currents immediately increased by 200 to 300 % in response to swelling, and decreased to approx. 50 % of control values after shinkage. These responses were almost totally abolished after injection of cytochalasin D into the oocyte cytoplasm (final concentration: 2 μ M) In contrast, BK channels showed only a minor sensitivity to volume changes; the BK channel activity decreased respectively increased approx. 20 % after swelling or shinkage. The opposite effects of volume changes on IK/SK channels versus BK channels suggest that the massive activation of IK and SK channels during volume changes is not mediated by changes in intracellular Ca^{2+}, but rather through interactions with the cytoskeleton.

Poster NO. 49

***Conus* peptide κ -PVIIA blocks *Shaker* K-channels: Identification of residues mediating the interaction**

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Predatory marine cone snails have evolved a sophisticated hunting strategy. Their venoms provide a rich source of different neurotoxins which are useful tools for the investigation of the structure and function of voltage gated ion channels. κ -conotoxin PVIIA (κ -PVIIA), a 27-amino acid peptide from the venom of *Conus purpurascens*, was identified and characterized as the first member of a new family of conotoxins blocking K-channels. The binding site in the *Shaker* K-channel appears to lie in the extracellular mouth of the ion channel pore. Introduction of single point mutations in the P-loop of the channel protein revealed that residues S421, S424, F425, D431, T449 and V451 are involved in the binding of κ -PVIIA as determined by measuring the reduction of whole cell peak currents of the mutant channels expressed in *Xenopus* oocytes.

Recently two groups independently solved the solution structure of κ -PVIIA using NMR; although the structures reported were very similar, the conclusions drawn about the key residues influencing toxin binding were quite different. By using the *Xenopus* oocyte expression system we tested alanine mutants of κ -PVIIA for their activity to block *Shaker* mediated whole cell currents. By this approach critical residues forming the interaction surface of κ -PVIIA have been identified. Additionally, the results of a mutant cycle analysis where mutant toxins were tested versus mutant *Shaker* isoforms provide data about interacting residues. In summary, our data obtained are consistent with a model developed by Menez and coworkers for polypeptide antagonists of K-channels, which predicts convergent functional features of K-channel blocking peptides from different species without apparent sequence homology.

Poster NO. 50

Transient and sustained HERG K^+ currents following premature stimuli

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Loss of function mutations in HERG K^+ channels increase the risk of arrhythmias and sudden cardiac death. It has been suggested that HERG K^+ channels, due to their rapid, voltage-dependent, C-type inactivation, may play a specific role in suppressing arrhythmias initiated by premature beats (Smith et al., Nature 379: 833). Since current responses to conventional voltage clamp steps do not necessarily permit the accurate prediction of current profiles during actual action potentials (APs) we examined the effects of premature stimulation on HERG K^+ currents in CHO cells using action potential clamp techniques. During a second (premature) AP, there is an initial large transient outward current, followed by an outward current during the repolarization phase that is significantly larger than the outward current observed during the first AP. When the premature stimulus was delivered at times varying from APD_{90}-100 ms to APD_{90}+30 ms, the magnitude of the transient outward current increased with longer interpulse intervals. However, at coupling intervals longer than APD_{90}+30 ms the magnitude of the transient outward current started to decrease. This biphasic response reflects the relative rates of recovery from inactivation and deactivation. Interestingly, the coupling interval for the two APs that produced the largest sustained current component, APD_{90}-50 ms, was significantly

shorter than that observed for the maximal transient outward current component (APD_{90} + 30 ms). The larger sustained current component during the second AP also resulted in the accumulation of both the transient and sustained components during multiple premature AP stimuli. These results suggest multiple anti-arrhythmic roles for HERG K^+ channels including suppression of arrhythmias initiated by early afterdepolarizations, premature beats and accelerated rhythm.

Poster NO. 51

A novel toxin-blocker of the ERG1 channel *(Oral presentation Thursday 14:30-15:00)*

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A novel peptide, named BeKm-1, has been isolated from the venom of the Central Asian scorpion *Buthus eupeus*. The toxin is unique and consists of 36 amino acids. It belongs to the family of the K^+ channel blockers isolated from scorpion venom. The highest degree of sequence homology is found between BeKm-1 and Ibtx (40% identity?). In patch-clamp whole-cell recordings the BeKm-1 toxin was applied to HEK-293 cells transiently transfected with hERG1-cDNA. BeKm-1 at 100 nM toxin was found to block the ERG1 channel in a fast and reversible manner. By application of [BeKm-1] from 0.1 to 300 nM, the dose-response relationship of the ERG1 tail current and the toxin was obtained. The IC_{50} value was determined at 3.3 nM, with a Hill coefficient of 0.9 (n=4). Co-transfection of the ERG1 channel with the β -subunits KCNE1 and KCNE2, respectively, did not alter the efficacy of the channel block by the toxin. This indicates that the mechanism underlying the effect of the toxin is restricted to interaction with the pore forming ERG1 protein alone.

The specificity of the BeKm-1 toxin was tested at a variety of K+ channels. Within the *ether-a-go-go* family of K^+ channels, the hEAG current was found to be unaffected by the presence of 100 nM BeKm-1. In contrast, the rELK1 current was slightly suppressed (9.4± 2.6%, n=3). For other members of the 6 TM K^+ channel superfamily, the toxin had no effect on any of the channels in the KCNQ-family (KCNQ1-4), nor on four members (hSK1, rSK2, hIK, hSlo) in the family of calcium-activated K+ channel were not toxin-sensitive.

Based on these results the toxin can be a useful tool in separation of the M-like current and ERG current in NG108-15 neuroblastoma cells.

Poster NO. 52

Modulation of voltage-gated potassium channels by tyrosine kinases and phosphatases: a molecular switch for myelination?

Peretz A[1]., Sobko A.[1], Gil-Henn H.[2], Elson A.[2] and Attali B.[1], Department of Neurobiology[1] and Molecular Genetics[2], The Weizmann Institute of Science, Rehovot 76100, Israel.

Phosphorylation and dephosphorylation are important processes through which the cell modulates ionic channel activity. We recently found that a Src family tyrosine kinase constitutively activates delayed-rectifier K^+ channels (I_K) in mouse Schwann cells. Using Fyn knockout mice (*Fyn-/-*), we show that Fyn kinase plays a major role in early myelination of peripheral nerve, since early postnatal *Fyn-/-* mice exhibit hypomyelination of sciatic nerves. As tyrosine phosphatases generically counter activities of tyrosine kinases, we sought to determine whether lack of protein tyrosine phosphatase ε ε (PTPε) which is expressed in Schwann cells, would modulate Kv channels and Schwann cell function *in vivo*. We generated gene-targeted (*Ptpre-/-*) mice which do not express PTPε . Lack of PTPε causes hypomyelination of sciatic nerve axons in newborn *Ptpre-/-* mice. Primary *Ptpre-/-* Schwann cells exhibit increased voltage-dependent potassium channel activity, concomitant with hyperphosphorylation of Kv1.5 and Kv2.1 α -subunits of voltage-gated, delayed-rectifier potassium channels. Co-expression of PTPε together with Kv1.5 or Kv2.1 in *Xenopus* oocytes markedly reduces Kv current amplitudes in a manner largely dependent upon PTPε catalytic activity. Our results suggest that PTPε down-regulates K^+ channel activity by dephosphorylating Kv channel α -subunits. In all, our data indicate that, similar to the Src family tyrosine kinases, PTPε is part of the finely-tuned molecular mechanism which regulates Kv channel activity during Schwann cell development and myelination of peripheral nerves.

Poster NO. 53

Cloning anD FUNCTIONAL CHARaCTERIzATION OF A Novel BRAIN SPECIFIC ACID SENSITIVE TANDeM PORE DOMAIN POTASSIUM CHANNEL

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TASK-3, a new subunit of the family of tandem pore-domain K^+ channels (2P K^+ channels) was identified and cloned from guinea-pig brain and an orthologous sequence was isolated from a human genomic library. The full length gpTASK-3 cDNA was found to encode a polypeptide of 366 amino acids with a topology of four putative transmembrane segments and two pore regions. Although the overall identity at the amino acid level between TASK-3 and its closest relative, TASK-1, is 62 %, the two subunits diverge substantially at the C-terminus, except for the extreme C-terminal sequence. Analysis of the gene structure confirmed the presence of an intron within the conserved GYG motif of the first P region, as found in other 2P K^+ channels. RT-PCR analysis demonstrated strong expression

of gpTASK-3 in brain and low mRNA levels in other tissues. *In-situ* hybridisation of rat brain sections with rTASK-3-specific ^{33}P-radiolabeled oligonucleotides showed a highly differential expression pattern in neurons with strongest signals in distinct nuclei of the brainstem and diencephalon. Expression of TASK-3 in *Xenopus* oocytes revealed an outwardly rectifying current that was sensitive to extracellular pH and was blocked by Ba^{2+} with a K_i of 210 μM (-130 mV). Cell-attached single-channel recordings of TASK-3 were carried out in HEK293 cells. The single channel current-voltage relation showed weak inward rectification in the presence of external Ca^{2+} and Mg^{2+}. The open-state probability increased markedly with depolarization. Removal of external divalent cations increased the mean single-channel slope conductance at -100 mV from 27 pS to 100 pS. Substitution of an extracellular histidine residue (His98) adjacent to the first selectivity filter, which is conserved in TASK-1 and TASK-3 subunits, identified a putative extracellular pH sensor at the outer part of the K^+-selective pore.

Poster NO. 54

Kv channel "disinactivators": identification and characterization of small molecule inhibitors of N-type inactivation

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Small molecules that slow or prevent rapid inactivation of voltage-gated (Kv) K^+ channels would be expected to keep these channels open longer and thereby reduce neuronal excitability and limit neurotransmitter release. In some Kv channels, rapid inactivation is mediated through binding of an N-terminal domain of the pore-forming α -subunit, or an auxiliary β -subunit, to a cytoplasmic acceptor located at or near the channel pore (N-type inactivation: Hoshi et al. (1990) Science 250:533; Isacoff et al., (1991) Nature 353:89; Rettig et al., (1994) Nature 369:289). We recently developed a yeast-two hybrid (YTH) counter selection (i.e. rescue) assay in which we reconstituted a protein-protein interaction between the human Kvβ 1 β -subunit and the S4-S5 cytoplasmic loop of the human Kv1.1 α -subunit. In this assay, a functional protein-protein interaction results in cycloheximide sensitivity, and yeast cell death, in media containing cyclohexamide. Inhibition of the protein-protein interaction, via small molecule application, rescues yeast cell growth in media containing cyclohexamide. We used this assay to screen over 175,000 synthetic organic molecules and identified several compounds that specifically inhibit the Kvβ 1/Kv1.1 S4-S5 interaction. Remarkably, several of these inhibitors potently eliminate N-type inactivation in CHO cells or *Xenopus* oocytes coexpressing Kv1.1 and Kvβ 1, converting the rapidly inactivating conductance into a sustained conductance. Moreover, several of these compounds dramatically modulate neuronal excitability and neutotransmitter release in an *in vitro* seizure model, and have potent activity *in vivo*. Our success with this YTH assay indicates that protein-protein interactions between Kv channel pore-forming and auxiliary subunits are viable small molecule drug targets, and that modulating these interactions offers novel and exciting approach to ion channel modulation.

Poster NO. 55

The potassium channel KcsA: characteristics during opening

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The discovery of the two-TM K^+ channel gene (*kcsA*) within the bacterium *Streptomyces lividans* allowed the purification of KcsA [1]. Four assembled subunits of the wildtype protein KcsA embedded in planar bilayers form a channel [1] with the following electrophysiological characteristics: (i) K^+ ions can cross the pore in a highly hydrated state ($n_{H2O} > \sim 6$), (ii) the selectivity for K^+ exceeds that for Na^+ ions by 11 times, and both Ca^{2+} and Mg^{2+} are permeant, (iii) the internal side is blocked by Ba^{2+} ions in a voltage-dependent manner, (iv) intrinsic rectification is due to gating, depending on the direction of the electric field, (v) the internal side is pH-sensitive, and (vi) the open pore has a diameter of ~ 5.8 Å [2]. By comparative analyses of mutant KcsA proteins assembly [3], gating and rectification properties could be elucidated [4].

[1] Schrempf, H., Schmidt, O., Kümmerlen, R., Hinnah, S., Müller, D., Betzler, M., Steinkamp, T., Wagner, R. (1995) EMBO J 14:5170-5178.

[2] Meuser, D., Splitt, H., Wagner, R., Schrempf, H. (1999) FEBS Lett 462:447-452.

[3] Splitt, H., Meuser, D., Borovok, I., Betzler, M., and Schrempf, H. (2000) FEBS Lett, in press.

[4] Splitt, H., Meuser, D., Wagner, R., and Schrempf, H. Submitted.

Poster NO. 56

A postsynaptic transient K^+ current modulated by arachidonic acid regulates synaptic integration in hippocampal pyramidal cells

Geert M.J. Ramakers and Johan F. Storm*, Institute of Physiology, University of Oslo, Oslo, Norway.

Voltage-gated ion channels in the dendrites of mammalian central neurons can modulate the impact of synaptic inputs. One of the ionic currents contributing to such modulation is the fast-inactivating A-type potassium current (I_A) in hippocampal pyramidal cells. We have investigated the role of I_A in synaptic integration in CA1 pyramidal cells in rat hippocampal slices by whole-cell patch clamp recording. Heteropodatoxin-3 (HpTX3), a selective blocker of the Kv4

channels underlying the somatodendritic I_A, reduced I_A by 60-70%, and strongly enhanced the amplitude and summation of trains of excitatory postsynaptic potentials (EPSPs) in response to stimulation of fibers in *stratum radiatum*. Arachidonic acid (AA), which is known to modulate Kv4 channels, mimicked the effects of HpTX, both by suppressing I_A recorded in voltage clamp, and enhancing trains of EPSPs to the extent that they started to trigger action potentials. The effects of both the toxin and AA were prevented by loading the postsynaptic cell with Cs^+ ions. We conclude that the Kv4-mediated A-current is activated during postsynaptic depolarizations and strongly regulates the somatodendritic integration of high-frequency trains of synaptic input. Because AA can be released by such input and enhances synaptic efficacy by suppressing I_A, such modulation of I_A could play an important role in frequency-dependent synaptic plasticity in the hippocampus. [*Supported by an EU Research Training Grant (BIO4CT975106), the Norwegian Research Council (NFR/MH), and the Nansen, Langfeldt and Odd Fellow foundations*.]

Poster NO. 57

Pharmacology of HERG and KCNQ1 channels coexpressed with KCNE1 in HEK 293 cells

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Inhibition of cardiac K^+ channels is the therapeutic action of class III antiarrhythmics but it also seems to be an explanation for the potential cardiotoxicity of non-cardiovascular drugs such as antihistamines, antipsychotics, antidepressives and antifungal agents. These agents can in very rare instances trigger life-threatening polymorphic ventricular tachycardias such as torsades de points.

HERG (human ether a-go-go related gene) and *KCNQ1* encode two types of voltage-dependent K^+ channels expressed in the heart. HERG and KCNQ1 have both been suggested to be associated with the beta-subunit KCNE1 (also called minK or IsK). Mutations in KCNQ1, HERG and KCNE1 are the cause of three types of inherited long QT syndromes (LQT1 , 2 and 5) indicating that the repolarizing current carried by these channels are important for the normal suppression of cardiac arrhythmias.

We have studied the basic biophysical characteristics of currents through HERG and KCNQ1 channnels coexpressed with KCNE1 in the mammalian cell line HEK 293. Further, the inhibition of these currents by a range of non-cardiovascular drugs were addressed. At HERG+KCNE1 the antipsychotics sertindole, haloperidole and pimozide were the most potent compounds tested with IC_{50} values below 200 nM. Two of these compounds, sertindole and pimozide, also blocked KCNQ1+KCNE1 with IC_{50} values of 0.55 uM and 1.5 uM, respectively. The antihistamines astemizole and terfenadine blocked HERG+KCNE1 at 180 nM and 300 nM, respectively.

Poster NO. 58

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The K_{ATP} channel is crucial for regulation of insulin secretion from pancreatic β-cells, and mutations in either the SUR1 or Kir6.2 subunit of this channel may lead to persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI). Here we report the functional properties of K_{ATP} channels containing a PHHI missense mutation, R1420C, which lies in the second nucleotide binding fold (NBF2) of SUR1.

Photoaffinity labeling of NBF1 with 8-azido-[α-^{32}P]ATP was inhibited by MgATP and MgADP with K_i of 1.6 and 17 μM, respectively, for SUR1, and of 1.5 and 13 μM, respectively, for SUR1-R1420C. This indicates the affinity of NBF1 for ATP and ADP is similar for wild-type (wt) and R1420C SUR1. However, the MgATP and MgADP affinity of NBF2 of SUR1-R1420C (ATP, 350 μM; ADP, 290 μM) were lower than wt SUR1 (ATP, 64 μM; ADP, 65 μM). MgATP and MgADP stabilized ATP binding at NBF1 of wt SUR1 by interacting with NBF2. This cooperative nucleotide binding was not observed for SUR1-R1420C. Both wt and R1420C SUR1 hydrolyzed ATP at NBF2 but not at NBF1, as indicated by labeling with 8-azido-[α-^{32}P]ATP but not 8-azido-[γ-^{32}P]ATP. Electrophysiological studies of wild-type and mutant K_{ATP} channels expressed in *Xenopus* oocytes, revealed that the R1420C SUR1 mutation does not affect ATP, diazoxide or tolbutamide sensitivity, but produces an increase in the EC_{50} for MgADP activation from 70 to 200 μM. These values agree well with those found for MgADP binding to NBF2.

We conclude, that cooperative nucleotide binding at the NBFs of SUR1 may not be essential for activation or inhibition of the K_{ATP} channel, and that the reduced affinity of NBF2 for MgADP may contribute to the impaired metabolic regulation of Kir6.2/SUR1-R1420C channels.

Poster NO. 59

Effects of early and delayed afterdepolarizations on HERG K^+ currents compared to inward rectifier K^+ currents

Yu Lu, Martyn P. Mahaut-Smith, Christopher L-H. Huang, Jamie I. Vandenberg. Department of Biochemistry, University of Cambridge, UK.

Life threatening cardiac arrhythmias are often initiated by early or delayed afterdepolarizations (EADs or DADs). HERG K^+ channels are voltage gated channels, that function as inward rectifiers and play an important role in phase 3 repolarisation. HIRK1 K^+ channels are strong inward rectifiers that contribute to phase 3 repolarization as well as maintenance of the resting membrane potential. To gain insights into the ability of these channels to suppress arrhythmias initiated by EADs and DADs we used action potential (AP) clamp techniques to study theses channels transfected in CHO cells. During an EAD, HERG currents are significantly larger than during a normal action potential by ~1.2 fold. In contrast HIRK1 currents are very similar during normal APs and EADs. During a series of DADs, HERG conductance is proportional to the amplitude of DAD voltage, but gets smaller with later DADs, due to the time dependent deactivation of HERG. When a series of 10 DADs were applied the HERG current during the 1^{st} DAD is about 10.5 fold larger than during the 10^{th} DAD. In contrast HIRK1 currents were equally large irrespective of the coupling interval between the end of the normal AP and the DAD. These results indicate that HERG K^+ channels will be more effective at suppressing EADs and DADs closely coupled to the preceeding AP whereas HIRK1 channels will help suppress any DADs. These differences are due to the different mechanisms of inward rectification of the two channels, i.e. HIRK1 channels undergo extrememly rapid rectification, whereas the inward rectification of HERG K^+ channels is due to voltage dependent C-type inactivation that has a timecourse of the order of a few to 10s of ms depending on the voltage.

BENZON SYMPOSIUM No. 47

MOLECULAR PHARMACOLOGY OF ION CHANNELS

AUGUST 13-17, 2000, COPENHAGEN, DENMARK

Organizing committee:
Jan Egebjerg, Søren-P. Olesen and Povl Krogsgaard-Larsen

Abstracts - THURSDAY, August 17, 2000

Cracking the puzzles of CRAC, Ca^{2+} release-activated Ca^{2+} channels in human T lymphocytes.
Michael D. Cahalan, Alla F. Fomina, Dept. Physiology, UCI, Irvine, CA USA.
Ca^{2+} release-activated Ca^{2+} (crac) channels provide the Ca^{2+} influx to sustain the $[Ca^{2+}]_i$ signal that is required for mitogenic activation in T lymphocytes. The use of Na^+ as a current carrier enabled Ca^{2+} release-activated Ca^{2+} (crac) channels to be detected and characterized with single-channel resolution during whole-cell recording in Jurkat T cells and in resting and PHA - activated human T cells. Passive Ca^{2+}-store depletion resulted in the opening of 41-pS crac channels characterized by high open probabilities, voltage-dependent block by extracellular Ca^{2+} in the μ M range, selective Ca^{2+} permeation in the mM range, and inactivation that depended upon intracellular Mg^{2+} ions. The number of crac channels per cell increased greatly from ~ 10 in resting T cells to ~ 130 in activated T cells. Treatment with the phorbol ester PMA also increased crac channel expression to ~ 60 channels per cell, whereas the immunosuppressive drug cyclosporin A suppressed the PHA-induced increase in functional channel expression. Capacitative Ca^{2+} influx induced by thapsigargin was also significantly enhanced in activated T cells. We conclude that a surprisingly low number of crac channels are sufficient to mediate Ca^{2+} influx in human resting T cells, and that the expression of crac channels increases ~ tenfold during activation, resulting in enhanced Ca^{2+} signaling. K^+ channels provide the electrical driving force for Ca^{2+} entry by maintaining the membrane potential. The *n*-type voltage-gated K^+ channel, encoded by *Kv1.3*, is required for the initial T-cell proliferation from the resting state to the activated state. Intermediate-conductance Ca^{2+}-activated K^+ channels, encoded by *IKCa1,* are particularly important in re-stimulation of previously activated human T cells where they are dramatically up-regulated.

Molecular Mechanisms of Gating in Cyclic Nucleotide-gated Channels
Zagotta W N, Department of Physiology and Biophysics, University of Washington, Seattle, Washington.
Rod cyclic nucleotide-gated channels (CNG1) are activated by the direct binding of cGMP to an intracellular domain in the carboxyl-terminal region. The binding of ligand initiates a sequence of molecular events that results in the opening of the channel pore. We asked the question: does the S6 region, which is predicted to line the inner vestibule of these channels, undergo conformational changes associated with gating? Guided by the structure of KcsA channels, we constructed a homology model of the CNG1 channel S5-pore-S6 regions. This model was used to make testable predictions of amino acid accessibility and side chain orientation. To test our predictions, we introduced cysteines into 12 sites in the S6 region of a cysteine-free alpha subunit of the rod CNG channel (CNG1c7). Consistent with our homology model, which predicts the cytoplasmic ends of the S6 helices are in close proximity to each other, we found spontaneous disulfide bonds form between S399C residues in neighboring subunits. Interestingly, these disulfide bonds only form when S399C channels are in the clo*sed state and not when they are in the open state. We also measured the rate of modification by internal MTS reagents of a number of cysteines introduced in the S6 helices. We found the rate of modification to be similar for both open and closed states of these channels. Our results suggest that the S6 region of CNG1 channels undergoes a conformational change associated with gating and the activation gate is within or beyond the selectivity filter in the permeation pathway.

Ion Channels in the Pain Pathway
David Julius, Department of Cellular & Molecular Pharmacology, University of California, CA – USA.
The capsaicin (vanilloid) receptor is a non-selective cation channel that is located on primary afferent sensory neurons of the "pain" pathway. When expressed in heterologous cell systems, this channel (VR1) can be activated by vanilloid compounds or structurally-related second messengers, by noxious heat (> 43°C), or by extracellular protons. Based on these in vitro studies, we have suggested that VR1 acts as a polymodal detector of noxious chemical and physical stimuli, thereby capable of integrating information at the sensory nerve terminal under normal or pathophysiological conditions. To test this hypothesis, we have generated VR1-deficient mice and examined their sensitivity to chemical and physical stimuli using a battery of cellular and behavioral assays. VR1-deficient mice are unaffected by vanilloids (capsaicin or resiniferatoxin), demonstrating that this protein is essential for mediating the actions of these compounds

in vivo. Sensory neurons or C-fibers from these mutant mice also show a marked reduction in proton (pH 5) sensitivity, suggesting that VR1 contributes significantly to the excitation or sensitization of nociceptors in the setting of injury-evoked tissue acidosis. VR1-deficient mice also show marked reductions in sensitivity to noxious heat at both cellular and behavioral levels. However, heat-evoked responses are not eliminated in these animals, demonstrating that thermal nociception involves multiple transduction mechanisms. VR1 mutant mice show little thermal hypersensitivity in the setting of inflammation, illustrating the importance of VR1 to injury-induced thermal hyperalgesia. Importantly, VR1-deficient mice show normal sensitivity to mechanical stimuli under normal or inflammatory conditions.
To understand how the capsaicin receptor functions as a polymodal signal transducer, we have looked for mutations in VR1 that alter responses to specific stimuli. This screen has highlighted positively charged residues near the putative pore-loop region of the channel that selectively reduce activation of VR1 by extracellular protons or alter the effects of acidification on heat- or vanilloid evoked responses

New Perspectives on Structure and Function of Voltage-gated Calcium Channels
Tsien RW, Cataldi M, Yang N, Aldrich RW, Perez-Reyes E, Pitt G, Zuehlke R, Reuter H, Piedras-Renteria E, Smith SM, Jun K, Shin H, Lee C-C, Zoghbi H. Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford CA 94305.
The last several years have seen remarkable progress in almost all aspects of the study of voltage-gated calcium channels, so important for linking membrane excitation to cellular responses. With the cloning of three new α_1 subunits for T-type channels, we have a subfamily of pore-forming principal subunits for each of the channel classes (T-, N- and L-type) defined in 1985. This presentation will focus on recent progress on aspects of how calcium channels function as signal transduction molecules. The "menu":
The role of acidic amino acids in the Ca^{2+} channel pore: relationship to pore size as well as divalent cation selectivity and permeation. What makes T-type channels different?
Control of Ca^{2+} channel gating: calmodulin as key player in both inactivation and facilitation of L-type Ca^{2+} channels. How can the same Ca^{2+} sensor molecule serve such different roles?
Physiological and possible structural roles of Ca^{2+} channels: effects of deleting α_{1A} subunits or modifying them as in spinocerebellar ataxia 6 or migraine.

Diseases Due to Chloride and Potassium Channel Mutations
Thomas J. Jentsch, Zentrum für Molekulare Neurobiologie Hamburg, Universität Hamburg, D-20246 Hamburg, Germany.
Ion channels have important functions in controlling nerve and muscle excitability, in transepithelial transport, and in cell homeostasis. Many ion channels have functions in the plasma membrane, although others function in intracellular organelles. During the past decade, it became apparent that mutations in several ion channel genes lead to human inherited disease. This demonstrates the importance of ion channels for diverse functions in the organism. In our lab, we identified the CLC family of voltage-gated chloride channels and identified several diseases that are due to CLC gene defects. Mutations in the skeletal muscle chloride channel ClC-1 lead to myotonia congenita, which is due to muscle membrane electrical hyperexcitability. In vitro electrophysiological analysis of mutations found in patients could well explain the phenotypical differences between mutations leading to dominant versus recessive disease, and the pathophysiology of myotonia is very well understood.
Mutations in the chloride channel ClC-5, which is predominantly expressed in kidney, cause Dent's disease. Patients present with low molecular weight proteinuria and with hypercalciuria, which in turn results in kidney stones, nephrocalcinosis and renal failure. ClC-5 is highly expressed in endocytotic vesicles in the proximal tubule. It probably serves to facilitate intravesicular acidification, which is necessary for the endocytotic uptake of proteins. While this explains proteinuria, the mechanism leading to hypercalciuria is currently unknown. Another kidney-specific chloride channel, ClC-Kb, is mutated in a form of Bartter's syndrome. ClC-Kb probably mediates transepithelial chloride reabsorption in the thick ascending limb of Henle's loop, explaining the massive renal salt loss observed in Bartter's syndrome.

Structure, Function, and Molecular Pharmacology Voltage-gated Sodium Channels
William A. Catterall, University of Washington, Seattle, WA.
The voltage-gated sodium channels are composed of a large α subunit of 260 kDa in association with a β 1 subunit of 36 kDa and, in neurons, a disulfide-linked β 2 subunit of 33 kDa. The α subunit is the pore-forming subunit. β 1 and β 2 are single membrane-spanning glycoproteins containing immunoglobulin-like folds in their extracellular domains. They interact with the α subunit through their extracellular domains and modulate channel expression and gating. The immunoglobulin-like folds have the structures of cell adhesion molecules and interact with extracellular proteins like tenascin.
The α subunits of sodium channels are organized in four homologous domains (I through IV) which each contain six transmembrane alpha helices (S1 through S6). The S4 segments contain positively charged residues which serve as

voltage sensors for channel activation and move outward under the influence of the electric field to initiate activation. The S5 and S6 segments and the short membrane-associated segments between them (SS1/SS2) form the pore.
The fast inactivation of the open sodium channel is mediated by closure of a hinged-lid-like inactivation gate formed by the intracellular loop between domains III and IV. The hydrophobic motif IFM within this loop serves as the inactivation particle. This motif moves from a cytoplasmic location into the channel structure during inactivation and becomes inaccessible to chemical modification. The three-dimensional structure of the core of the inactivation gate, including the IFM motif, has been determined by NMR spectroscopy and forms the basis for a mechanistic interpretation of site-directed mutagenesis studies of the inactivation process. The inactivation gate folds into a receptor region formed by the IIIS4-S5 loop, the IVS4-S5 loop, and the intracellular end of the IVS6 segment.
Local anesthetics and related drugs block the pore of sodium channels by binding to a receptor site formed by amino acid residues in transmembrane segment S6 in domains III and IV. Site-directed mutations of critical amino acids at similar positions in these segments greatly reduce the affinity for local anesthetic block and specifically disrupt high affinity binding to the inactivated state. Many different structural classes of sodium channel blocking drugs interact with this site in the pore. In contrast, peptide scorpion toxins which alter gating of sodium channels bind to the extracellular ends of S4 segments and trap them in either an activated or non-activated state. α -scorpion toxins trap the IVS4 segment in its inward position and slow or prevent inactivation; β -scorpion toxins trap the IIS4 segment in its outward position and greatly enhance activation. Voltage sensor-trapping may be a general mechanism of action of peptide toxins which affect ion channel gating.

Poster NO. 60

Identification of an amino acid responsible for acid-mediated potentiation of vanilloid receptor (VR1) function.

Smith, G.D., Clarke, C. E., Meadows, H. J., Benham C.D., Randall, A.D., Davis, J.B.
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We have investigated the basis of the acid-mediated potentiation of vanilloid receptor-mediated capsaicin responses. Experiments were performed using electrophysiological analysis of both human (hVR1) and rat (rVR1) receptors expressed in Xenopus oocytes. Application of capsaicin produced concentration-dependent activation of an outwardly rectifying conductance, in both rVR1 and hVR1 expressing oocytes. This was completely reversed upon agonist removal. As previously reported by others, weak acid challenges (i.e. to pH 6.5) produced no receptor activation alone but substantially facilitated capsaicin responses. Stronger acid challenges (i.e. to pH<6) produced direct activation gating of VR1. The potentiation of capsaicin responses at pH 6.5 involved both an increase in the maximum response achievable and a leftward shift in the concentration-response curve. At negative holding potentials the acid-dependent potentiation of capsaicin responses appeared much greater for hVR1 than rVR1. For both rat and human forms however the pH 6.5-mediated potentiation of VR1 was strongly voltage-dependent, decreasing e-fold for 53 mV of membrane depolarisation with hVR1. Mutation of the only extracellular histidine residue in rVR1 produced no significant change in VR1 activation by capsaicin or the enhancement of capsaicin-mediated currents at pH 6.5. In contrast, mutation of glutamate 478 to alanine (rVR1-E478A) completely eliminated the acid-mediated potentiation of rVR1 without altering capsaicin activation itself. It also appears glutamate 478 may contribute to direct acid-mediated gating of VR1 since its mutation to alanine altered, but did not eliminate, the activation of rVR1 by stronger acid challenges ($\leq$ pH 6.0).

Poster NO. 61

Modulation of Ryanodine Receptor Type 1 by Adenosine and its Catabolites. An Structure-Activity Approach.

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Ryanodine receptor (RyR) plays a key role in the excitation-contraction coupling in skeletal muscle acting as a calcium release channel. RyR is an allosteric-regulated receptor-channel formed by 4 subunits of high molecular weight. Among multiple modulators, purines such as ATP and caffeine, have been shown to activate RyR. ATP promotes an enhanced open probability in the RyR, whereas caffeine increases its sensitivity to be activated by [Ca^{2+}] at nM range. The aim of this project was to evaluate the modulation on the RyR properties of other purines such as adenosine and its catabolites, making a correlation between the purines´ structural and electronic features (calculated through semiempirical methods) and their modulatory effectiveness.
Using equilibrium [^{3}H]-ryanodine binding assay as a probe to estimate RyR´s activity, we showed that adenosine and its catabolites, at nM and μ M ranges, are activators of the RyR with the next potency order: xanthine>adenosine>inosine>uric acid>hypoxanthine. To define if adenosine and its catabolites are recognized by the RyR´s sites for ATP or caffeine, experiments of calcium dependence and competition were done. Calcium-dependence results showed a similar pattern of RyR activation for ATP, adenosine and xanthine, whereas data from competition experiments indicate a mutual interaction among these 3 purines. It is probable that due to its polarity, xanthine fits at the hydophillic sites for ATP and not in the hydophobic sites for caffeine. Our conclusions are: 1) RyR presents low and high affinity binding sites for purines; 2) Activation of RyR is dependent on the type of purine tested and its concentration; 3) ATP, adenosine and adenosine´s catabolites are recognized at the same site(s).

Poster NO. 62

Changes in mRNA for ryanodine receptors after transient cerebral ischemia and tolerance induction

Christina Dahl, Nils Henrik Diemer, Laboratory of Neuropathology, University of Copenhagen, Denmark.

The ryanodine receptor (RyR) is involved in the regulation of intracellular Ca2+ concentration via Ca2+-induced Ca2+ release. Moreover, disturbance of the intracellular Ca2+ homeostasis is assumed to precede ischemia-induced neuronal death. Using the rat 4-vessel occlusion model of cerebral ischemia and tolerance induction, we studied the changes in the mRNA levels for the RyR subtype 1, 2 and 3 by means of in situ hybridisation with oligonucleotides. The mRNA level for the RyR1 was unchanged in the different ischemic group. RyR3 mRNA level decreased in DG after 9 minutes of ischemia and after 3 + 8.5 minutes of ischemia. In contrast, RyR2 mRNA level was altered in the ischemic vulnerabel CA1 region. Thus, 3 minutes of ischemia increased the mRNA level in both CA1 and CA3, whereas 9 minutes of ischemia increased the mRNA level in CA1, but decreased the level in CA3. 3 + 8.5 minutes of ischemia reduced the mRNA level only in CA3. The RyR immunoreactivity remained unchanged in all the ischemic groups compared to sham-operated animals. These changes do neither support a protective or degenerative mechanism mediated via the RyR after ischemia and tolerace induction, which corresponds to the lack of effect of dantrolene against ischemic induced damage in the rat brain.

Poster NO. 63

Auxiliary Subunits of the Voltage-Activated Calcium Channel

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Voltage-activated calcium channels are formed by heterooligomeric complexes consisting of various combinations of an α 1 subunit with auxiliary ß, α 2δ and γ subunits. The α 1 subunit accounts for the pore and contains the voltage sensor of the channel. The current through the α 1 subunit is modulated by interactions with different auxiliary subunits. Recently we identified and characterized two novel α 2δ subunits (Klugbauer et al., 1999, J. Neurosci.). Coexpression studies with α 1C and α 1E revealed that these subunits shift the voltage dependence of channel activation and inactivation in a hyperpolarizing direction and accelerate the kinetics of current inactivation.

Genetic diversity can also be observed for γ subunits. We found that there are at least five γ subunits that are differentially expressed in tissues, γ -1 is expressed only in skeletal muscle, γ -2, -3 and -4 in different regions of the brain, while γ -5 is more ubiquitously expressed. Transient transfection of the novel γ subunits together with different high and low voltage-gated calcium channels revealed their modulatory effects. The neuronal γ subunits shift the steady-state inactivation of α 1A to hyperpolarized potentials, whereas γ -5 accelerates current activation and inactivation of the T-type α 1G calcium channel. The expression pattern of the γ subunits together with their electrophysiological effects implicate that γ -2 could be associated with P/Q-type α 1A, γ -4 with α 1E and γ -5 with the T-type α 1G calcium channel.

Poster NO. 64

Regulation of low-voltage-activated α1G calcium channel by auxiliary subunits and channel blockers

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Cloning of three members of low-voltage-activated calcium channel family enabled to investigate directly their electrophysiological and pharmacological profile as well as their putative subunit composition. We have expressed α 1G channel subunit in HEK 293 cells alone or in combination with members of families of α 2δ or γ auxiliary subunits. Whole cell patch clamp method was used to characterize channel properties. α 2δ -2 and γ -5 subunits significant and systematically modified activation and/or inactivation of the current. In contrast, α 2δ -1, α 2δ -3, γ -2 and γ -4 subunits failed to modulate the current or had only minor effects.

The pharmacological profile of the α_{1G} channel only partly resembled that of native T-type calcium channels. I_{Ca} was blocked by Ni^{2+} and Cd^{2+} with IC_{50}´s of 1130± 60 μM and 658± 23 μM, respectively. Nickel modified channel activation and both Ni^{2+} and Cd^{2+} accelerated channel deactivation. Channel was potently blocked by mibefradil in a use- and voltage-dependent manner (IC_{50}´s 0.39 and 0.12 μM at holding potentials –100 and –60 mV, respectively), moderately blocked by phenytoin (IC_{50} 73.9± 1.9 μM) ethosuximide (20% block by 3 mM) and resistant to the block by valproate. 5 mM amiloride inhibited I_{Ba} by 38% and significantly slowed current activation. The α_{1G} channel was not affected by 10 μM tetrodotoxin. Antagonistic (1 μM isradipine and 10 μM nifedipine) and agonistic (1 μM Bay K 8644) dihydropyridines had only minimal blocking or activating effect on the current through the α_{1G} channel, respectively.

Poster NO. 65

Facilitation Of Plateau Potentials In Turtle Motoneurones By A Pathway Depending On Calcium And Calmodulin

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1. The involvement of intracellular calcium and calmodulin in the modulation of plateau potentials in motoneurones was investigated by intracellular recordings in a spinal cord slice preparation.
2. Chelation of intracellular calcium or inactivation of calmodulin reduced the amplitude of depolarization induced plateau potentials. Inactivation of calmodulin also inhibited facilitation of plateau potentials by activation of group I metabotropic receptors for glutamate or muscarine receptors.
3. In low sodium medium and in the presence of tetraethylammonium (TEA) and tetrodotoxin (TTX), evoked calcium action potentials were followed by an afterdepolarization mediated by L-type calcium channels. The amplitude of the afterdepolarization depended on the number of calcium spikes.
4. This calcium induced plateau potential was reduced by blockade of calmodulin.
5. It is proposed that plateau potentials mediated by L-type calcium channels in spinal motoneurones are facilitated by activation of a calcium and calmodulin depending pathway.

Poster NO. 66

Electrophysiological analysis of the vanilloid receptor (VR1): studies of recombinant receptors and knockout mice *(Oral presentation Thursday 13:30-14:00)*

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Since its initial cloning in 1997, the capsaicin-gated ion channel VR1 has been the topic of much novel research. We have cloned both rat (rVR1) and human (hVR1) versions of this protein and stably transfected them into HEK293 cells. In addition, we have generated a mouse line in which the gene encoding VR1 has be genetically ablated. Expression of both rVR1 and hVR1 in HEK293 cells lead to the appearance of ionic conductances that could be activated by capsaicin, anandamide, extracellular acidification and temperatures in excess of ~44° C. For each of these stimuli, the VR1-mediated currents could be inhibited by capsazepine, in a voltage-dependent manner. Irrespective of the stimulus used to activate the receptor, the current-voltage relationships of VR1-mediated responses exhibited reversal potentials close to 0 mV, substantial outward rectification and a region of negative slope conductance at very negative potentials. The rectification properties of the VR1 conductance were not instantaneous but exhibited clear time-dependence. Divalent ions such as Cd^{2+} and Zn^{2+} produced multifaceted actions on VR1 which included effects permeation, activation and desensitization. Genetic ablation of the mouse VR1 gene completely eliminated the capsaicin responsiveness of small diameter sensory neurones cultured from dorsal root ganglia; responses to GABA and ATP, in contrast, were unaltered. VR1 deletion also removed a slowly activating, non-desensitizing, acid-gated current present in capsaicin-sensitive wild-type sensory neurones. Furthermore VR1 knockout eliminated a strongly temperature-dependent cation conductance that was observed at temperatures in excess of a threshold of ~44° C. These data indicate that VR1 may be important in nociceptive responses to both acid and heat.

Poster NO. 67

Possible involvement of calcium and potassium fluxes in the effects of idazoxan in isolated rat aorta

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Idazoxan (IDA) is an α_2 adrenoceptor antagonist, but also blocks imidazoline receptors. Others have shown that in rat aorta contracted by phenylephrine (PHE) IDA induces endothelium dependent ($>10^{-8}$ M) and independent ($>10^{-6}$ M) relaxation (the latter inhibited by TEA). We further investigated this, using phentolamine (PHA) as α_2 antagonist, D600 to block Ca_L, angiotensin II (ANG) and prostaglandin $F_{2\alpha}$ (F2A) as nonadrenergic agents, high K^+ for depolarization. Aorta rings (2 mm wide, ± endothelium, male adult Wistar rats) were mounted in oxygenated PSS (HCO_3^- buffer, pH 7.2-7.4) at 37^0 C and force was recorded (PC-based system); results as % of PHE 10^{-5} M effects (mean ± SEM; n=6). IDA $>10^{-5}$ M induces small (<25%) contractions of resting preparations, that are partially D600-sensitive. IDA does not inhibit contractions induced by high K^+ or by ANG and shows weak relaxation of F2A effects. IDA relaxation in rings contracted by PHE was somewhat stronger in the presence of endothelium, increased with lower PHE, was only slightly reduced by TEA 10^{-2} M and was absent in the presence of D600. IDA effects may not involve α_2, very few in the rat aorta and PHA inhibited them only at very high doses. Some imidazolines contract the rat aorta by a nonadrenergic, nonimidazolinic mechanism involving Ca_L. Although α_2 determine smooth muscle depolarisation, they are also able to open K channels. Idazoxan and clonidine are also α_2/α_1 partial agonists in the rat aorta. IDA ineffectiveness in high K^+ argues against the proposed cGMP-mediated mechanism in the presence of endothelium and does not favor the idea of K^+-dependent hyperpolarization for the direct relaxing effect, which may involve interaction with Ca_L.

Poster NO. 68

Distinct contribution to Ca^{2+}-sensitive inactivation and facilitation of L-type Ca^{2+} channels by individual amino acids in a consensus calmodulin-binding motif of α_{1C}

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Voltage-dependent L-type calcium channels play important roles in cardiovascular and neuronal excitability and signal transduction. Calcium flux through these channels regulates their own activity by linking channel inactivation and facilitation to the previous entry of calcium ions. The molecular basis of these autoregulatory gating mechanisms remains unclear. Calmodulin (CaM) is a critical calcium sensor for both inactivation and facilitation. The nature of the CaM effect depends on residues within a CaM-binding isoleucine-glutamine (IQ) motif. Alanine substitutions of all residues at five key positions (I1624, Q1625, F1628, R1629, and K1630) abolished all Ca^{2+}-dependence, while corresponding individual alanine replacements behaved similarly to the wild-type channel (77wt) in four of five cases. Only the I1624A mutant removed calcium-dependent inactivation and unmasked a strong facilitation. Both effects were further amplified by additional substitution of alanine for the adjacent glutamine. Replacement of I1624 by 13 other amino acids produced graded and distinct patterns of change in the two forms of modulation. The extent of Ca^{2+}-dependent facilitation was monotonically correlated with the affinity of CaM for the mutant IQ-motif, determined in peptide binding experiments *in vitro*. Ca^{2+}-dependent inactivation also depended on strong CaM binding to the IQ-motif, but showed an additional requirement for a bulky, hydrophobic side chain at position 1624. Abolition of Ca^{2+}-dependent modulation by IQ-motif modifications mimicked and occluded the effects of over-expressing a dominant negative CaM mutant. (Supported by grants from SNF and from NIH).

Poster NO. 69

Oral administration of novel blocker of erythrocyte Cl-conductance (NS3623) reduces sickle cell dehydration and sickling in transgenic sickle (SAD) mice *(Oral presentation Thursday 15:00-15:30)*

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A distinguishing feature of sickle cell disease is erythrocyte dehydration, which enhances sickling due to the steep concentration dependence of HbS polymerization. These dense cells exhibit low filterability, impaired capillary flow and may cause stroke and painful ischemic crises. Thus, inhibition of sickle cell dehydration is a therapeutic strategy for this disease. K^+-loss via the Ca^{++}-activated K^+ (Gardos) channel is a main cause for sickle cell dehydration and depends on a concomitant Cl^--current. Therefore, dehydration could be reduced by inhibition of the Cl^- conductance (g_{Cl}). The prototype of a new class of reversible g_{Cl} blockers, NS1652, inhibited *in vitro* deoxygenation-induced salt loss from human sickle cells, inhibited mice erythrocyte g_{Cl} *in vivo* after IV administration, and was well-tolerated (Bennekou et al, Blood 2000; 95:1842). NS3623, a NS1652 analogue, can be administered orally and exhibits long-term blocking effects in mice, with 70% g_{Cl} block persisting 5 hours after administration of 100 mg/kg. SAD-mice (a transgenic mouse model of sickle cell disease) were treated for 3 weeks with NS3623. Oral doses between 20 and 200 mg/kg/day significantly increased the erythrocyte volume and cation content and decreased cell density and cell Hb concentration. Significant blockade of the Gardos channel mediated KCl transport could be demonstrated ex vivo. With NS3623, the fraction of circulating sickled cell decreased significantly, with a predominance of well-hydrated, non-sickled forms. The present experiments demonstrate the feasibility of this novel therapeutic strategy based on *in vivo* reduction of sickle cell dehydration and sickling by using a g_{Cl} inhibitor.

Poster NO. 70

Modulation of Chloride-Dependent Gating of ClC-1 by Internal pH and Hydrophobic Anions

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It is believed that in the skeletal muscle chloride channel, ClC-1, the permeating anion serves as a gating charge, and voltage-dependence of the gating can be attributed to the voltage-dependent binding of Cl^- in the channel pore. Gating of ClC-1 is also dependent on the internal pH, but the nature of this dependence remains unclear. In the present work we used whole-cell patch-clamping of Sf9 cells expressing rat ClC-1 to further study the mechanisms of ClC-1 gating. Addition of benzoate or hexanoate to the internal solution resulted in a faster and more complete deactivation of the inward currents; at the same time, the apparent P_{open} curves were shifted to more positive potentials. Almost identical changes in the parameters describing ClC-1 current kinetics could be achieved by increasing the internal pH with a faster and more complete deactivation of the inward current and a shift of the apparent P_{open} curves to more positive potentials. Relative amplitudes of the inward current components were also affected in a very similar way by internal hydrophobic anions and high internal pH. Accepting the hypothesis that ClC-1 is gated by the permeating anion we propose that benzoate binding from the internal side of the pore can modulate Cl^- affinity for the gating site. Similarly,

modulation of that site by OH^- or some intrinsic "blocking particle" binding at the internal mouth of the channel seems to be one of the most probable reasons for ClC-1 gating dependence on the internal pH.

Poster NO. 71

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Our goal is to determine the 3-D structure of the outer surface of voltage-gated sodium channels (NaChs) in its various states. In the first phase, we have been studying the effects of complementary mutagenesis on Site 3 scorpion α-toxins and rat skeletal muscle isoform 1 (rSkM1) on the toxin-channel dissociation constants (K_d) and the k_{on} and k_{off} kinetic constants relevant to the resting state. Using chimeric and site-specifically mutated NaChs, we have shown that a relatively new class of *Leiurus quinquestriatis hebraica* (Lqh) toxins, which cause slowing of current decay and create a residual current, bind to the S3-4 loop of Domain 4 of rSkM1. Our studies indicate: 1. Insect- and mammalian-specific toxins are both Site 3 toxins binding to S3-4D4 since each of their affinities is markedly affected by the D1428N and/or K1432Q mutations in rSkM1. 2. The Lqh toxins bind to overlapping but not identical sites (*i..e.*, the bioactive surfaces are quite distinguishable) because there is great variation in affinity ratios ($K_d\{Ch^{mut}\}/K_d\{Ch^{WT}\}$) of the different toxins caused by the same channel mutations (*e.g.*, Asp ^{1428} and Lys^{1432}). 3. More detailed studies with LqhαIT provide interaction energy values that suggest the following electrostatic bonds: D1428(NaCh) .... K25(LqhαIT) and K1432(NaCh) .... R63 (LqhαIT). Modeling the conformation of the rSkM1 S3-4D4 loop and docking it with LqhαIT we have arrived at a postulated structure for this region of the Na channel. Scorpion α-toxin binding to Site 3 is voltage-dependent due changes in the conformation of the channel associated with progression of the channel from resting through closed to open, fast and slow inactivated states. Using perfusion and voltage-jump protocols with wild-type and mutant rSkM1s we have made measurements of K_d values and k_{on} and k_{off} kinetic constants over the voltage-range -140 to +150 mV. The differences in toxin affinities associated with the different states of the channel, which can be accessed by different experimental protocols, has enabled estimates of the relative energy differences between these various channel conformations. Investigations of channels with mutations in voltage-sensors and those that effect fast- or slow-inactivation are consistent with the calculated energy differences between states.

Poster NO. 72

Endogenous expression of the sodium channel subtype rH1 in the glial stem cell line HiB5

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A neuronal stem cell line HiB5 derived from rat hippocampus used for transplantation and with a site-specific differentiation pattern, was evaluated electrophysiologically. Using the patch-clamp technique in the whole cell configuration, a voltage-gated sodium current was observed. The current voltage (IV) relationship of this channel gave rise to the characteristic bell-shaped curve with a potential of maximal activation at -15 mV. Although being a channel expressed by a brain derived cell type the sensitivity to Tetrodotoxin was found to be low with an IC_{50} = 1 μ M. Steady state inactivation for this channel gave a steady-state inactivation curve with a half maximal potential of inactivation V_{50} = -78 mV. The inactivation curve was shifted in the hyperpolarised direction compared to the classical brain types like rBIIA. These characteristics resembled the cardiac subtype rH1. This was confirmed by reverse transcription polymerase reaction on mRNA isolated from HiB5 cells using degenerated primers in regions with high homology among different sodium channel subtypes and amplification of a region with low homology. The effects of two classical sodium channel blockers, Lamotrigine and Sipatrigine, were compared pharmacologically. The tonic block of either was state- and voltage dependent with Sipatrigine being the more potent. In a test situation mimicking use-dependency of action Sipatrigine showed a more pronounced effect compared to Lamotrigine. In conclusion, we have shown that rH1 is expressed in a brain derived cell and provided that the channel is expressed also in differentiated neurons, classical antiepileptic and neuroprotective compounds may in part be effective due to modulation of the rH1 channel.

Poster NO. 73

Relaxation by phenamil in the isolated rat aorta may involve Na^+ influx

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The amiloride derivative phenamil is a smooth muscle relaxant and inhibitor of the epithelial type Na^+ channel. An amiloride-resistant homologue of this channel is present in the smooth muscle of aorta and brain microvessels. The vasorelaxant properties of phenamil have been rather poorly investigated so far. In a study of amiloride derivatives phenamil has been shown to relax the isolated rat aorta contracted by high K^+, with a stronger effect in the presence of endothelium. We further investigated this, by comparison between phenamil effects upon high K^+ and alpha1 adrenergic contractions.

Aorta rings (2 mm wide; ± functional endothelium) from male adult Wistar rats were suspended between wire hooks in oxygenated physiological saline solution (bicarbonate buffer, pH 7.2-7.4) at 37^0 C. Contractile activity in isometric conditions was recorded on a PC-based system. Submaximal contractions were induced by 10^{-5} M phenylephrine and

by 40 mM K^+. Carbachol 10^{-5} M was used to test the functional integrity of the endothelium. Cumulative dose-effect curves for phenamil confirmed the potentiating effect of endothelium and showed a lower sensitivity of the alpha1 adrenergic contraction to inhibition by phenamil. Phenamil pretreatment did not influence the rapid phase (1 min) of force development. Phenamil effects were highly reproducible, but slowly reversible at concentrations above 10^{-5} M. Phenamil effects were inhibited by iso-osmolar substitution of Na^+ with sucrose.
The results reveal a pro-contractile involvement of epithelial type Na^+ channels during the aortic sustained contraction. Other mechanisms, such as phenamil interaction with Na^+/Ca^{2+} or Na^+/H^+ antiport, or with Na^+/K^+ ATPase, are not consistent with the dose-effect curves or even with relaxation itself.

Poster NO. 74

RNA editing and alternative splicing of the pre-mRNA encoding the α 1 subunit of the *Drosophila* voltage gated calcium channel.

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In *Drosophila melanogaster* there exists only one gene of the ADAR family of RNA editing enzymes responsible for converting adenosine to inosine in RNA. Flies mutant for this gene survive but have major behavioural, locomotory and mating defects and show neurodegeneration in the brain that increases with age. The pre-mRNA that encodes the alpha-1 subunit of the CNS voltage gated calcium channel is edited by ADAR at 10 different positions, the consequence of which is the insertion of 9 different amino acids other than those encoded by the DNA at these editing sites. Eight of the editing events target residues in or near helices of the voltage sensor domains. The frequency of editing is different at each site and increases from embryo to adult. Our analysis of *cac* transcripts also identified new alternatively spliced exons and one micro-exon that encodes only one amino acid, an aspartate residue situated in IVS3-S4 adjacent to the voltage sensor helix

Poster NO. 75

Developing New Pharmacophores for Sodium Channel Blockers

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We have a long standing interest in developing new, selective blockers of neuronal sodium channels for a variety of therapeutic indications. In pursuit of this we have begun to develop a pharmacophore to represent those compounds which are active in our in vitro models of sodium channel blockade. Our initial models appear to be quite generic and we therefore questioned to what extent the ability to block sodium channels occurs undetected in drugs under development in unrelated areas. To answer this question we have used our pharmacophore to query the Derwent World Drug Index for drugs which conform to the generic structure we find predictive of sodium channel blocking activity. A representative set of these compounds has been tested in our in vitro models and the resulting data will be presented in our poster.

Poster NO. 76

Heterocyclic AMPA Analogues: Stereochemistry and Molecular Pharmacology

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The purpose of this work was to resolve and to investigate the enantiopharmacology of the potent AMPA receptor agonist (RS)-2-amino-3-[3-hydroxy-5-(2-methyl-2*H*-tetrazol-5-yl)isoxazol-4-yl]propionic acid ((RS)-2-Me-Tet-AMPA).
(RS)-2-Me-Tet-AMPA was resolved using a Chirobiotic T chiral preparative HPLC column. The configuration of zwitterionic (–)-2-Me-Tet-AMPA was assigned to possess the (R)-configuration based on an X-ray crystallographic analysis supported by the elution order of (–)- and (+)-2-Me-Tet-AMPA using four different chiral HPLC columns and by circular dichroism spectra. The two enantiomers did not show detectable affinity for NMDA receptor sites, and (R)-2-Me-Tet-AMPA was essentially inactive in all of the test systems used. Whereas (S)-2-Me-Tet-AMPA showed low affinity in the [^{3}H]KA binding assay, it was significantly more potent than AMPA in the [^{3}H]AMPA binding assay. In concord with these findings, (S)-2-Me-Tet-AMPA was markedly more potent than AMPA in the electrophysiological cortical wedge model. In contrast to AMPA, which showed comparable potencies at cloned receptors formed by the AMPA receptor subunits (GluR1– 4) in *Xenopus* oocytes, more potent effects and a substantially higher degree of subunit selectivity were observed for (S)-2-Me-Tet-AMPA.
It is concluded that (S)-2-Me-Tet-AMPA is a subunit-selective and highly potent AMPA receptor agonist and a potentially useful tool for studies of physiological AMPA receptor subtypes.

Poster No. 77

Molecular Structure and Expression of SK-Potassium Channels from Trout CNS

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Small-conductance Ca^{2+}-activated potassium channels (SK-channels) play a crucial role in the regu- lation of neuronal firing activity in that they control interspike intervals and spike-frequency adaption. In contrast to BK-channels SK-channels manifest Ca^{2+}-sensitivity without voltage-dependence. As yet four different genes encoding SK-type channels were cloned from mammals, whilst very little is known about the molecular structure of SK-channels in lower vertebrate species. By RT-PCR-cloning and RACE-techniques we isolated a full-length cDNA encoding a fish homologue of mammalian SK2 and a partial sequence sharing structural similarities with SK1, termed TSK2 and TSK1 respectively. Comparison of the fish SK-homologues with their mammalian counterparts revealed a conserved asparagine in the outer vestibule of TSK2 which determines apamin-sensitivity of SK2-channels, whereas in the case of TSK1 this residue was replaced by a histidine. As observed by RT-PCR-analysis transcripts of TSK2 and TSK1 were widely distributed throughout excitable tissues, including cerebellum, tectum opticum, retina and in muscle but not in liver. During development expression of both TSK-transcripts was detected initially at stage 30 (hatching). To generate polyclonal antibodies against the TSK2-channel, the amino-terminal tail of the TSK2-protein was overexpressed in E.coli as a his-tag fusion protein. On frozen tissue sections the anti-TSK2-antibodies showed a bright fluorescence staining of selected neurons: in the visual center of trout (tectum opticum), e.g., a prominent labeling of of pyramidal cells and a strong staining of the optic nerve fiber tracts was detected. In addition, by single cell RT-PCR transcripts encoding TSK1 and TSK2 were revealed in retinal ganglion cells. To allow for an electrophysiological characterization of the TSK2-channel it was fused to GFP and cloned into mammalian expression vectors for transient cell expression. As an alternative strategy for heterolo-gous expression recombinant baculoviruses were constructed and used for infection of insect cells, that provide a convenient system for the electrophysiological characterization of recombinant ion channels.

Supported by the graduate college of Lower Saxony.

Poster No. 78

Development of Conotoxin Calcium Channel Antagonists by Combinatorial Library.

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Synthetic peptides derived from conotoxins have been successfully applied as selective antagonists of N-type calcium channels in a variety of pathological conditions. In particular, the ω -type of conotoxins are beneficial in treatment of specific pain syndromes and some of which are >100 fold more potent than morphine.

The ω -conotoxins contain thrree disulfide bonds and a four looped structure. Although this scaffold is maintained among interspecies of *Conus*, their amino acid sequences are hypervariable. We herein report the development of a chemical combinatorial library strategy to prepare constrained conotoxin analogs to improve their metabolic stable but maintaining their structural fold with fewer cystine constraints.

Our approach is based on ligation chemistry of unprotected peptides. In particular, the chemistry involves the use of a single unprotected peptide to generate different shapes though various intramolecular constraints. Furthermore, the chemistry can be applied by solid-phase method based on the split-and –mix strategy to increase diversity. The advantages, limitations, and success of our strategy will be discussed.