2610-Pos

Development of an *in vivo* Model to Investigate the Role of the Heavy Metal Efflux System Sil from *cupriavidus Metallidurans* Ch34 in Resistance to Silver and Copper Ions

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The RND-type (Resistance Nodulation and cell Division) efflux systems play an important role in the ability of bacteria to survive in the presence of a broad range of toxic compounds. Two major subfamilies are represented by the systems involved in Heavy Metal Efflux (HME-RND) or Hydrophobic and Amphiphilic compounds (e.g. antibiotics) Efflux (HAE-RND). These tripartite protein complexes are composed of an inner membrane (RND) and an outer membrane (member of the Outer Membrane Factor family - OMF) component linked together by a periplasmic adaptator protein (member of the Membrane Fusion Protein family - MFP). The RND protein is a cation/proton antiporter and is responsible for the substrate specificity. SilABC is an uncharacterized HME-RND system from *C. metallidurans* CH34. Using a proteomic approach, we have previously demonstrated the induction of Sil proteins in response to the presence of silver or copper in the culture medium. We report here on the development of an *in vivo* model to investigate the role of the SilABC proteins in the active transport of these heavy metal ions. The metal-sensitive *E. coli* strain GR17 was transformed with the *sil*ABC genes. Silver and copper tolerance of transformed bacteria was evaluated by the determination of minimal inhibitory concentration (MIC) values in a minimal Tris-glucose medium. A twofold increase in MIC values was observed when the three proteins were expressed demonstrating that the SilABC system transports efficiently silver and copper ions *in vivo*. In addition, we have demonstrated that the RND protein SilA alone is able to mediate partial resistance which is compatible with its cation/proton antiporter function.

2611-Pos

Effects of Various Inhibitors on Nitrate Uptake Mediated by *Bacillus* sp. GS2

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Nitrate is one of the major components causing salt stress in Korean farm lands. In order to remove the excess amount of nitrate from the cultivating soils, soil bacteria having nitrate uptake activity were isolated. One of them showed a high capability of nitrate uptake. In the PCR analysis, 1373 bp of 16S rRNA gene were sequenced and compared to those of various microorganisms. The strain has identified as *Bacillus* sp. GS2. Growth of GS2 was not much facilitated by nitrate; however, high capacity of nitrate uptake was measured by removing 40 mM nitrate within 12 h. Nitrate transporter and nitrate reductase are useful enzymes to remove excess soil nitrate. GS2 showed high activity of nitrate reductase and the amount of nitrite formation was directly proportional to the amount of nitrate uptake. In order to characterize the bacterial nitrate uptake, the effect of chlorate was measured on the nitrate uptake activity of GS2 since chlorate was reported to inhibit nitrate transporter. While bacterial growth was not much inhibited by chlorate, the nitrate uptake was inhibited by 80% at the concentration of 50 mM chlorate. The effects of vanadate and phenylglyoxal (PGO) were measured on nitrate uptake. Both vanadate and chlorate showed similar patterns of inhibition on nitrate uptake and 50% inhibitions were obtained at 10-30 mM. PGO, an inhibitor of microbial nitrate transporter, completely inhibited the nitrate uptake at 1 mM. These results suggest that the nitrate reduction by GS2 is mediated by both membrane nitrate transporter and nitrate reductase in cytosol rather than by periplasmic enzyme.

2612-Pos

Physical and Functional Coupling of Electrogenic Na^+/HCO_3^- Cotransport and Carbonic Anhydrases in the Myocardium

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The Na^+/HCO_3^- cotransport (NBC) is an important sarcolemmal acid extruder in cardiac muscle. Functionally electroneutral (NBC3, 1 Na^+:1 HCO_3^-) and electrogenic (NBC1 and NBC4, 1 Na^+:2 HCO_3^-) forms of the transporter have been characterized in the heart. Mammalian cardiac muscle expresses membrane bound carbonic anhydrases (CA) IV (CAIV), IX (CAIX) and XIV (CAXIV), and cytosolic CAII. Association of CAII and CAIV, and NBC1, was previously demonstrated in the kidney and heterologous systems. NBC1 and CA physical and functional interaction was explored in the rat heart by co-immunoprecipitation and intracellular pH measurement (pH_i) experiments, respectively. CAII, CAIV, CAIX, and CAXIV were immunoprecipitated with anti-NBC1 antibody, using rat ventricular lysates. Conversely, non immune serum and irrelevant anti-glial fibrilar acidic protein antibodies failed to co-immunoprecipitated CAs with NBC1. NBC1 activity was investigated in isolated rat cardiomyocytes, using intracellular fluorescent measurements of BCECF-AM, to monitor pH_i. Cardiomyocyte membrane potential depolarazing pulses (MPDP) were applied by addition of 45 mM extracellular K^+, to study NBC1 activity. After 10 minutes of MPDP a significant intracellular alkalinization was detected (0.17 ± 0.03 pH units; n=6, $P<0.05$). The alkalinization was fully cancelled with specific anti-NBC1 functionally inhibitory antibodies (0.02 ± 0.02 pH units; n=5), indicating activation of NBC1 isoform. Similarly, the NBC1-mediated increase of pH_i (0.17 ± 0.02 pH units; n=11, $P<0.05$), was inhibited with a poorly membrane-permeant CA inhibitor, benzolamide (100 μM, 0.09 ± 0.02 pH units; n=6, $P<0.05$), and a potent membrane-permeant CA inhibitor, ethoxyzolamide (100 μM, 0.05 ± 0.01 pH units; n=6, $P<0.05$), demonstrating a functional coupling between NBC1 cotransport and extracellular CAs, and NBC1and intracellular CAs in the cardiac muscle, respectively. We demonstrated that the NBC1 Na^+/HCO_3^- cotransport is functionally and physically coupled to both plasma membrane-anchored CAs and cytosolic CAII, forming a HCO_3^- transport metabolon in the myocardium.

2613-Pos

Role of Na^+/Ca^{2+} Exchanger (NCX1) in Aldosterone-Induced Cardiac Remodeling

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Recent clinical and experimental studies have indicated the utility of eplerenone, a selective mineralocorticoid receptor antagonist, in cardiovascular and renal injuries. Actually, chronic treatment with aldosterone and salt can induce cardiac hypertrophy and renal injuries in experimental animals. However, its underlying mechanism is still unknown. To investigate whether the Na^+/Ca^{2+} exchanger type 1 (NCX1) would be implicated in aldosterone-induced cardiac remodeling, we administered aldosterone (0.3 μg/h) and NaCl (1%) in NCX1 heterozygous mice (N1-KO), heart-specific transgenic mice overexpressing NCX1 (N1-Tg), and wild-type mice (WT). After 4 weeks of treatment, WT and N1-Tg showed a significant increase in heart weight/body weight (HW/BW) ratio with evidence of mild contractile dysfunction, whereas N1-KO maintained average HW/BW ratio and normal cardiac function. More importantly, NCX1 inhibitors (SEA0400, KB-R7943) attenuated aldosterone-induced cardiac hypertrophy and mild dysfunction when administrated to WT for 4 weeks. On the other hand, prolonged treatment with eplerenone (200mg/kg/day) prevented cardiac hypertrophy that was spontaneously induced in N1-Tg. These results suggest that NCX1 participates in aldosterone-induced cardiac remodeling.

2614-Pos

Sodium-Dependent Inactivation of NCX1.3: Aortic Smooth Muscle Cells Versus Transfected CHO Cells

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Despite the importance of Na/Ca exchange (NCX) activity in Ca homeostasis in blood vessels, there have been few detailed studies of the regulation of NCX activity in smooth muscle cells. Here we investigated the regulation of NCX by allosteric Ca activation and Na-dependent inactivation in rat aortic smooth muscle cells (ASMC) and compared the results with those of the smooth muscle isoform NCX1.3 expressed in CHO cells. Fura-2 loaded ASMC or transfected CHO cells were treated with ATP + thapsigargin to release Ca and prevent subsequent Ca re-accumulation in internal stores. Reverse exchange activity was initiated by applying 0.1mM Ca in Li-PSS or K-PSS. In both ASMC and transfected CHO cells treated with 1 mM ouabain, Ca uptake occurred following a 10-20sec lag period attributable to the positive feedback of allosteric Cai activation. To examine the Na-dependence of NCX activity, cells were treated with gramicidin and preincubated in 10-140 mM Na-PSS before activity was initiated by applying 0.1 mM $CaCl_2$. In ASMC, NCX activity peaked at 20 mM Na but declined at higher Na concentrations; essentially no Ca uptake was seen at 140 mM Na. In contrast, robust activity was seen throughout that entire Na concentration range in transfected CHO cells. At 20 mM Na, Ca uptake in ASMC increased to a peak value and then declined sharply. After removing Ca with EGTA in 20 mM Na, subsequent pulses with 0.1 mM Ca revealed no activity until a 10 min recovery period had occurred. In contrast, NCX activity in the transfected CHO cells recovered

within 30 s after EGTA treatment. We conclude that NCX activity in ASMC is much more susceptible to inactivation at high concentrations of Na (Na-dependent inactivation) than in transfected CHO cells.

2615-Pos

Scallop Muscle Na - Ca Exchanger can be Activated by Either AMPK and PK-A Through Phosphorylation of Ser621 in CBD2

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Site directed mutagenesis has shown that Ser^{621} in the CBD2 sub-domain of the Ca^{2+}-regulatory domain of scallop muscle Na^+ - Ca^{2+} exchanger (NCX-SCA) is a substrate for cAMP-PK (PK-A) in the native membrane - bound enzyme [1, 2], and under the same conditions Na^+-driven $^{45}Ca^{2+}$ uptake is stimulated [3]. Examination of the amino acid sequence of NCX-SCA (AY567834, GenBank) shows a consensus sequence for AMP-PK in CBD2 in the Ca^{2+}-regulatory domain, with Ser^{621} as the target residue. The consensus sequence for cAMP-PK in CBD2 (K^{618}RG**S**V) is embedded within that for AMP-PK (L^{616}LKRG**S**VEDL). Exposure of native scallop muscle membranes to constitutively active AMP-PK [4] activated Na^+ - Ca^{2+} exchange by approximately the same factor as with cAMP-PK. These results suggest that both intra- and extracellular signals may stimulate the activity of NCX-SCA through phosphorylation of the Ca^{2+}-regulatory domain in the large cytoplasmic loop.

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2616-Pos

Cardiac-Specific Overexpression of NCX1.1-Xip Mutant Causes Dilated Cardiomyopathy in Mice

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The Na^+/Ca^{2+} exchanger (NCX1.1) plays the primary role in Ca^{2+} extrusion from cardiac myocytes during diastole. There have been many reports showing that NCX1.1 expression levels are elevated in heart failure; however, the importance of NCX1.1 in the pathophysiology of cardiac disease is not well understood. To determine the *in vivo* cardiac function of NCX1.1, we generated transgenic mouse lines with cardiac-specific overexpression of wild-type or mutated NCX1.1, with Na^+-dependent inactivation eliminated. Here we show that NCX1.1 mutant transgenic mice develop dilated cardiomyopathy, whereas wild-type NCX1.1 transgenic mice produce hypertrophic cardiomyopathy. Six-month-old mutant transgenic mice showed contractile dysfunction, ventricular arrhythmias, interstitial fibrosis, and mitochondrial defects. Cardiac myocytes isolated from these mice showed reduced Ca^{2+} transients, suggesting greater Ca^{2+} extrusion via overexpressed mutant exchangers. Microarray analysis of their ventricles clearly indicated molecular changes generally associated with the heart disease phenotype. Thus, these mouse models will be useful for understanding the role of NCX1.1 in cardiac diseases.

2617-Pos

Ibogaine Stimulates Ionic Currents Mediated by Serotonin Transporter

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Ibogaine is a non-competitive inhibitor of serotonin transporter (SERT) that is thought to stabilize a conformation of SERT in which the substrate binding site is open to the cytoplasm. Cocaine is a competitive SERT inhibitor thought to stabilize a conformation with the substrate site open to the extracellular medium. As measured by whole-cell patch clamp, the substrate, serotonin (5-hydroxytryptamine, 5-HT), induced inward currents in HEK-293 cells stably expressing SERT. These currents were previously shown to be uncoupled from 5-HT flux. Cocaine, like other previously tested competitive inhibitors, blocked this current and also blocked a small leak current observed in the absence of 5-HT. Ibogaine, in contrast to competitive inhibitors, stimulated an inward current in the absence of 5-HT. This current was, like the 5-HT induced current, blocked by inhibitory concentrations of cocaine. Concentrations of ibogaine that largely inhibited transport stimulated smaller currents than did 5-HT, and those currents were slower to develop than the 5-HT currents. When ibogaine was added after 5-HT, the current decreased to the level observed with ibogaine alone. This decrease occurred with a time course similar to the time course of the appearance of the ibogaine current when ibogaine was added alone. These results suggest that SERT-mediated currents are associated with conformations of SERT that are different from the conformation stabilized by competitive inhibitors such as cocaine. Both ibogaine and 5-HT apparently alter SERT conformation and increase the proportion of transporter conformations that mediate uncoupled ion permeation.

2618-Pos

Overexpression, Purification and Functional Characterization of the Human Serotonin Transporter

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The human serotonin transporter (SERT) regulates the concentration of serotonin, a neurotransmitter, in the synaptic cleft through the reuptake of serotonin. SERT is a member of SLC6 (Solute Carrier) family with 12 transmembrane helices and two N-linked glycosylation sites. We synthesized the SERT gene from 90 oligo DNAs by PCR-based gene assembly. After transient transfection of the codon-optimized synthetic SERT gene to mammalian HEK293 cells and single-step immunoaffinity purification using N-terminal Flag-tag, full-length expression was confirmed by nano-ESI MS/MS analysis. Then, we constructed a mammalian stable cell line which inducibly expresses the human SERT gene. The glycosylation profile showed that the unglycosylated species expressed at an early stage after tetracycline induction eventually decreased and glycosylated species came to be dominant at a later stage (32hr or later). It turned out that the N-linked glycosaccharides at N208 and N217 were deglycosylated with PNGase F without denaturation, which implied that the N-linked glycosaccharides were well exposed. Blue Native PAGE showed that the purified SERT was dominantly in a tetrameric state. It is consistent with a previously published result of the dopamine transporter, other SLC6 family transporter. We further investigated the functionality of SERT both at a cellular level and in a purified form. HEK293 cells overexpressing the SERT on the plasma membrane uptake the neurotransmitter analogues in time dependent manner. Ligand binding assay with [3H] imipramine showed that the purified SERT was fully functional and the functional expression level was determined as mg level per litter culture volume. Those result shows that mammalian overexpression system along with the use of synthetic gene would be useful in elucidating structure and function relationship of the human transporters.

2619-Pos

Inhibition of SERCA1 by a Novel Antimalarial Compound

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Malaria remains one of the most important diseases in developing countries. *Plasmodium falciparum* (*Pf*), the etiological agent of malaria, is developing an increasing resistance to traditional drugs, like chloroquine (CQ). Consequently, the development of new antimalarial agents able to overcome CQ-resistance is an urgent task to be accomplished.

The antifungal agent clotrimazole (CLT) has been shown to have a moderate growth-inhibiting effect on different *Pf* strains (1), and it has been taken as a model for the rational design of innovative classes of antimalarial agents (2). We have previously shown that CLT is able to inhibit the SERCA1 (SR-CaATPase) (3). Here we present some results concerning the effect on SERCA1 of NF1058 (4), a novel compound with potent antimalarial properties that possesses key structural elements of both CLT and CQ. We used an approach that combines biochemical and electrical techniques. Our data show that NF1058 inhibits the steady-state hydrolytic activity of the pump with a medium affinity ($K_{0.5}$ = 41 ± 1 microM), but it does not interfere with Ca-binding to the enzyme. We therefore suggest that the reduction of steady-state hydrolytic activity of SERCA1 by NF1058 may be due to an interference with the Ca-release process. We are planning to explore the possibility that NF1058 can inhibit *Pf*ATP6, a SERCA-type ATPase expressed by the plasmodium.

Ente Cassa di Risparmio di Firenze is acknowledged for financial support.

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