within the open-closed cycle. This reveals that the outermost transmembrane domain is important for appropriate gating and shows a high conservation of M4 function among members of this superfamily.

2511-Pos Board B481

Targeted Delivery of Glycine Receptors to Peripheral Neurons as Treatment for Pain

Michael Cascio[1], James R. Goss[1], Shaohua Huang[1], David M. Krisky[1], Richard J. Clarke[2], Jon W. Johnson[2], Joseph C. Glorioso[1].
[1]University of Pittsburgh School of Medicine, Pittsburgh, PA, USA,
[2]University of Pittsburgh, Pittsburgh, PA, USA.

Neurotransmitter-gated receptors play a vital role in the regulation of nociception. In the adult CNS, the ionotropic glycine and GABA receptors are typically inhibitory and act to silence neurons by transiently permitting inward Cl^- currents upon activation. However, depending on the Cl^- equilibrium potential, these receptors may instead depolarize neurons. We hypothesized that targeted expression of the α1 subunit of glycine receptor (GlyR) in peripheral sensory neurons using a non-replicating herpes simplex virus (HSV)-based vector might reduce nociceptive behavior upon subsequent GlyR activation by exogenously-applied glycine. HSV-directed expression of the human α1 subunit of GlyR alone is sufficient to produce glycine-gated chloride currents in whole-cell patch clamp studies of infected mammalian cells. In cultured dorsal root ganglion neurons, expressed GlyRs are diffusely localized in the neuronal plasma membrane. In both a formalin footpad model of inflammatory pain or an osteosarcoma pain model, rats inoculated with the GlyR-expressing vector (vHGlyRα1) exhibited significantly reduced nociceptive behavior following subcutaneous injection of glycine into the footpad. This reduction in pain-related behavior was reversed following injection of the GlyR antagonist strychnine. Targeted expression of GlyRs and their modulation via mutagenesis and/or exogenous application of agonists, antagonists, and allosteric modulators offer a novel and potentially powerful method to alter neuronal activity. Here we show that selective activation of HSV-directed α1 GlyR expression in peripheral neurons eliminates pain-related behavior in two pain models, and has the potential to be used therapeutically for pain management.

2512-Pos Board B482

Simultaneous Recording Of Ligand-binding And Channel-gating On Individual Nicotinic-acetylcholine Receptors In Living Cells

Ralf Schmauder, Davor Kosanic, Ruud Hovius, Horst Vogel.
Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland.

Simultaneously measuring both the ligand-binding and the gating of ligand-gated ion channels will provide important novel insight in receptor and channel function (see e.g. Edelstein et. al.). Such measurements require a fluorescently labeled agonist as ligand, single molecule sensitive optical and electrical detection, micro-second time-resolution, and optimal alignment of the optical and electrophysiological single-channel acquisition parts.

Here we present first results from simultaneously measurements of ligand-binding and channel gating of individual prototypical ligand-gated ion channels in living cells, using APD-based fast confocal detection and conventional patch-clamping in single channel mode. In order to reach a reasonable throughput of experiments, it was essential that the sample, patch-pipette and the confocal volume could be moved independently and be aligned with micrometer precision. We show data from nicotinic-acetylcholine receptors, expressed in HEK293 cells and fluorescently labeled epibatidine as agonist, to elucidate details of the channel gating.

S.J. Edelstein, O. Schaad & J.P. Changeux: Biochmistry;1997; 36(45), 13755

2513-Pos Board B483

Functional Characteristics of alpha3beta3gamma2 and alpha1beta2-gamma2 GABA-A receptors

Angelo Keramidas, Neil Harrison.
Weill Medical College of Cornell University, New York, NY, USA.

The $GABA_A$ receptor is a member of the cys-loop family of ligand gated ion channels and the major mediator of neuronal inhibition. Each receptor is a pentameric protein complex composed of homologous subunits, the combination of which gives rise to numerous $GABA_A$ receptor subtypes. Within the thalamus, two synaptic $GABA_A$ receptor isoforms predominate; the $\alpha_1\beta_2\gamma_2$ channels, which are found at the synapses of relay neurons, and the $\alpha_3\beta_3\gamma_2$ channels that are located at synapses in the reticular nucleus. We endeavoured to characterize the kinetic properties to these two $GABA_A$ receptors. Our preliminary data suggest that both receptors open to a single channel conductance level of 26 pS, irrespective of the GABA concentration used to elicit activity. $\alpha_3\beta_3\gamma_2$ $GABA_A$ receptors, however, exhibit longer active periods (bursts) at saturating (5 mM) and low (2 μM) concentrations of GABA compared to $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. The mean burst length and intra-burst open probability (P_o) for the $\alpha_3\beta_3\gamma_2$ channels was 136.7 ± 16.8 ms and 0.84 ± 0.05 (n = 4), respectively and for the $\alpha_1\beta_2\gamma_2$ channels was 87.4 ± 12.0 ms and 0.82 ± 0.02 (n = 3), respectively, when exposed to 5 mM GABA. At 2 μM GABA, the mean burst length and P_o for $\alpha_3\beta_3\gamma_2$ channels was 25.8 ± 4.7 ms and 0.78 ± 0.03 (n = 3), respectively and for $\alpha_1\beta_2\gamma_2$ channels was 8.4 ± 1.9 ms and 0.73 ± 0.02 (n = 3), respectively. These measurements are consistent with the reported slower deactivation phase of ensemble, $\alpha_3\beta_3\gamma_2$ mediated synaptic currents and suggest that GABA has a longer occupancy at $\alpha_3\beta_3\gamma_2$ channels than at $\alpha_1\beta_2\gamma_2$ channels.

2514-Pos Board B484

DPNI-caged Gaba As A Tool For Investigating The Kinetic Properties And Distribution Of Gaba Receptors And For Silencing Neurons In Situ

David Ogden.
Univ Paris Descartes, Paris, France.

The nitroindoline caged amino acids are hydrolytically stable and have photochemical and pharmacological properties suitable for characterising neurotransmitter receptors. Although the NI and MNI - caged glutamates show no interference in the activation of glutamate receptors at 10 mM concentration range, the corresponding GABA and glycine NI derivatives showed evidence of binding and interference in the amplitude and kinetics of activation at their respective receptors at 0.1 mM concentrations. Similarly interference with GABAergic transmission by 50 μM CNB-caged GABA has also been reported. We describe here a nitroindoline caged GABA modified by addition of a biphosphate on the caging group with the idea that the high negative charge will impede binding of the cage to the GABA receptor. Experiments to test the inhibition of miniature GABA mediated synaptic events in cerebellar molecular layer interneurons showed much reduced inhibition, approximately 66% inhibition of amplitude at 1 mM DPNI-GABA. However, with laser pulses of 0.1 ms at 1 mM DPNI-GABA the 10-90% risetimes were comparable with synaptically evoked currents, indicating that equilibration of the cage with the receptor is fast and interferes little with kinetic measurements. The decline of laser evoked currents was prolonged compared with spontaneous events suggesting that the effective volume of GABA release in the laser spot is larger than synaptic release. The precision of locating GABA 'hot spots' in the focal plane was estimated to decline 50% in 1.75 microns, permitting detailed mapping of the distribution of receptors over neuronal compartments. Furthermore, at 100 μM DPNI-GABA, where there is no interference with receptor activation, photolysis localised to the soma of Purkinje neurons or interneurones suppressed spiking with millisecond precision, providing a useful tool for network analysis.

2515-Pos Board B485

A Single Steroid-binding Site is Sufficient for Potentiation of GABA-A Receptors

Joe Henry Steinbach, John R. Bracamontes.
Washington University, Saint Louis MO, MO, USA.

Neuroactive steroids are efficacious potentiators of GABA-A receptors. Recent work identified a site in the α1 subunit of the GABA-A receptor which is essential for potentiation by steroids. However, each receptor contains two copies of the α1 subunit, leading to questions of whether both sites are required and whether the sites mediate distinguishable effects. We generated subunit concatemers so the α1 subunits could be mutated separately, and examined consequences of a mutation which removes potentiation by most neurosteroids (α1 Q241L) and a mutation which mimics the presence of bound steroid (α1 Q241W). Concatemers were expressed in *Xenopus* oocytes, and activation by GABA, potentiation by neuroactive steroids and the agonist activity of the partial agonist P4S were determined. When the α1 Q241L mutation is made in both α1 subunits the EC_{50} for activation by GABA is shifted to higher concentration and the ability of neurosteroids to potentiate responses to a low concentration of GABA is lost. Conversely, when the α1 Q241W mutation is made in both subunits the EC_{50} for GABA is shifted to lower concentration, the relative ability of P4S to activate the receptor is increased and potentiation by neurosteroids is lost. For both, mutation of only one α1 subunit did not produce the full effect of mutating both sites. Hence, the presence of a single wild-type site is sufficient to mediate all effects seen in macroscopic responses. Overall, mutation of the α1 subunit between the γ and β subunits had a larger effect, which might reflect a subtle difference between sites or a consequence of subunit position. The data demonstrate that at a macroscopic level the presence of a single wild-type steroid-binding site is sufficient to mediate the responses to steroid.

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2516-Pos Board B486

MTS-Etomidate Selectively Reacts Within the $GABA_A$ Receptor Etomidate Binding Site

Deirdre S. Stewart, Shaukat S. Husain, Keith W. Miller, Stuart A. Forman.
Mass General Hospital, Boston, MA, USA.

The general anesthetic etomidate exerts its major clinical actions through potentiation of $GABA_A$ receptor activation. $GABA_A$ receptors are pentameric,

usually consisting of combinations of alpha, beta, and gamma subunits. A photoreactive structural analog of etomdiate ([3H]azietomidate) labels amino acids on transmembrane domains in both alpha (M236) and beta (M286) subunits. This suggests the presence of two interfacial anesthetic binding sites per $GABA_A$ receptor, consistent with receptor structural homology models based on *Torpedo* nicotinic acetylcholine receptor. To further characterize the etomidate binding site, we have created a number of Cys substitutions within the α/β intersubunit region and have synthesized a novel etomidate derivative, 2-(methylsulfonyl) thio-etomidate (MTS-etomidate), designed to covalently modify cysteines. Human $GABA_A$ receptors (α1, β2, γ2L) were expressed in *Xenopus* oocytes and current responses were measured using two-electrode voltage clamp. Using pCMBS and MTSEA, we found that cysteine substitutions at both α1M236 and β2M286, were accessible to modification. Cysteine modification was also evident with pCMBS at α1L232C, one helical turn above α1M236 in TM1. In contrast, modification by MTS-etomidate was evident only at α1M236C, but not at β2M286C or, α1L232C. These results suggest that MTS-etomidate orients itself in a precise conformation within its binding pocket and acts as a highly selective structural probe, yielding information not only about the residues with which it interacts but also about the orientation of etomidate within the biding pocket consistent with its mechanism of action.

2517-Pos Board B487

Investigations of Ligand-gated Ion Channels Using Caged Neurotransmitters Reveal Mechanism-based Approaches to Modulating Receptor Function

George P. Hess.
Cornell University, Ithaca, NY, USA.

Recognizing the problem of rapidly equilibrating membrane-bound proteins on cell surfaces with activating ligands due to the water layer that tightly adheres to the surface of spherical objects (1), we began to develop caged (2) neurotransmitters (3,4). A caged neurotransmitter can be equilibrated with receptors on a cell surface before photolysis releases the neurotransmitter in the microsecond time region (3,4). Using caged neurotransmitters in whole-cell current recordings enables the rate constants for the opening (k_{op}) and closing (k_{cl}) of the receptor channel, and thus the channel-opening equilibrium constant (Phe^{-1}), to be measured without interference from diffusional delays or receptor desensitization.

The rate constants and the channel-opening equilibrium constant of the excitatory nicotinic acetylcholine receptor (5) are altered in the presence of noncompetitive inhibitors, such as cocaine, as are those of a mutated inhibitory gamma-aminobutyric acid (GABA) receptor (6) linked to epilepsy (7). Based on the mechanisms of the two receptors, we developed compounds that bind with higher affinity to the open-channel than to the closed-channel conformation of each receptor, alleviating (8,9) the inhibition of the acetylcholine receptor and potentiating (10) the function of a mutated $GABA_A$ receptor linked to epilepsy.

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2518-Pos Board B488

Toward CFTR Structural Dynamics During Ion Channel Gating

Andrei A. Aleksandrov, Luing Cui, John R. Riordan.
University of North Carolina, Chapel Hill, NC, USA.

While CFTR catalyses the irreversible hydrolysis of its physiological ligand (ATP), there is disagreement about whether CFTR channel gating is a thermally driven process (Aleksandrov & Riordan 1998 FEBS Lett. 431:97, Csanady et al., 2006 JGP128:509). Knowing the answer to this question is of fundamental importance to understand problems leading to CFTR functional failure in cystic fibrosis, an inherited disease of high morbidity and mortality. Although CFTR single channel recordings do not provide a direct measure of enzymatic activity, they do provide an opportunity to establish the relationship between ATP binding and hydrolysis at the nucleotide binding domains (NBDs) and channel gating. The use of Rate Equilibrium Free Energy Relationship (REFER) is a possible way to probe structural dynamics in an ion channel gating (Auerbach, 2005 PNAS 102:1408). We use this conceptual framework to reveal the role of ATP hydrolysis in CFTR ion channel function. The REFER graphs for the set of nucleotide ligands have slope $\Phi \approx 1$ for the openings and $\Phi \approx 0$ for the closings. This means that the nucleotide triphosphate interaction with the binding sites is required for channel opening. In contrast, the ion channel transition from the open to the closed state occurs independently of any events in the binding sites. This could not be interpreted by conventional mechanical models of molecular devices where the mobile species are levered from the initial to the final position by force generated in the binding sites. However this behavior fits very well to Brownian ratchet models where internal thermal diffusion is a key element of the process, and specific interactions induce no mechanical movements directly but instead are used to trap favorable fluctuations.

2519-Pos Board B489

Use Of Isolated Vesicles From *Xenopus* Oocytes For Planar Patch Clamp Recordings

Annette Nicke[1], **Andrea Brüggemann**[2].
[1]MPI for Brain Research, Frankfurt/Main, Germany, [2]Nanion Technologies, Munich, Germany.

Oocytes from the *Xenopus laevis* toad represent an efficient expression system. The two-electrode voltage-clamp technique (TEVC) as well as patch clamp recordings from excised patches are powerful tools to study ion channels following expression in *Xenopus* oocytes. The TEVC technique allows the measurement of macroscopic currents from whole cells. It is widely used in basic research and also applied for compound screening in pharmaceutical industry. Recordings from excised patches enable the analysis of ion channels under cell free conditions and even single channel analysis. In contrast to the TEVC technique, its application requires highly skilled electrophysiologists.

A publication by Yong Zhang et al. * describes a technique to prepare isolated membrane vesicles from *Xenopus* oocytes by incubating them, after removal of the vitelline membrane, in a hypertonic ''blebbing'' solution. This treatment induces the formation of plasma membrane blebs that eventually detach from the oocyte surface to form isolated plasma membrane vesicles (PMVs).

Here we used these PMVs to create macro patches onto a planar borosilicate glass surface using the Port-a-Patch (Nanion) automated patch clamp device. The PMVs formed readily gigaseals and showed small capacities as expected for macro patches. Currents could be recorded from voltage-gated as well as ligand-gated ion channels.

In conclusion, we present an application that has the potential to overcome the practical limitations associated with the generation of excised patches and might enable the automated analysis of such patches.

*Mechanically gated channel activity in cytoskeleton-deficient plasma membrane blebs and vesicles from Xenopus oocytes.
Yong Zhang, Feng Gao, Vsevolod L. Popov,* Julie W. Wen,* and Owen P. Hamill J Physiol. 2000 February 15; 523(Pt 1): 117-130.

2520-Pos Board B490

Ultrafast Applications For Determining the Kinetics Of Ligand-gated Channels

Jerónimo Auzmendi, Lucí López, Carla Pallavecini, Estefanía Pieagari, Leandro Radusky, **Luciano Moffatt**.
University of Buenos Aires, Buenos Aires, Argentina.

Determining kinetic models that describe and predict the behavior of ligand-gated channels is a daunting task. It can be done by the analysis of a considerable amount of single channel recordings or, as it has recently shown for P2X2 receptors, by the analysis of macrocurrents after the application of very short pulses of different concentrations of the agonist. Here we present some advances towards a general strategy for determining a kinetic model for any particular ligand-gated channel using ultrashort pulses. First is the problem of generating the agonist pulses. For that purpose we optimize the movement of the liquid interface between agonist solution and saline. This movement is generated by driving a PZT actuator, whose movement is measured by the deflection of a laser that hits a four quadrant movement detector. The movement of the piezo was optimized to produce pulses as large as 20 micrometers and as brief as 20 microseconds of duration. The piezo drives the movement of a theta tube that eject the agonist and the saline, the movement of whom is measured using stroboscopic microscopy. On the other hand, in order to achieve high speed of solution exchange, a high velocity of fluid is necessary at the right times, so a valve-controlled pressurized system was developed. On patch controls have to be made by switching from saline to a low cation solution using the drop in current as an indicator of the time profile of agonist concentration as seen by the patch. Finally, the gathered data is analyzed by contrasting the likelihood of different allosteric models. For that purpose, an extension of the Macroscopic Recursive method is presented that allow its application to time averaged recordings.