AuPS/ASB Meeting - Adelaide 2010

Free communications: Calcium signalling

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Chair: Yue-kun Ju

Expression of STIM and Orai in liver disease

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Liver disease is one of the leading causes of death in people with type II diabetes. Disruption in Ca^{2+} homeostasis has been detected in numerous liver diseases. In hepatocytes, Ca^{2+} regulates glucose homeostasis, protein synthesis and lipid metabolism amongst other functions. Store-operated calcium entry (SOCE) plays a key role in maintaining intracellular Ca^{2+} and SOCE plays an important role in growth and differentiation of hepatocytes. Defects in this process may lead to the development of liver disease ranging from non-alchoholic steatosis, cirrhosis and cancer. STIM and Orai1 proteins are key components of SOCE and STIM1 and Orai are required for SOCE in primary hepatocytes (Jones *et al.*, 2008). STIM1 and Orai1 isoforms, STIM2, Orai2 and Orai3 may also be involved in disease altered SOCE. We hypothesize that defects in Ca^{2+} homeostasis associated with liver disease are due to abnormal expression, localization, and interaction of Orai1 with STIM1. In hepatocytes isolated from genetically obese (*fa/fa*) Zucker rats, a model of obesity and insulin resistance that develop steatosis, we previously detected a significant decrease in the amplitude of ISOC activated by 25 μM ATP compared to the ISOC of lean Hooded Wistar rats. To assess whether this observation was due to altered gene expression, the mRNA levels of STIM1, Orai1 and their isoforms STIM2, Orai2 and Orai3 were compared between primary hepatocytes of Zucker obese and lean control rats. In addition, gene expression was determined in cultured H4IIE rat liver cancer cells treated with or without 100 nM insulin/dexamethasone to influence differentiation. Hepatocytes were isolated from Zucker rats anaesthetized with xylazine/ketamine followed by collagenase perfusion of the liver. Gene expression was measured by relative quantitative polymerase chain reaction (qPCR) and expressed relative to B-actin. Results revealed no difference in expression of STIM1, STIM2, Orai1 and Orai3 in hepatocytes from obese and lean Zucker rats. This suggests that the observed difference in SOCE is due to changes in the distribution of STIM and Orai proteins, not altered expression. Orai2 is only expressed at low levels in liver tissue and was difficult to detect in all samples. From measurements obtained it appears that there is a small increase in Orai2 expression in hepatocytes from obese Zucker compared with lean Zucker rats, suggesting that this might contribute to the observed altered SOCE current. Insulin and dexamethasone treatment of H4IIE cells resulted in a significant ($p<0.05$) decrease in the levels of STIM2 and Orai3 accompanied by a decrease in STIM1 level, and increase in Orai1 levels. Orai2 was not detected. These initial findings suggest that STIM and Orai expression is altered in liver cancer and indicates their possible involvement in cellular differentiation. The complete absence of Orai2 indicates its possible loss in cancer tissue. Future assessment of expression levels in diseased human liver tissue samples compared to normal tissue will test whether this finding is physiologically relevant. Moreover, imunofluoresence microscopy will help determine if altered distribution of STIM1 is responsible for changes in ISOC of obese zucker rats hepatocytes. Further work will be needed to determine whether a change in distribution occurs in livers of Type II diabetes patients that might lead to disruption of liver Ca^{2+} homeostasis and development of liver disease.

Jones BF, Boyles RR, Hwang SY, Bird GS, Putney JW. (2008). Calcium influx mechanisms underlying calcium oscillations in rat hepatocytes. *Hepatology* **48(4):** 1273-81.

Molecular mechanisms of paracetamol induced liver damage

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Paracetamol (acetaminophen) is the most commonly used analgesic and antipyretic drug that is available over the counter in many countries, including Australia. At the same time, paracetamol overdose is the most common cause of acute liver failure and the leading cause of liver failure requiring liver transplantation in developed countries (Chun *et al.*, 2009). Paracetamol overdose causes a multitude of interrelated biochemical reactions in liver cells producing multitude of outcomes. Among those are covalent modification and inhibition of enzyme activity, protein oxidation, lipid peroxidation, DNA fragmentation, and deregulation of Ca^{2+} homeostasis, each contributing to paracetamol-induced liver damage (Jaeschke & Bajt, 2006). It has been known for a long time that paracetamol overdose causes a Ca^{2+} raise in hepatocytes; however, the importance of Ca^{2+} in paracetamol-induced liver toxicity is not well understood, primarily due to lack of knowledge about the source of Ca^{2+} rise (Thomas, 1993).

In this work we investigated the molecular pathways of Ca^{2+} entry activated by paracetamol in hepatocytes. Primary rat hepatocytes were isolated by liver perfusion with collagenase under general anaesthesia (intraperitoneal injection of pentobarbitone 50 mg/kg body mass). The experiments were conducted on cells maintained in culture for 24-48h. Cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) was measured using Fura-2 with the aid of a Nikon TE-300 inverted fluorescence microscope. Measurements of ion currents were conducted by standard patch clamping in whole cell mode using a computer-based EPC-9 patch-clamp amplifier and PULSE software.

Fura-2 experiments showed that application of 5 mM acetaminophen to the bath caused slow increase in $[Ca^{2+}]_{cyt}$ reaching levels above 1 μM in 45 min. Application of paracetamol in the absence of Ca^{2+} in the bath solution did not cause any changes in $[Ca^{2+}]_{cyt}$ suggesting that it activates Ca^{2+} entry across plasma membrane through Ca^{2+} permeable channels. In patch clamping experiments incubation of hepatocytes with 5-10 mM paracetamol for 30-60 min resulted in activation of a large linear non-selective cation current, which was inhibited by 20 μM clotrimazole, 100 μM chlorpromazin, 100 μM 2-APB, 5 μM N-(p-amylcinnamoyl)anthranilic acid (ACA), and 100 μM La^{3+}. Similar current was activated by treatment of hepatocytes with 100-500 μM of H_2O_2 for 15-30 min. The selectivity and pharmacological profile of the channels activated by paracetamol and H_2O_2 were consistent with those of the Transient Receptor Potential Melanostatin (TRPM) 2 channel. TRPM2 is a non-selective cation channel expressed in many tissues and is activated by ADP ribose and H_2O_2. We confirmed the expression of TRPM2 in primary rat hepatocytes by western blotting and RT-PCR. To ascertain the molecular identity of the channel mediating non-selective cation current activated by paracetamol, the expression of TRPM2 protein was suppressed by a specific siRNA. SiRNA-mediated knockdown of TRPM2 in rat hepatocytes resulted in almost complete elimination of the current activated by either paracetamol or H_2O_2. These results suggest that TRPM2 may play a significant role in paracetamol toxicity and oxidative damage in the liver.

Chun LJ, Tong MJ, Busuttil RW, Hiatt JR. (2009) *Journal of Clinical Gastroenterology* **43**, 342-349.
Jaeschke H & Bajt ML. (2006) *Toxicological Sciences* **89**, 31-41.
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Mitochondria-induced hyperpolarization in mouse locus coeruleus neurons is dependent on Ca^{2+} entry but not intracellular Ca^{2+} release

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Locus coeruleus (LC) neurons are known to play a fundamental role in brain function, impacting on many physiological processes such as regulation of sleep and vigilance, learning and memory, behavioural flexibility, and a range of other functions (for review see Sara, 2009). Mitochondria are intracellular organelles that appear to be involved in vast range of different pathways, including energy production, neuronal death, oxidative stress, neurodegenerative diseases and their role in buffering intracellular Ca^{2+} and resultant impact on Ca^{2+}-dependent pathways (Ishii, Hirose & Iino, 2006; Lehninger, Nelson & Cox, 2008). In rat LC neurons, it has been demonstrated that mitochondrial disruption caused an increase in intracellular Ca^{2+} and activation of Ca^{2+}-activated K^+ channels and resultant membrane hyperpolarization (Murai *et al.*, 1997). Here, we demonstrate that the hyperpolarization caused by mitochondrial disruption in mouse LC neurons is dependent on external Ca^{2+} entry and not dependent on increases in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$). The methods used for euthanizing mice were approved by the Animal Care and Ethics Committee at the University of Newcastle. The brain was rapidly removed with a slice containing the LC then prepared, allowed to equilibrate and placed on the stage of an upright microscope in a bath perfused with artificial cerebrospinal fluid (ACSF) at 35°C (de Oliveira *et al.*, 2010). Recordings were made from LC neurons using patch electrodes in whole cell recording mode. Mitochondrial disruption with the protonophore CCCP (1 μM) caused hyperpolarization or outward current in current and voltage clamp modes, respectively. This outward current was likely to be dominantly generated by Ca^{2+} activated K^+ channels of the SK type, as the conductance was largely blocked by Apamin (1 μM). The outward conductance was dependent on external Ca^{2+} entry, as determined using Ca^{2+}-free (0.5 mM EGTA) ACSF and Co^{2+} ACSF (Co^{2+} substituted for Ca^{2+}) solutions. This conductance was not inhibited when an internal pipette solution containing a high concentration of the Ca^{2+} buffer EGTA (15 mM) was used, suggesting that $[Ca^{2+}]_c$ was not involved in its activation. Ca^{2+} imaging demonstrated that CCCP increased intracellular Ca^{2+} in both ACSF and the Ca^{2+}-free ACSF. The latter observation combined with the finding that the CCCP-generated outward conductance was not activated in Ca^{2+}-free ACSF confirmed that increases in cytosolic $[Ca^{2+}]_c$ *per se* did not activate the outward conductance. Taken together, these results demonstrate that hyperpolarization induced by mitochondrial disruption using the protonophore CCCP causes Ca^{2+} entry and resultant Ca^{2+}-activated K^+ conductance that is independent of intracellular Ca^{2+} release from stores, but is dependent on external Ca^{2+} entry. This suggests that activation of this conductance occurs in a microdomain.

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Ishii K, Hirose K & Iino M. (2006). Ca^{2+} shuttling between endoplasmic reticulum and mitochondria underlying Ca^{2+} oscillations. *EMBO Reports* **7:** 390-396.

Lehninger AL, Nelson DL & Cox MM. (2008). *Lehninger Principles of Biochemistry*. W.H. Freeman, New York.

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Calcium influx-activating action of P-EPTX-Ar1a: an isolated neurotoxin from the venom of Irian Jayan death adder

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We have isolated a fraction from Irian Jayan death adder (*Acanthophis rugosus*) venom, that we have shown displays pre-synaptic neurotoxic activity in chick neuromuscular junction *via* phospholipase A_2 activity (Chaisakul *et al.*, 2010). We called this fraction P-EPTX-Ar1a. Past studies have reported that many pre-synaptic snake neurotoxins increase cellular calcium by hydrolyzing the plasma membrane and generating lysophosphatidylcholine and fatty acids (Rigoni *et al.*, 2007; Tedesco *et al.*, 2009). In this study, we investigated whether P-EPTX-Ar1a changes cytoplasmic calcium in rodent dorsal root ganglion cells (DRG).

DRGs were isolated from embryonic day (E) 19 Wistar rats or E18 Swiss mice. Cells were isolated by gentle trituration, in the absence of digestive enzymes, and the cells were plated onto 9mm poly-ornithine/laminin coated glass coverslips and rested for 2 h in DMEM/F12 containing 5% fetal calf serum, 0.5 ng/ml nerve growth factor, 1:100 dilution N2 hormone supplement and 2ng/ml glial-cell derived nerve growth factor. The cells were washed and incubated in the calcium fluorophore Fluo-4-AM at 22°C for 10 min. The cells were then continuously superfused with Hanks solution flowing at 1ml/min at 35°C, and the experiment started after a 10 min wash. The snake toxin was added to the superfusate for 4 min. In separate experiments, the patch clamp technique was used in whole cell mode to record the effects of P-EPTX-Ar1a on membrane currents in DRG cells.

P-EPTX-Ar1a, 74nM, caused a prompt increase in cytoplasmic calcium in approximately one third of DRG neurons. The smaller diameter DRG cells were more vulnerable than those of larger diameter. Bursts of cytoplasmic calcium continued to occur throughout the 4 min application of the snake toxin. Following removal of the toxin, calcium bursting abated slowly. DRG cells were then exposed to calcium-free Hanks solution for 1min prior to and during snake toxin exposure. In this situation the increase in cytoplasmic calcium in response to toxin application was delayed by about 3min. Normal (1.3mM) calcium was re-introduced during the washout period and this caused prolonged bursts in cytoplasmic calcium in 98±2% of toxin-sensitive cells. Furthermore, the increase in cytoplasmic calcium in this situation was so large (equal to or exceeding that evoked by exposure to 100 K solution for 10 s) that many cells promptly withdrew projections and discontinued association with the glass coverslip. The increase in cytoplasm calcium induced by snake toxin was unaffected by nifedipine or ω-conotoxin, which block L-type and N-type voltage-gated calcium channels, respectively. However, the response was blunted by agatoxin. Pretreatment with tetrodotoxin, which blocks voltage-gated sodium channels 1.1-1.4, 1.6, 1.7, completely prevented both the immediate increase in cytoplasmic calcium induced by snake toxin in calcium-containing Hanks solution and to the large delayed response following restoration of extracellular calcium, as described above. P-EPTX-Ar1a induced an inward current in DRG cells.

Like many other snake toxins, P-EPTX-Ar1a increases cytoplasmic calcium in neurons. The response is very difficult to reverse and the extent of the increase in cytoplasmic calcium can be sufficient to cause cell death within minutes. The underlying mechanisms may involve alteration of the activity of ion channels.

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Alcohol-induced pancreatic trypsinogen activation depends on calmodulin-sensitive inositol trisphosphate receptors types 2 and 3

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One of the major causes of acute pancreatitis is excessive alcohol intake; however the molecular mechanism of this severe inflammatory disease is not completely understood. Acute pancreatitis is generally initiated by premature trypsinogen activation in pancreatic acinar cells mediated by excessive intracellular calcium release from internal stores. We now show that in two-photon permeabilized mouse pancreatic acinar cells even a relatively low ethanol concentration elicits calcium release from intracellular stores and also induces intracellular trypsin activation. Adding the calcium sensor calmodulin (at a normal intracellular concentration) to the solution surrounding the permeabilized cells markedly reduced ethanol-induced calcium release and trypsin activation. Both ethanol-elicited calcium liberation and trypsin activation were significantly reduced in acinar cells from mice in which type 2 inositol trisphosphate receptors had been knocked out. Double knock out of inositol trisphosphate receptors of both types 2 and 3 further reduced ethanol-induced calcium release and trypsin activation to low levels. Thus the inositol trisphosphate receptor calcium release channels, that are responsible for normal pancreatic stimulus-secretion coupling, also play a major role in the toxic action of ethanol. Calmodulin provides a protective mechanism, regulating the sensitivity of the calcium release process.