2620-Pos

Alternative Splicing of Exons Encoding Portions of the Carboxy-Terminus of K-Cl Cotransporter-4 (KCC4)

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K-Cl cotransporter-2 (KCC2) is expressed only in neurons of the central nervous system, and its transcript contains two exons (22 and 24) encoding portions of the carboxy-terminus that have long been thought to be unique to this KCC isoform. Here we report that KCC2's closest ortholog, KCC4, exhibits alternative splicing of these two exons. From database analysis and RT-PCR, we identified an alternatively spliced exon encoding 41 amino acids (similar to exon 22 of KCC2) in KCC4 of chicken brain. This same exon was also identified in KCC4 of the zebrafish, pufferfish, and protherian mammal, platypus, but remarkably it was absent from KCC4 genes of mouse, rat, and human, indicating evolutionary pressure to remove this exon from therian mammals. Further database analysis and RT-PCR, identified a second alternatively spliced exon encoding 5 amino acids (similar to exon 24 of KCC2) in KCC4 of all vertebrates. Using an antibody that recognizes a peptide encoded by exon 22 of chicken KCC4, we examined the localization of the "long form" of KCC4 (KCC4-S1) in chicken tissues. KCC4-S1 exhibited robust expression in heart with much lower expression in colon and kidney. When expressed in HEK293 cells, KCC4-S1 exhibited activation by cAMP (20μM forskolin), whereas mouse KCC4 or chicken KCC4 which lack exon 22 did not. Sequence analysis of both exons from KCC2 and KCC4 revealed that they encode regulatory elements and/or trafficking motifs. Hence, we hypothesize that the regions encoded by these two alternatively spliced exons regulate the activity or membrane localization of KCC4.

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Phosphatidic Acid Association with the Bovine Mitochondrial ADP/ATP Carrier

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The beef heart adenine nucleotide carrier protein (Anc) of the inner mitochondrial membrane can be purified in a form stabilized by binding the inhibitor carboxyatractyloside. The protein is copurified with bound lipid. We show for the first time that phosphatidic acid is one of the lipids bound to Anc. The short spin lattice relaxation time found by ^{31}P MAS/NMR for this lipid indicates that it is tightly bound to the protein. However, this lipid also has a comparatively small chemical shift anisotropy suggesting that it can undergo reorientation in space. The lipid bound to Anc is in a bilayer arrangement, but the phosphatidic acid shows rapid isotropic motion. Phosphatidic acid is also shown to be present in mitochondria, prior to the isolation of Anc. In Triton-solubilized mitochondria, phosphatidic acid, cardiolipin, phosphatidyethanolamine and phosphatidylcholine exhibit resonance lines in the static ^{31}P NMR spectra, but in the purified Anc only the phosphatidyethanolamine and phosphatidylcholine can be detected by this method, even though the other lipids are still present. This demonstrates that the phosphatidic acid and cardiolipin are interacting with the Anc.

The thermal denaturation of the Anc was determined by differential scanning calorimetry. The protein denatures at 74°C with a calorimetric enthalpy corresponding to a deeply membrane inserted integral membrane protein and a van't Hoff enthalpy indicative of a dimeric state for the protein.

2622-Pos

Functional Characterization of Recombinant Arabidopsis Thaliana Mitochondrial Adenine Nucleotide Translocator 2

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This work describes the recombinant expression, purification, functional reconstitution, transport properties and substrate specificity of the *Arabidopsis thaliana* mitochondrial adenine nucleotide translocator (ANT2). Using RT-PCR, a cDNA clone encoding ANT2 was obtained from *Arabidopsis thaliana* seedlings and expressed as a fusion protein with C-terminal His6 and V5 epitope tags in *Saccharomyces cerevisiae* under the control of the glucose inducible yeast *GAL1* promoter. Localization of the expressed recombinant protein was confirmed in yeast membrane extracts by Western analysis. The time course of the protein expression indicated an optimum induction time of 7 hours. The recombinant protein was solubilized from *S. cerevisiae* extracts using Triton X-100 detergent. Purification was by one-step immobilized metal affinity chromatography. The enriched ANT2 fraction was reconstituted into egg yolk L-α-phosphatedylcholine liposomes containing 3% cholesterol and 20 mM internal ATP. Functionality of the reconstituted protein was confirmed by demonstration of pyridoxal 5'-phosphate-sensitive [^{3}H]-ATP uptake. Transport assays showed that the reconstituted recombinant protein mediates specific ATP-transport using first order kinetics with an apparent *K*m value of 15 μM for ATP-exchange. External application of ATP and ADP led to 62% and 39 % inhibition of [^{3}H]-ATP uptake respectively compared to the pyridoxal 5'-phosphate inhibitor (100% inhibition), indicating a high specificity for ATP/ADP compared to other nucleotides. These characteristics are further compared to [^{3}H]-ATP-transport and specificities measured for plant mitochondria extracts. This work opens new prospects toward further deciphering the different physiological roles and structure/function relationships of plant mitochondrial ATP-translocators.

2623-Pos

Structural and Functional Studies of Bacterial TrkG Potassium Transporters

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The TrkG/TrkH family K^+ transporters are essential for survival of bacteria in low potassium media. Active transport of K^+ is likely achieved by coupled transport of either Na^+ or H^+. Based on sequence homology and mutational studies, it has been proposed that a TrkG or TrkH protein has four homologous repeats that assemble in a K^+ channel-like architecture. Since the putative selectivity filter region of the transporter has very little sequence identity to that of a K^+ channel, it is unclear how K^+ selectivity is achieved. Furthermore, how a channel-like architecture supports the function of active transport of K^+ remains a mystery. In addition, all TrkG/TrkH proteins assemble with a cytosolic domain, TrkA, which binds to ATP or NAD, and the function of TrkA is unknown. To address these questions, we first took a structural genomics approach to identify proteins suitable for structural studies. 90 TrkG/TrkH genes were cloned from 57 prokaryotic genomes and their expression was examined in *E. coli*. 5 of the clones expressed higher than 0.25 mg/liter cell culture and the stability of the purified protein in various detergents was then analyzed by size-exclusion chromatography. 3 of them exhibited a mono-disperse chromatography profile, and were pursued for crystallization. For these three transporters, their corresponding TrkA protein was cloned and co-expressed, and one yielded a stable complex which could be purified to homogeneity. Crystallization of the transporters, alone or in complex with TrkA, is ongoing. In parallel, functional studies confirmed their role as K^+ transporters in complementation studies using a K^+ uptake deficient *E. coli* strain transformed with a plasmid carrying the cloned TrkG/TrkH gene(s). Equilibrium binding studies using ^{35}S-ATP, ^{3}H-NAD, or $^{86}Rb^+$ with the purified complex further showed that it retained its function in detergent-solubilized form.

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Structure-Function Relationships of Inhibitors for the EAAC1 and ASCT2 Amino Acid Transporters

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The SLC1 family of sodium-dependent amino acid transporters includes the high-affinity neuronal glutamate transporter EAAC1 and the closely related neutral-amino-acid transporter ASCT2, which exchanges neutral amino acids, such as alanine, serine, cysteine and glutamine across the cell membrane. ASCT2 may be important for glutamate release from astrocytes, but no high-affinity inhibitors are known that would allow one to test this hypothesis.

ω-aryl substituted aspartamides and 2,3-diaminopropionates have been found to be powerful inhibitors of the EAAT1-3 glutamate transporters.[1] We have synthesized a series of ω-aryl substituted amino acid amides, esters and ethers as inhibitors of these transporters to map out the pharmacophore and achieve selectivity for the ASCTs or EAATs. The properties of these new compounds were studied by electrophysiological recording from HEK293 cells expressing ASCT2 and EAAC1. We found that compounds of this class are competitive inhibitors of amino acid binding. In particular, ω-dithienyl-2,3-diaminopropionate is a potent inhibitor of ASCT2 (apparent $K_i = 33$ μM), with a K_i one order of magnitude lower than the best inhibitor known to-date.

1) Greenfield A., Grosanu C., Dunlop J., McIlvain B., Carrick T., Jow B., Lu Q., Kowal D., Williams J., Butera J., Bioorg. Med. Chem. 2005, 15, 4985-8.