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The True Ionic Nature of the Na+/Glucose Cotransporter Leak Current

Jean-Philippe Longpré[1], Maxime G. Blanchard[2], Dominique G. Gagnon[3], Jean-Yves Lapointe[4].
[1]Université de Montréal, Montréal, QC, Canada, [2]Universite de Lausanne, Lausanne, Switzerland, [3]University of Chicago, Chicago, IL, USA, [4]Universite de Montreal, Montreal, QC, Canada.

Expression of the Na^+/glucose cotransporter (SGLT1, SLC5A1) in *Xenopus* oocytes is characterized, by a phlorizin(Pz)-sensitive leak current (observed in the absence of glucose) which represents 5 to10% of the maximal Na^+/glucose cotransport current. This current was considered to represent the glucose-independent steps of the cotranport mechanism and was originally called a Na-leak even if its reversal potential (Vr) was 80 mV more negative than E_{Na}. Using a human SGLT1 mutant (C292A) displaying a large leak current (-290 nA at −155 mV), the leak Vr showed only a modest negative shift when external Na concentration ($[Na^+]_e$) was lowered and was insensitive to changes in external [Cl^-]. When external pH (pH_e) was decreased from 7.5 to 6.5 and 5.5, the leak current (at −155 mV) increased to −600 and −1500 nA and its Vr shifted by +15 and +40 mV, respectively. If protons appear to be the main charge carrier at low pH_e's, other ions need to be involved at pH_e 7.5 since rising pH_e to 8.5 produced no further reduction of the leak current. Starting from a $[Na^+]_e$ of 15 mM (pHe=7.5), adding 75 mM of either Na^+, Li^+, Cs^+ or K^+ generates a similar increase in the leak current amplitude. This is in sharp contrast with the cotransport activity which accepts only Na^+ and, to a lesser extent, Li^+ as driving cations. This demonstrates that a cationic pathway clearly distinct from the cotransport mechanism exists through SGLT1. This is reminiscent of the Pz-sensitive passive water permeability of SGLT1. Interestingly, breaking disulfide bridges in SGLT1 or the C292A mutant with dithiothreitol produces a decrease in both the leak current and the Pz-sensitive water permeability. This suggests that the cationic leak may share a common pathway with water across the cotransporter.

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The Transport Mechanism of the Human Sodium Myo-Inositol Cotransporter 2 (hSMIT2)

Louis J. Sasseville, Jean-Yves Lapointe.
Université de Montréal, Montréal, QC, Canada.

The myo-inositol molecule (**MI**) is isomerous to glucose and serves as a compatible osmolyte involved in cell volume regulation and as a precursor of important signalling molecules (inositol phosphates, phosphatidylinositols etc.). hSMIT2 (the product of *SLC5A11*) is a secondary active MI transporter expressed in the apical membranes of small intestine and proximal tubule, and the two-electrode voltage-clamp technique was used to characterize hSMIT2's electrogenic properties after expression in *Xenopus* oocytes. As previously observed for the Na/glucose cotransporter (SGLT1, 49% identical with hSMIT2), hSMIT2 has a 2 Na^+: 1 sugar stoichiometry, is inhibited by phlorizin and, in the absence of substrate, exhibits large phlorizin-sensitive pre-steady-state currents with fast (~2 ms), medium (~10 ms) and slow components (~150 ms). The fact that the charge movement recorded during a negative voltage pulse (Q_{on}) can be up to 4 times larger than the charge movement upon return to the normal holding potential (Q_{off}) indicates that at least a component of the transient current does not represent displacement of a charge associated with the transporter across a fraction of the membrane electrical field. If the slow component of the transient current is omitted from the analysis, Q_{on} equals Q_{off} and a 5 state kinetic model can accurately reproduce the electrogenic properties of hSMIT2. The overall resulting model indicates that SMIT2 experiences a slow conformational change of the empty transporter with a voltage-dependent intermediate state followed by a fast Na binding reaction and a slower MI binding step. The slow component of the transient currents is thought to represent a slow relaxation of the hSMIT2 leak current which is relatively large (i.e. 4 times the amplitude of typical SGLT1 leak currents).

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Roles of Calcium Binding Domains 1 and 2 in Ca^{2+} Regulation of the Na^+-Ca^{2+} exchanger

Michela Ottolia, Debora A. Nicoll, Kenneth D. Philipson.
UCLA, Los Angeles, CA, USA.

The crystal structures of two Ca^{2+} binding domains (CBDs) in the Na^+-Ca^{2+} exchanger (NCX) have been recently resolved. We generated full-length NCXs with mutations in CBD1 and CBD2 to determine their roles in Ca^{2+}-dependent regulation of NCX.

The effects of regulatory Ca^{2+} on the mutant exchangers were measured in giant excised patches. Currents were elicited by applying Na^+ to the cytoplasmic side of the patch in the presence of different regulatory Ca^{2+} concentrations. The pipette contained Ca^{2+} at all times. Under these conditions, NCX current peaks and then decays due to inactivation triggered by high cytoplasmic Na^+. Regulatory Ca^{2+} relieves this inactivation.

Glu 385 coordinates one (Ca3) of the four Ca^{2+} found in CBD1. Peak NCX current of mutant E385A displayed a 4-fold decrease in Ca^{2+} affinity demonstrating the important role of Ca3. NCX-M7 has mutations at 7 of the 10 amino acids that coordinate Ca^{2+} binding to CBD1 which should eliminate Ca^{2+} binding to this region. This mutant showed a further decrease in apparent Ca^{2+} affinity but retained regulation confirming a contribution of CBD2 to Ca^{2+} regulation. Addition of the mutation K585E (located in CBD2) into the NCX-M7 background resulted in an increase in apparent Ca^{2+} affinity. K585E appears to increase Ca^{2+} binding-affinity of CBD2.

Additional mutations within CBD2 were also tested. Previously we have shown that NCX mutants E516L, D578V and E683V lack Ca^{2+} regulation. Introduction of a positive charge either at position 516 or 683 partially rescued NCX Ca^{2+} regulation. The positive charge may mimic Ca^{2+}. These results stress the importance of CBD2 in NCX Ca^{2+} regulation. Additional mutants (including D578R) are under investigation. Our final goal is to gain a better understanding of the interaction between CBD1 and CBD2.

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Is ATP Required For Activities Of The Na^+/Mg^{2+} Exchange?

Michiko Tashiro.
Tokyo Medical University, Tokyo, Japan.

Cytoplasmic Mg^{2+} concentration ($[Mg^{2+}]_i$) was measured with the fluorescent indicator furaptra in rat ventricular myocytes at 25°C. In intact cells loaded with Mg^{2+}, introduction of extracellular Na^+ induced a rapid decrease in $[Mg^{2+}]_i$; the initial rate (initial $\Delta[Mg^{2+}]_i/\Delta t$) was thought to represent the rate of Na^+-dependent Mg^{2+} efflux (putative Na^+/Mg^{2+} exchange). To study whether the Mg^{2+} efflux depends on energy derived from ATP, in addition to transmembrane Na^+ gradient, we estimated the initial $\Delta[Mg^{2+}]_i/\Delta t$ after metabolic inhibition. In the absence of extracellular Na^+ and Ca^{2+}, treatment of the cells with 1 μM FCCP (~10 min) or 5 mM KCN ($\geq$90 min) caused a increase in $[Mg^{2+}]_i$ from ~1 mM to ~2.5 mM (probably due to breakdown of MgATP) and cell shortening by ~50% (probably due to formation of rigor cross-bridges). The initial $\Delta[Mg^{2+}]_i/\Delta t$ was largely reduced, on average by 90% in FCCP-treated cells and 92% in KCN-treated cells. Intracellular Na^+ concentration ($[Na^+]_i$) measured with a Na^+ indicator SBFI was, on average, 5.0-10.5 mM within the time range for initial $\Delta[Mg^{2+}]_i/\Delta t$ measurements, which is lower than that required for 50% inhibition of the Mg^{2+} efflux (~40 mM, Tashiro et al., Biophys J 89:3235-3247, 2005). Normalization of intracellular pH by application of 10 μM nigericin did not reverse the inhibition of the Mg^{2+} efflux. These results suggest that elevation of $[Na^+]_i$ or intracellular acidosis are not primarily responsible for the slowed Mg^{2+} efflux. Instead, in seems likely that a decrease in ATP below the threshold of rigor cross-bridge formation (~0.4 mM estimated indirectly) inhibits the Mg^{2+} efflux, suggesting that absolute requirement of ATP for the Na^+/Mg^{2+} exchange.

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The K+/Ca2+ Exchanger from Human RBC: Effect of oxidative stress on one or both sides of the membrane

Daniel A. Galue Karmowsky, Angeles Zambrano, **Jesus G. Romero**.
Universidad Central Venezuela, Instituto de Biologia Experimental, Lab. Fisiologia, Caracas, Venezuela.

A internal Ca2+ concentration rise has been associated with the senescence process of the Human Red Blood Cell. Since very little is known about it, using the Patch Clamp Technique we are focused in the study of a current associated with the transport of Ca2+. In this regard, we have already presented direct evidences of the existence of a novel transporter capable to account with the Ca2+ entry during ageing process, the K+/Ca2+ Exchanger (1)(2). At the microcirculation level these membrane are exposed to an oxidative stress by mean of the O2/CO2 interplay, to this respect we have already presented some preliminary results (3). Here, we present a completed study of the differential effect of the oxidative stress in one or both sides of the RBC membrane: No matter how the stress is applied the currents are diminished in a concentration dependence fashion by 28% (inside only) against 40% (both side affected), this effect depends in the fluxes directions. Same dependence is presented by the time course developed, presenting a tmax= 40 min for out to inside Ca2+ flux vs. 16 min for in to outside. On the other hand, when the effect is presented without the activation mechanism influence, the overall effect remains, but there is practically no dependence on the sidedness of the oxidative stress, and in fact, there is no dependence on the direction of the fluxes in any condition, suggesting an effect on the activation mechanism. Interestingly, there is no effect on the kinetic of the deactivation process, no matter how the oxidative stress is applied. Structural implication are discussed.

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3537-Pos Board B584

β-adrenergic Regulation of a Novel Isoform of NCX: Sequence & Expression of Shark Heart NCX in Human Kidney Cells

Einsley Janowski[1], Regina Day[2], Alexander Kraev[3], Lars Cleemann[1], Martin Morad[1].
[1]Georgetown University, Washington, DC, DC, USA, [2]Uniformed Services University of the Health Sciences, Bethesda, MD, USA, [3]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada.

Overexpression of the Na^+/Ca^{2+} exchanger (NCX) in mammals is associated with arrhythmias in cardiac hypertrophy and failure. The function and regulation of NCX genes varies significantly among vertebrates likely reflecting differences in molecular structure. We have previously reported that β-adrenergic suppression of the Ca^{2+}-efflux and -influx modes of amphibian cardiac NCX1.1 is associated with specific molecular motifs. In contrast, cardiac NCX of shark *(Squalus acanthias)* shows 'bimodal' adrenergic regulation with preferential suppression of the Ca^{2+}-influx mode (Woo and Morad, PNAS 98:2023, 2001); and its sequence (DQ 068478) reveals two novel proline/alanine-rich AA-insertions. Here we examined the effects of deleting the longer of these inserts.

Shark and mutant shark cardiac NCX were expressed in mammalian cells (HEK 293 and FlpIn 293), and their activity was measured as Ni^{2+}-sensitive Ca^{2+}-fluxes (Fluo-4) and membrane currents (I_{NaCa}) by changing $[Na^+]_o$ and/or membrane potential (V_m). Bimodal regulation, defined as differential regulation of Ca^{2+}-efflux and influx pathways with a strong suppression of its Ca^{2+}-influx mode and no change, or enhancement, of the Ca^{2+}-efflux mode, persisted in the shark NCX regardless of Ca^{2+} buffering, closely resembling the β-adrenergic regulation of native shark cardiomyocytes. In contrast, β-adrenergic stimulation of the shark mutant NCX produced an equal suppression of the inward and outward currents as well as the Ca^{2+} fluxes (as found with frog NCX), thereby abolishing the bimodal nature of the regulation. Control experiments were carried out with untransfected and dog cardiac NCX expressing cells.

We conclude that shark NCX is physiologically functional in mammalian cells, retaining the essentials of its bimodal β-adrenergic regulation. In addition, the deleted shark-specific insert was found to affect the modality of cAMP-dependent regulation, possibly because it provides essential intramolecular flexibility and/or binding sites.

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Calmidazolium Inhibits Na+/ca2+ Antiporter In Mitochondria

Alexey V. Berezhnov[1], Vitaly A. Kasymov[2], Evgenia I. Fedotova[1], Valery P. Zinchenko[1].
[1]ICB RAS, Pushchino, Russian Federation, [2]UCL, London, United Kingdom.

A major function of Ca2+ in mitochondria is the regulation of intramitochondrial enzyme activity. A rise of Ca2+ in mitochondria can stimulate the oxidative metabolism and ATP production through activating Ca2 ± sensitive matrix dehydrogenases and controlling the synthesis of ATP. The main mechanism of the Ca2+ efflux from mitochondria in the cells of heart, brain, skeletal muscles, pancreas, and the majority of tumor cells is the Na+/Ca2+ exchange (NCXmito). Application of the NCX inhibitors (tetraphenylphosphonium cation, CGP37157) increases the concentration of Ca2+ ([Ca2+]m) and NADH level in mitochondria, and stimulates the oxidative metabolism. Little is known about the regulation of NCXmito. In this work it is shown, that calmodulin (CaM) participates in the regulation of the antiporter activity. To study the NCXmito activity, we used the Ca2 ± signal generating purinoreceptors of Ehrlich ascites tumor cells (EATC) and an inhibitor of CaM, R24571. R24571- and ATP-induced changes in the [Ca2+]i, [Ca2+]m, NADH fluorescence, and the membrane potential of mitochondria were recorded. ATP produced a transitory increase in [Ca2+]i, [Ca2+]m and NADH fluorescence. A preliminary addition of Na+/Ca2+ exchange inhibitors slightly changed the cytosolic signal, but suppressed the efflux of Ca2+ from mitochondria. Addition of ionomycin (0.5μM) in the time of the plateau in the mitochondrial Ca2 ± signal caused a decrease in Rhod-2 and NADH fluorescence up to the initial level, which indicated the Ca2 ± efflux from mitochondria. R24571 (3-5μM) caused a transitory Ca2 ± signal in the cytosol and an irreversible increase of NADH and Rhod-2 fluorescence. Ionomycin caused NADH and Rhod-2 fluorescence to recover to the initial level. Thus, R24571 inhibits the NCXmito in EATC, which suggests that its activity is regulated by CaM.

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High-Yield Expression and Purification of a Plasma Membrane Citrate Transport Protein

Jiakang Sun, June A. Mayor, Rusudan Kotaria, Ronald S. Kaplan.
Rosalind Franklin University of Medicine and Science, North Chicago, IL, USA.

Citrate is prominently and uniquely involved in metabolic crossroads that govern the energy balance of human and other organisms, yet little is known about the molecular mechanisms determining its entry into cells. The unique ability of plasma membrane citrate transporters (PMCTs) to import extracellular citrate into the cytoplasm confers upon them an essential role in determining an organism's energy state. Thus, PMCTs located in liver and brain figure prominently in: i) fatty acid, lipid, and cholesterol biosyntheses; ii) control of glucose metabolism via the citrate-mediated allosteric inhibition of PFK-1; and iii) neuronal cell synthesis of neurotransmitters. Therefore, inhibition of human PMCT function may prove to be a novel strategy for altering energy balance in a manner that mimics caloric restriction. With this background in mind, we recently cloned PMCTs from *C. elegans, Drosophila,* mouse, rat, and human into the *Pichia pastoris* expression system and developed a biofermentation protocol for high-yield expression of the human and the *C. elegans* PMCTs. We have been able to successfully purify detergent-solubilized *C. elegans* PMCT via sequential chromatography on Ni-NTA, Talon, and Superdex, resulting in material that is > 90% pure. Mass spectrometry confirms that full-length *C. elegans* PMCT was the purified entity. Preliminary studies indicate that we have been able to successfully reconstitute detergent-solubilized *C. elegans* PMCT function in liposomal vesicles. Sulfhydryl reagents such as NEM and pCMB effectively inhibit the reconstituted transporter. These studies provide the foundation for a detailed structure/function analysis of this metabolically essential plasma membrane transport protein. Supported by NIH grant GM-054642 to R.S.K.

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What are the structural determinants of Cr transport regulation?

Lucia K. Santacruz-Toloza, Marcus D. Darrabie, Rajashree Mishra, Danny O. Jacobs.
Duke University Medical Center, Durham, NC, USA.

Phosphocreatine (PCr) and Creatine Cr play key roles in energy metabolism by replenishing ATP levels via creatine kinases and ADP. The sole source of Cr to skeletal and cardiac myocytes is transport by the Cr transporter (CrT), a membrane protein belonging to the neurotransmitter transporter family (SLC6) that also includes the serotonin, dopamine and norepinephrine transporters. Previous work suggests that Cr transport is modulated by AMPK and PKC. For example, Cr transport in HL-1 cardiomyocytes expressing the human CrT is decreased when the cells are treated with β-PMA, or incubated in culture media enriched for Cr. Cr transport increases if these cells are incubated in culture media depleted of Cr or supplemented with AICAR, an AMPK activator. To identify phosphorylation sites that regulate CrT function, we created single and multiple site mutants lacking putative intracellular phosphorylation sites that scored highly (greater than 0.7) when the transporter protein sequence was evaluated by the NetPhos 2.0 algorithm (CBS, Technical University of Denmark). These sites tended to cluster at the beginning of the N-terminus and the end of the C-terminus of the protein. Ablation of these potential phosphorylation sites had no effect on transport capacity, or the ability to respond to β-PMA, AICAR or substrate availability. We also generated mutants with incremental deletions of the N-or C-termini of the CrT protein. Deletions that remove the entire N or C-terminus resulted in the expression of an inactive transporter protein, while smaller truncations yielded functional Cr transporters that still responded to PKC and AMPK activation and Cr availability like the phosphorylation mutants. Therefore, either low-probability phosphorylation site(s) modulate CrT activity and/or a yet to be identified signalling molecule or interacting protein(s) are responsible for modulation of Cr transport by AMPK, PKC and substrate availability.

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What Localization Of UCP4 Can Tell Us About Its Function?

Elena E. Pohl.
Institute of Cell Biology and Neurobiology, Berlin, Germany.

UCP4 is a member of the mitochondrial uncoupling protein subfamily und is suggested to be mainly involved regulation of reactive oxidative species and/or calcium concentration. Here we investigate for the first time the subcellular, cellular and tissue distribution of UCP4 at physiological conditions using antibodies against UCP4. Affinity purificated polyclonal anti-body against UCP4 was first designed and evaluated using recombinant protein UCP4-GFP. We present evidences that UCP4 is expressed in central nervous system, as previously shown at mRNA level. The described distribution of UCP4 mRNA in kidney was not confirmed an the protein level. Protein are expressed in both fetal and adult murine brain tissue. UCP4 dis-tribution pattern in neuronal cell bodies does not support the idea of thermogenesis in syn-apses. The hypothesis that proapoptotic stress may induce transient UCP4 expression as a component of an apoptotic pathway during neuronal differentiation is discussed.