used to monitor the formation of such architectures and to study of their ion permeability.

Smooth & Skeletal Muscle Electrophysiology

854-Pos Board B733

ATP Regulates Mammalian Neuromuscular Transmission by Dramatically Decreasing the Resting Muscle Chloride Conductance via P2Y1

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In skeletal muscle, extracellular ATP arises locally from released synaptic vesicles and broadly from active muscle fibers. We examined the direct effect of ATP on developed, innervated mammalian skeletal muscle (*ex vivo* rat *levator auris longus*) using measurements of individual muscle fibers with two intracellular microelectrodes. Near the neuromuscular junction (NMJ), 20μM ATP prolonged the decay of miniature endplate potentials (mEPP tau) by 31 ± 7.5% and extended the membrane potential responses induced by step current pulses ($\Delta E_{m(Ipulse)}$) by 59 ± 3.0%. These responses correlated with an increased input resistance (R_{in}) of 31 ± 2.0%. Analogous increases in non-synaptic regions reveal that ATP acts throughout the muscle fiber. In contrast, 50μM adenosine, a well-characterized metabolite of ATP, induced no apparent increase in mEPP tau, $\Delta E_{m(Ipulse)}$ or R_{in}. Applying established pharmacology showed that the ATP receptor mediating these effects is likely the G-protein-coupled P2Y1, since 20μM ATPγS and ADPβS (slowly hydrolysable analogs of ATP and ADP) and 10μM 2-methylthioadenosine-5′-O-diphosphate (2MeSADP) mirrored the effect of ATP. Furthermore, 20μM MRS2179 blocked activation by 20μM ADPβS. The significant effect of ATP on R_{in} was presumably achieved by closing chloride channels, which maintain the largest conductance of resting muscle. This was confirmed with the chloride channel blocker anthracene-9-carboxylic acid (200-500μM), which mimicked ATP and prevented additional increases by 20μM ATPγS. This ATP response appears unique to mammals, as 50μM ATP induced no analogous increase in the archetypical frog NMJ. Our work outlines a novel mechanism by which physiological levels of ATP regulate synaptic transmission and dramatically alter the resting membrane properties of mammalian skeletal muscle. This has potential implications for the physiology of muscle excitability and fatigue, and the pathophysiology of Thomsen and Becker myotonias.

855-Pos Board B734

Cycloxygenase-2 Inhibitor Celecoxib Is A Potent Activator Of Vascular KCNQ K^+ Channels And An Inhibitor Of L-type Ca^{2+} Channels

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Cyclooxygenase-2 (COX-2) inhibitors are important members of the family of non-steroidal anti-inflammatory drugs (NSAIDs). Celebrex® (celecoxib) and Vioxx® (rofecoxib) were introduced in 1999 and rapidly became the most frequently prescribed new drugs in the United States. Vioxx® was voluntarily withdrawn from the market because of a reported increased risk of myocardial infarction and stroke in patients taking the drug for prolonged periods of time. Celecoxib has been reported to inhibit several classes of ion channels, but its effects on vascular smooth muscle ion channels have not been described. Using whole-cell perforated patch clamp techniques we examined effects of celecoxib on K^+ and Ca^{2+} currents in A7r5 rat aortic smooth muscle cells. Application of 10μM celecoxib enhanced K^+ current by 2-3 fold and substantially inhibited Ca^{2+} currents with an apparent positive shift in the voltage-dependence of activation. Both effects were reversible on washout. Nether rofecoxib (10μM), another selective COX-2 inhibitor, nor diclofenac (10μM), a nonselective COX inhibitor, affected Ca^{2+} or K^+ currents in A7r5 cells. We previously reported that KCNQ5 channels are the predominant K^+ channels determining outward potassium current at negative membrane potentials in A7r5 cells. We estimated cumulative dose-response curve of celecoxib on isolated KCNQ5 currents. Celecoxib enhances KCNQ5 current in 3.5 fold with an EC_{50} of 6.9 ± 1.5 μM, without shifting the activation curve. Celecoxib (10μM) was unable to restore KCNQ current inhibited by 100 pM vasopressin (AVP, vasoconstrictor hormone) or 1nM PMA (PKC activator) but inhibition of L-type Ca^{2+} currents (with a positive shift of activation) persisted. The effects of celecoxib, but not rofecoxib, on vascular Ca^{2+} and K^+ channels may explain the differential risks of cardiovascular diseases in patients treated with Celebrex® or Vioxx®.

856-Pos Board B735

Basal Ca^{2+} Entry Controls NFAT Transcriptional Activity, Proliferation And Migration Of Human Vascular Smooth Muscle Cells

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We have previously shown that in the rat the sarco/endoplasmic reticulum calcium ATPase, SERCA2a, controls vascular smooth muscle cell (VSMC) proliferation through modulation of the activity of the transcription factor NFAT. Here we tested the hypothesis that SERCA2a, controls human VSMC proliferation by inhibiting voltage-independent Ca^{2+} entry and the NFAT transcription pathway.

Single-channel recording showed that proliferating VSMC possessed high spontaneous voltage-independent basal channel activity (nPo: 0.415 ± 0.050; n=31). SERCA2a gene transfer, using an adenoviral vector, to proliferating VSMCs abolished the activity of these channels (0.091 ± 0.096; n=26) compared to control cells infected with an adenovirus encoding GFP (0.315 ± 0.035; n=42). SERCA 2a gene transfer also down-regulated expression of transient receptor potential channels TRPC4, TRPC5 and stromal interacting molecule 1 (STIM1) suggesting their involvement in the channel activity.

SERCA2a gene transfer also inhibited VSMC proliferation and migration as well as the NFAT activity. Furthermore, NFAT activity was inhibited by depolarization-induced Ca^{2+} influx and by several calcium channel inhibitors such as nifedipine, mibefradil, carboxyamidotriazole and 2-aminoethoxydiphenyl, suggesting involvement of different types of Ca^{2+} channels in the control of NFAT and proliferation. Our data suggest that different types of Ca^{2+} channels are involved in the control of NFAT transcriptional activity and proliferation. By controlling submembrane Ca^{2+} concentration, SERCA2a finely regulated basal Ca^{2+} current *via* regulation of translocation and/or expression of STIM1 and TRPCs.

Acetylcholine Receptors

857-Pos Board B736

Cellular Basis Of Nicotine-induced nAChr Upregulation

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The upregulation and preferential assembly of high sensitivity $(\alpha4)_2(\beta2)_3$ neuronal nicotinic acetylcholine receptors (nAChRs) contribute to aspects of nicotine addiction such as sensitization and tolerance. The cellular mechanisms of these events, however, remain elusive. We employed fluorescently tagged nAChRs to study plasma membrane upregulation by total internal reflection fluorescence microscopy (TIRFM) as well as changes in intracellular receptor stoichiometry using pixel-based Förster's resonance energy transfer (FRET). To delineate the effect of β2 on α4 nAChR trafficking in the absence of nicotine, mouse neuroblastoma (N2a) cells were transiently transfected with either α4-meGFP/wildtype β2 or α4-meGFP/wildtype β4 subunits (m = monomeric; e = enhanced) and imaged at 48 h post-transfection by TIRFM. To set TIRFM parameters, cells were co-transfected with the pCS2-mcherry plasmid, which served as a reference probe. pCS2-mcherry expresses mcherry with a lyn kinase membrane localization signal, allowing visualization of the PM using red emission from mcherry. The α4-meGFP reporter was used to detect receptor expression at the PM. Results showed that the α4-meGFP/wildtype β2 receptors trafficked to the PM in ~10 % of the cells while ~90 % of imaged cells displayed α4-meGFP/wildtype β4 at the PM. In the presence of nicotine (0.1 μM for 48 h), α4-meGFP/wildtype β2 transfected N2a cells displayed a clear increase in receptor trafficking to the PM when visualized using TIRFM. Pixel-based sensitized emission FRET studies on N2a cells transiently transfected with an α4-mcherry and β2-meGFP FRET pair showed that chronic nicotine exposure (1 μM, 24 h) resulted in an increase in assembly of the high sensitivity $(\alpha4)_2(\beta2)_3$ population of receptors, a phenomenon that was blocked by co-incubation with the competitive nAChR antagonist, DhβE (1 μM). These preliminary results point to a modulatory role of β2 subunits as well as a possibly important role for activity-dependent receptor upregulation.

858-Pos Board B737

α-7 Nicotinic Receptor Positive Allosteric Modulators have Varying Kinetic Effects on Desensitization and Current Amplitude

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The α-7 nicotinic receptor is an important neuronal subtype of ligand gated ion channels. It forms a pentameric homomer that is activated by acetylcholine or nicotine to evoke rapidly activating and desensitizing currents. Activation of α-7 receptors has been implicated as a therapeutic strategy in schizophrenia and Alzheimer disease. Small molecule positive allosteric modulators (PAM's) have been shown to enhance α-7 currents, and are classified as type 1 or type 2 PAMs. The type 1 PAMs enhance the current amplitude but do not alter

desensitization, while the type 2 PAMs enhance the current amplitude but also slow the desensitization of the receptor. In order to understand the mechanism of action of the type 1 and type 2 PAM's, we tested three modulators on the QPatch automated patch-clamp system using GH4C1 cells stably expressing the rat α-7 receptor. We also explored the effect of repeated applications of these agents on their modulatory activity. Consistent with previously published data, PNU-120596 showed type 2 PAM activity accompanied with a decreasing magnitude of potentiation with repeated applications. Estimated EC50 values for PNU-120596 were stable with repeated compound application. NS-1738 produced a type 1 PAM activity, and a cumulatively increased potentiation following repeated applications, accompanied by an approximate 3-fold increase in EC50. In contrast, SB-206553 had similar potency and effects on the potentiation with repeated application. These results indicate that the different α-7 receptor PAMs have different rates of activation and desensitization, in addition to their type 1 or 2 effects on receptor desensitization.

859-Pos Board B738

Channel Blocking Properties Of Tetramethylammonium At The Human Muscle Acetylcholine Receptor

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Agonists at muscle acetylcholine receptors (AChR) all seem to be able to block the channel as well as activate it. In particular, many partial agonists block the channel pore at concentrations close to those that activate it, e.g. choline or tetramethylammonium (TMA). We recorded TMA-activated single- channel currents in the cell-attached configuration from HEK293 cells expressing human adult AChR. The amplitude of openings recorded at -80 mV appears to decrease progressively with agonist concentration because of fast channel block. The equilibrium constant, K_B, for open channel block was about 9 mM as estimated from the reduction of apparent single channel amplitude. This is comparable with the EC_{50} of 2 mM. Several records obtained at different TMA concentrations were fitted simultaneously with the HJCfit program[1]. Because the blockages were undetectable, the open state and the open-blocked state were treated as a single compound open state in the analysis. In the first instance fits were done with a mechanism that allows block of channels only when they are open. The predicted distribution of apparent open times at the lower concentrations TMA matched the observations quite well, but at the higher concentration the prediction was poor (the predominant mean apparent open time was about 1.5 times smaller than predicted). Then fits were done of a mechanism in which the block is not selective for the open state, but can occur from any state. In this case the distributions of apparent open times were predicted accurately at both low and high concentrations of TMA. The present data suggest that TMA does not act as a pure open channel blocker, but AChRs blocked by TMA can close and return to the resting state without re-opening.

1. Colquhoun *et al. J Physiol* 547, 699, 2003.

860-Pos Board B739

Temperature Dependence And Activation Energy of nAChR Gating

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Neuromuscular acetylcholine receptors (AChRs) are ion channels that alternately adopt conformations that either allow or prohibit the flow of ions across the membrane. These two stable end states are separated by an energy barrier, the peak of which is called the transition region. The energy of the transition region relative to the end states is the reaction activation energy (E_{act}). To quantify E_{act}, we studied the temperature dependence of single-channel gating rate constants (k_o, opening; k_c, closing) for wt and mutant AChRs, activated by different ligands or without any added ligand, over a range of temperatures (5-35 ^oC, HEK cells, cell attached, -110 mV, mouse $\alpha_2\beta\delta\epsilon$). The results were fitted by the Arrhenius equation: $k(T)=A*\exp(-E_{act}/RT)$. Increasing the temperature from 5^0 C to 35^0 C increased k_c for wt and δL265T AChRs activated by choline, each by ~35-fold (E_{act}=20.5 and 23.6 kcal/mol, respectively). We also estimated the temperature sensitivity of k_o and k_c in four more constructs with one or more point mutations in both α subunits. For all four, k_o and k_c increased with temperature: E_{act} (k_o and k_c; kcal/mol)=21 and 23 (Y127E activated by ACh); 24 and 27 (D97A + Y127F + S269I, unliganded); 29 and 23 (D97A + Y127F + S269I + W149F, unliganded); 29 and 25 (G153S activated by choline). For these six constructs the average activation energy was ~24.6 kcal/mol for both closing and opening. This quantity did not change with the agonist (including water) or the mutations. This suggests that the energy barrier for the gating isomerization is not significantly determined by the ligands at the transmitter binding sites or by the gating motions of the mutated residues, and that unliganded and diliganded AChR gating likely proceed by similar reaction pathways.

861-Pos Board B740

Interaction Between Two Domains in the AChR Gating Reaction

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The AChR is a large ion channel that isomerizes between non-conducting and conducting conformations. Residue αA96 is located in loop 5 (loop A) near the agonist-binding site, which moves at the outset of the channel-opening process (the Φ value for the adjacent residue αD97 is 0.93). Side chain substitutions at nearby (<0.45 nm, 2QC1.pdb) residue αY127 (β-strand 6) change the gating equilibrium constant (K_{eq}) by up to 290,000-fold (Φ=0.77). This suggests that αY127 moves in concert with the lower part of the extracellular domain, after the motion of loop 5. αD97 and αY127 are not coupled energetically. We tested the hypothesis that αA96 and αY127 energetically link the first two Φ-blocks, to propagate the opening conformational wave from the upper to the lower part of the extracellular domain. We mutated residue αA96 (C, F, K, L, N, Q) and measured single-channel gating kinetics (mouse $\alpha_2\beta\delta\epsilon$, cell-attached, -100 mV, 20 mM choline, PBS, 23°C). The Φ-value for αA96 is 0.90, indicating that it moves at the onset of channel gating along with other residues in loop 5. αA96N showed the largest change in K_{eq} (~900-fold) and markedly increased unliganded gating. Next, we performed mutant cycle analysis to test for energetic coupling between αA96 and αY127. K_{eq} for the double mutant αA96K+αY127E is 18-fold *greater* than the wt, where the effects of the single mutants, if additive, predict one that is 9.2-fold *smaller*. This corresponds to a coupling free energy of -3.1 kcal/mol. Similarly, K_{eq} for the double mutant αA96C+αY127C is 213-fold *greater* than the wt, whereas a value 1.7-fold *smaller* is predicted assuming independence (coupling free energy of -3.6 kcal/mol). These are large interaction energies that suggest αA96 and αY127 form a key energetic link between the first and second Φ-blocks.

862-Pos Board B741

βM2 of The Neuromuscular AChR: Gating, Desensitization and Orientation

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The M2 helix of each of the five acetylcholine receptor (AChR) subunit forms the narrow region of the ion conduction pathway. As part of an overall project of trying to understand the mechanisms that underlie two reactions - the C(losed)↔O(pen) conformational change ('gating') and desensitization - we studied single-channel currents from AChRs with a point mutation in the β subunit (mouse $\alpha_2\beta\delta\epsilon$, HEK cells, cell-attached, -100 mV, 23^{o}C, activated by 30 μM ACh). From measurements of cluster open probabilities (P_o) and durations (τ) we could make *qualitative* inferences about the effects of the mutations on gating (P_o; increase, decrease, no effect) and desensitization ($\tau_{cluster}$; altered, no effect). So far, 58 different mutations of 14 different βM2 residues have been examined. For some of these we also quantified the single-channel current amplitude of the R substitution (i_R; small, no effect). The results are as follows. 1) P_o (by mutation): 26 increased, 13 decreased, 18 no effect. The increases were most apparent in the equatorial 9′-12′ region. 2) $\tau_{cluster}$ (by residue): 6 altered and 8 no effect. The altered bursts were mostly prolonged, with the effects being largest at 9′-12′ and 14′-15′. 3) i_R (by residue): 5 small (8′-10′, 13′, 15′), 3 no effect (6 positions not tested). By examining mutants of all βM2 positions, using a saturating concentration of either choline or ACh, we hope to build maps of the energetic consequences with regard to gating and desensitization, and learn the orientation of residues in the Open conformation of the protein.

863-Pos Board B742

The Unliganded Gating Mechanism Of Nicotinic Acetylcholine Receptors

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The nicotinic acetylcholine receptor (AChR) switches between C (low agonist affinity and low conductance) and O (high agonist affinity and high conductance) conformations ('gating'). The probability of channel opening is very low in the absence of agonist, but when agonists are present at the two transmitter-binding-sites opening increases rapidly (~20μs), transiently to a high probability (~0.95). We observe that 'gain-of-function' mutants that increase the diliganded gating equilibrium constant (without affecting agonist binding to C) also increase the frequency of spontaneous openings. Unliganded openings occur in clusters in AChRs having several of such mutations. We analyzed the intra-cluster interval durations to estimate that the unliganded gating equilibrium constant is $\sim 1.15 \times 10^{-7}$ (mouse, $\alpha_2\beta\delta\epsilon$, -100 mV). The agonist affinity ratios (C vs. O) for acetylcholine, carbamylcholine, tetramethylammonium and choline are ~15,600, ~6700, ~6700 and ~600. The monoliganded (with ACh) gating equilibrium constant is $\sim 1.7 \times 10^{-3}$. Acetylcholine provides only ~0.9 k_BT more binding energy per site than tetramethylammonium, but ~3.1 k_BT more than choline. Mutations of binding site residue αW149 *increase*