OF α7 NICOTINIC RECEPTOR BY PHOSPHORYLATION

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α7 is one of the most abundant nicotinic receptors in the nervous system. It is highly expressed in the brain and contributes to cognition, attention, and working memory. The receptor contains an extracellular domain, which carries the agonist binding sites; a transmembrane domain, which forms the ion pore and the gate; and an intracellular domain (ICD), which contains sites for modulation and intracellular signaling. The concept of α7 as a dual metabotropic/ionotropic receptor is attracting increasing attention. Reciprocal crosstalk between phosphorylation-dependent signaling and receptor function has been proposed. However, the regulation of ion channel function by phosphorylation remains unclear. We here explored how tyrosine phosphorylation at ICD affects single-channel function of human α7 by combining site-directed mutagenesis and mammalian cell expression with patch-clamp recordings. We generated two mutant α7 receptors to prevent phosphorylation of key tyrosine residues (α7Y386F and α7Y442F). Wild-type α7 channel activity elicited by ACh consists of brief and isolated openings and less often as few brief openings in quick succession (bursts). We found that the mutations do not affect single-channel amplitude. However, visual inspection of the recordings showed sporadically long-duration bursts that were not detected in the wild-type receptor. To quantify these differences, we analyzed the burst duration histograms. Whereas wild-type α7 histograms are fitted by 2 components, about 40% of the recordings of α7Y386F and 20% of α7Y442F show a third component. This component corresponds to a novel population of long-duration bursts. Our results show that the decrease of phosphorylation increases open-channel duration, probably by reducing fast desensitization, which is the main pathway for terminating an opening event in α7.These findings reveal a novel regulatory mechanism that may be important in receptor function.

Keywords: ion channel, α7 nicotinic receptor, phosphorylation, patch-clamp, mutagenesis.

(757) **STUDY OF THE CALCIUM BINDING SITE IN THE PLASMA MEMBRANE CALCIUM PUMP BY THE PHOTOREACTIVE PROBE AZIDO-RUTHENIUM**

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Abstract: The Plasma Membrane Calcium ATPase (PMCA) is a P-type ATPase that maintains the homeostasis of Ca^{2+} in eukaryotic cells. It couples the transport of Ca^{2+} with the hydrolysis of ATP. The structure of PMCA is still not solved, and only limited information is available of ligand binding sites. The purpose of this work is to identify and characterize the calcium binding site of PMCA. We synthesized azido-ruthenium (AzRu), a photoactivatable reagent designed to obtain structural information, which binds covalently and specifically to Ca^{2+}-binding proteins after irradiation at 290 nm. The experiments were performed with purified PMCA from human erythrocytes. The results show that AzRu irreversibly inhibits PMCA activity. The time course of production of inorganic phosphate was measured before and after the addition of AzRu, in conditions where photolysis of the inhibitor was prevented. It was observed that the rate of inhibition by AzRu decreases by increasing $[Ca^{2+}]$ in the medium, whereas this rate remains unaffected by increasing $[Mg^{2+}]$. These results suggest that the calcium binding site would be involved in the inhibition process. Assays with $[\gamma\text{-}^{32}P]$ ATP indicate that inhibition of the ATPase activity was accompanied by an increase in the phosphorylated intermediate levels, which suggests that AzRu could be blocking the dephosphorylation of the pump. The photolabeling experiments with AzRu were observed by mass spectrometry using calmodulin (CaM) and lysozyme. CaM-AzRu adducts were observed but not with lysozyme. The results confirm the specific interaction of AzRu with calcium dependent proteins. Photolabeling mass spectra of PMCA with AzRu showed the disappearance of signals from certain peptides from the cytoplasmic domains suggesting an interaction with AzRu. Due to the difficulty of studying membrane proteins by mass spectrometry, we are evaluating an optimization of the study of the transmembrane domain where the PMCA-AzRu adduct would be found.

Keywords: Plasma Membrane Calcium ATPase, Azido-Ruthenium reagent, Ca^{2+} binding site, mass spectrometry

(692) **STUDY OF THE MECHANICAL GATING OF THE AQUAPORIN FAPIP2;1 FROM STRAWBERRY**

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Recently, we published the first works that experimentally demonstrate the direct regulation of both plant and animal aquaporins by membrane tension (s) changes. Our recent work with *Bv*TIP1;2 and *Bv*PIP2;1 from red beet show that the first one is mechanosentive while the second one is not. This different behavior could be related to the differential distribution of GxxxG sequences (suggested to be responsible for mechanosensitivity in ion channels) observed by homology modeling. Previously we demonstrated that hAQP1 is a mechanosensitive channel. Both the water permeability (P_f) and the elastic volumetric coefficient (E) are negatively correlated in experiments with hAQP1 and *Bv*TIP1;2 (R^2>0.98), indicating that these aquaporins close with s increments. Phylogenetics analysis indicate that AQP1 and PIPs share a common ancestor and that divergence of the AQP1-PIP and TIPs groups occurred earlier in evolution. Therefore, three hypotheses arise for mechanosensitivity: 1) it would had been present in the ancestor of AQP1-PIPs and TIPs and was lost in PIPs; 2) it appeared in AQP1-PIPs and TIPs by separately; 3) *Bv*PIP2;1 is a mechanosensitive channel but less sensitive than hAQP1 and *Bv*TIP1;2. Now, we are studying the mechanosensitive properties of *Fa*PIP2;1 from strawberry. The homology model of *Fa*PIP2;1 shows differences with *Bv*PIP2;1 and similarities with *Bv*TIP1;2, suggesting that *Fa*PIP2;1 could behave as a mechanosensitive aquaporin. By means of simultaneous V and P measurements in Xenopus oocytes expressing *Fa*PIP2;1 we determine P_f and E under osmotic gradients. Our previous results with osmotic gradients up to 200 $mOmol.Kg_{H2O}^{-1}$ (E=0.5-0.8 KPa) showed that *Bv*PIP2;1 is not regulated by membrane tension changes. Preliminary results with *Fa*PIP2;1 show that this aquaporin does not behave as a mechanosenstive channel, at least up to changes induced with 200 $mOmol.Kg_{H2O}^{-1}$.

Keywords: water permeability; aquaporin; PIP; membrane tension; volumetric elastic modulus

(1757) **AMYLOID β PEPTIDE DECREASES α7 RECEPTOR POTENTIATION**

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Amyloid β peptide (Aβ) is a key player in the development of Alzheimer disease (AD). Aβ is visible as the primary component of senile plaques in the brains of Alzheimer's patients. Cholinergic activity mediated by human α7 nicotinic receptors is decreased in AD, and potentiation of α7 by positive allosteric modulators (PAMs) is emerging as a novel therapeutic strategy for improving memory and cognition. There are reports showing functional interaction of Aβ with α7, but the reported effects are very varied and the underlying mechanisms are not clear. Here we explored the effect of Aβ1-40 and Aβ1-42 on human α7 at the patch-clamp single-channel level. α7 channel activity elicited by 100 μM ACh consists of brief and iso-

lated openings. In the presence of PAMs, open channel lifetime is increased and openings appear grouped in long activation episodes. The type II PAM PNU-120596 (1 μM) prolongs open durations and elicits activation episodes of ~2 s. In the presence of Aβ there is a statistically significant decrease in the mean duration of the potentiated activation episodes, which is 2.6-fold at 100 nM Aβ1-40 ($p<0.001$, n=11) and 2-fold at 100 nM Aβ1-42 ($p<0.05$, n=10). To determine if the effect is specific for PNU-120596, we also tested NS-1738, which is an α7 type I PAM. Again, a 2-fold reduction in the duration of the activation episodes is observed ($p<0.001$). Complementary fluorescence spectroscopic studies using a fluorescent channel blocker, crystal violet, that binds with different affinities to resting and desensitized receptors provide insights into the functional changes. Our results demonstrate that Aβ inhibits potentiation of human α7, probably through an allosteric mechanism which involves slow block or increased desensitization. Deciphering the functional interaction between α7 and Aβ contributes to the understanding of the involvement of α7 in the pathophysiology of Alzheimer disease.

Keywords: Amyloid β peptide, nicotinic receptor, Patch-clamp, ion channel

(547) **ALL YOU NEED IS COFFEE**

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Cholinergic deficit is regarded as an important factor responsible for Alzheimer's disease symptoms. Two molecular targets for the treatment of this disease are acetylcholinesterase (AChE) and nicotinic receptor (nAChR). Caffeine (CAFF) acts as a non-competitive inhibitor of AChE but its mechanism of action on nAChR is still unknown. To this end, we first explored if CAFF influences the nAChR conformational state using the AChR conformational-sensitive probe crystal violet (CrV) and AChR-rich membranes from *T. californica*. CAFF induced changes in the KD value of CrV in a concentration-dependent manner taking the nAChR to a state close to the desensitized one. In the presence of α-bungarotoxin, a specific nAChR competitive antagonist, high concentrations of CAFF increased the KD value of CrV, compatible with a competition for the CrV site in the channel pore. The same effect was seen with galantamine, an AChE inhibitor and partial agonist of nAChR. To understand the molecular mechanism underlying the conformational changes of the nAChR, we expressed adult muscle or neuronal a7 nAChRs in BOSC cells, and performed single channel recordings with different CAFF concentrations in the presence or absence of ACh. At low concentrations (1-300 μM), CAFF activated muscle and a7 nAChRs, and the activation profile was independent of CAFF concentration. On the other hand, at high CAFF concentrations (up to 20 mM), the mean open duration decreased, the relative area of the briefer closed component and the cluster duration increased, and a flickering behavior was observed, these suggesting that CAFF acts as an open channel blocker. Thus, we here demonstrate a dual effect of CAFF on muscle and a7 nAChRs, behaving as a weak agonist at low concentrations and as a negative modulator at high concentrations. Our results bring new information about the mechanism of modulation of pharmacology targets for the design of new therapies for the intervention in neurological diseases.

Key words: Caffeine, nicotinic receptor, crystal violet, single channel recordings.

STRUCTURAL AND FUNCTIONAL BIOCHEMISTRY 2

(224) **CHARACTERIZATION OF THE CHDL DOMAINS OF RapA, AN EXTRACELLULAR LECTIN FROM *Rhizobium leguminosarum* INVOLVED IN BIOFILM MATRIX ASSEMBLY**

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Abstract: In natural environments microbes live in multicellular structures called biofilms, in which cells are embedded in a matrix of self-produced biopolymers. The extracellular matrix determines the immediate conditions of life of biofilm cells, and also provides adhesion to surfaces and mechanical stability. Despite the importance of the matrix in the biofilm mode of life, very little is known about the mechanisms leading to matrix assembly and the extracellular proteins involved in this process. We have recently characterized the RapA lectin secreted by *Rhizobium leguminosarum*, which has a profound impact in the organization of the biofilm matrix. The RapA lectin interacts specifically with the acidic exopolysaccharides (EPS/CPS) produced by *R. leguminosarum* in a calcium-dependent manner. The protein is composed of two CHDL domains that are similar to the extracellular domains of eukaryotic cadherins. Aiming to obtain a tool to study the development of the matrix during biofilm formation, we dissect the protein in its two halves, and study the properties of the individual CHDL domains. The domains were amplified by PCR using specific primers, cloned as His tag fusions and purified from the soluble fraction of *Escherichia coli* BL21(DE3) induced cells. The purified domains were analyzed by CD spectroscopy with the addition of calcium ions, and in a functional test by means of a binding inhibition assay (BIA) with the EPS. Our results show that the lectin activity is confined to the carboxy terminal CHDL domain of RapA, which contains the calcium binding site and is able to bind to the EPS, although with less affinity than the entire RapA lectin.

Keywords: lectin, calcium, biofilm matrix, exopolysaccharide.

(279) **STRUCTURE OF DIFFERENT PHOSPHORYLATED STATES OF PLASMA MEMBRANE CALCIUM PUMP**

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The control of the cytoplasmic calcium homeostasis relies on many transporters like the Plasma Membrane Calcium Pump (PMCA) and the Sarcoplasmic Reticulum Calcium Pump (SERCA) which belong to the P-ATPase family. At difference with SERCA, structure and function of PMCA has not been fully elucidated yet, because obtaining suitable preparations for X-ray crystal diffraction and NMR techniques was unsuccessful.

To elucidate the structural changes produced in PMCA during the reaction cycle we tested a purified preparation of the pump with fluoride complexes of beryllium, aluminum and magnesium, each one stabilizing different analogues of phosphorylated intermediates in P-ATPases. These blockers were previously assayed in SERCA leading to different conformational states. To follow the binding of fluoride complexes by fluorescence we employed eosin, which binds to the N domain of PMCA. Quantum yield of the bound probe decreased in the presence of fluoride complexes indicating more exposure to solvent. The magnitude of this change depended on the presence of calcium and the complex identity.

Following the kinetics of the conformational change, we propose a model to explain how these complexes stabilize phosphorylated states in PMCA. The detectable conformational change associated with an intermediate of the reaction cycle also allows us to develop a method to measure PMCA activity using a fluorescence approach. Finally, we prepared and refined PMCA structural models based on homology with SERCA and Na^+/K^+ ATPase at different states, to understand the changes at the nucleotide-binding domain during the catalytic cycle of PMCA.

This work was supported by Agencia Nacional de Promoción Científica y Tecnológica PICT 2014 0065, Consejo Nacional de Investigaciones Científicas y Técnicas PIP 11220150100250CO, and Universidad de Buenos Aires Ciencia y Técnica grant 2014-2017: 20020130100254B

Keywords: fluoride complexes, fluorescence, homology modelling

(727) **HOW DEEP IS YOUR BLUE? A NOVEL COPPER CONTAINING NITRITE REDUCTASE FROM A THERMO-**