AuPS/ASB Meeting - Newcastle 2007

Symposium: Calcium channels, microdomains and muscle function

Monday 3 December 2007 – Hunter Room

Chair: Dirk van Helden, James Brock

Store operated Ca^{2+} channels and microdomains of Ca^{2+} in liver cells

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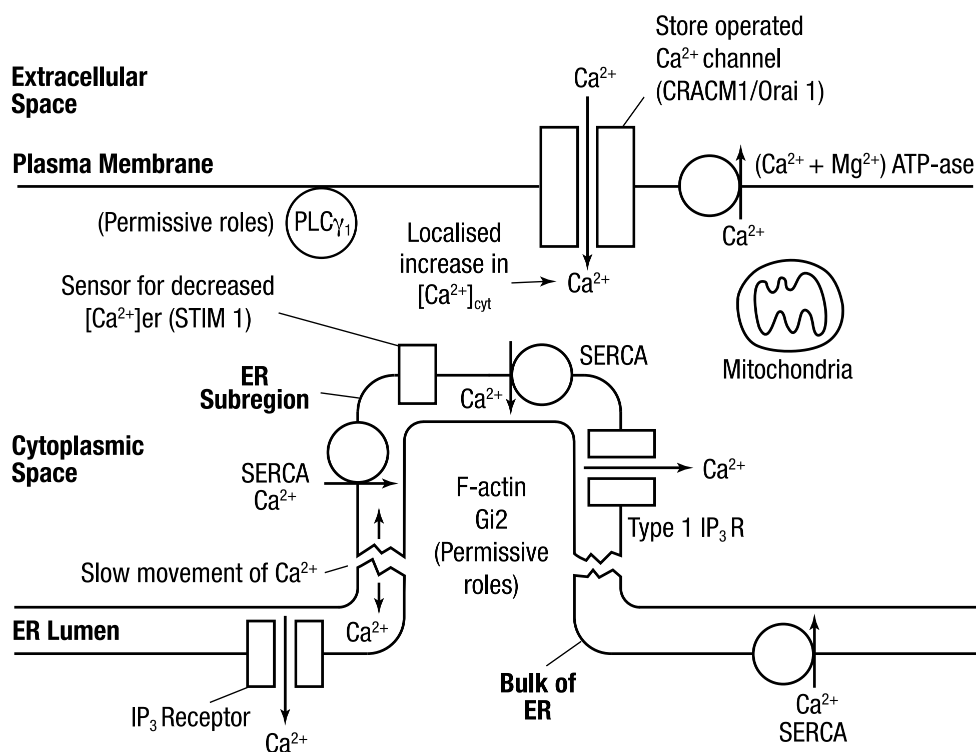
Oscillatory increases in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) play essential roles in the hormonal regulation of animal cells. Increases in $[Ca^{2+}]_{cyt}$ require the release of Ca^{2+} from the endoplasmic reticulum (ER) and Ca^{2+} entry across the plasma membrane. Store-operated Ca^{2+} channels (SOCs), activated by a decrease in Ca^{2+} in the lumen of the ER, are responsible for maintaining adequate ER Ca^{2+}. We are studying the nature and mechanism of activation of SOCs in liver cells. Patch clamp recording and fura-2 experiments indicate there is only one type of SOC in hepatocytes. These SOCs have a high selectivity for Ca^{2+} and properties essentially indistinguishable from those of Ca^{2+} release-activated Ca^{2+} (CRAC) channels. Orai1, a CRAC channel pore protein, and Stim1, a CRAC channel Ca^{2+} sensor, are components of liver cell SOCs. Recent studies in this laboratory have been directed to an investigation of the role of a sub-region of the ER in liver cell SOC activation (shown schematically in the figure). Experiments employing ectopically expressed TRPV1, localised in intracellular membranes, as an alternative method to deplete ER Ca^{2+}, have provided evidence that only a small component of the ER is required for the activation of SOCs. Consistent with this conclusion are the results obtained with choleretic bile acids, which activate SOCs without detectable Ca^{2+} release from the ER. Three aspects of Ca^{2+} microdomains appear to be important in SOC action. (i) The activation process probably requires a decrease in Ca^{2+} near the SOC channels at the cytoplasmic side of the plasma membrane. (ii) There is strong feedback of Ca^{2+} entry through Ca^{2+} localised at the mouth of the channel. (iii) A number of experiments indicate that Ca^{2+} in microdomains near the SOC channel has specific regulatory functions such as regulation of adenylate cyclase. Current experiments are directed to further elucidation of these microdomains of Ca^{2+}.



Store-operated Ca^{2+} entry and TRPC expression; possible roles in control of heart rate

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Store-operated Ca^{2+} channels (SOCCs) were first identified in non-excitable cells by the observation that depletion of Ca^{2+} stores caused increased influx of extracellular Ca^{2+} (Putney, 1986). Recent studies have suggested that SOCCs might be related to the transient receptor potential (TRPC) gene family (Vazquez *et al.,* 2004). In a previous study, we found that activation of the $P2Y_1$ purinergic receptor by ATP results in modulation of pacemaker firing due to receptor-coupled phospholipase C (PLC) activation and depletion of sarcoplasmic reticulum (SR) Ca^{2+} stores (Ju *et al.,* 2003). Since activation of SOCCs also involves PLC, we speculated that SOCCs might also be present in pacemaker tissue (Ju & Allen, 2007).

To study SOCC in pacemaker tissue, we first developed a method for recording intracellular Ca^{2+} signals from mouse sinoatrial nodes (SAN) in which the structural integrity and activity of the node is preserved. Store-operated Ca^{2+} entry was investigated in isolated mouse sinoatrial nodes (SAN) dissected from right atria and loaded with Ca^{2+} indicators. Incubation of the SAN in Ca^{2+}-free solution caused a substantial decrease in resting intracellular Ca^{2+} ($[Ca^{2+}]_i$) and stopped pacemaker activity. Reintroduction of Ca^{2+} in the presence of cyclopiazonic acid, a selective sarcoplasmic reticulum Ca^{2+}-ATPase inhibitor, led to sustained elevation of $[Ca^{2+}]_i$; a characteristic of store-operated Ca^{2+} channel (SOCC) activity (Ju *et al.,* 2007). Transcripts for all TRPC isoforms, except TRPC5 have been detected in SAN preparation. Immunohistochemistry studies also revealed the localizations of TRPC1, 3, 4, and 6 proteins in both the central and peripheral SAN (Ju *et al.,* 2007).

The mechanism of cardiac pacemaking involves voltage-dependent pacemaker current; in addition there is growing evidence that intracellular sarcoplasmic reticulum (SR) Ca^{2+} release plays an important role. The SOCC antagonist, SKF-96365 (10 µmol/L) that inhibited Ca^{2+} influx reduced the spontaneous pacemaker rate and stopped pacemaker firing in the present of CPA (Ju *et al.,* 2007). These newer findings suggest that Ca^{2+} entry and inward current triggered by store depletion might contribute to the pacemaker current and may play a role in control of heart rate.

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What's where and why at a vascular myoendothelial signaling complex?

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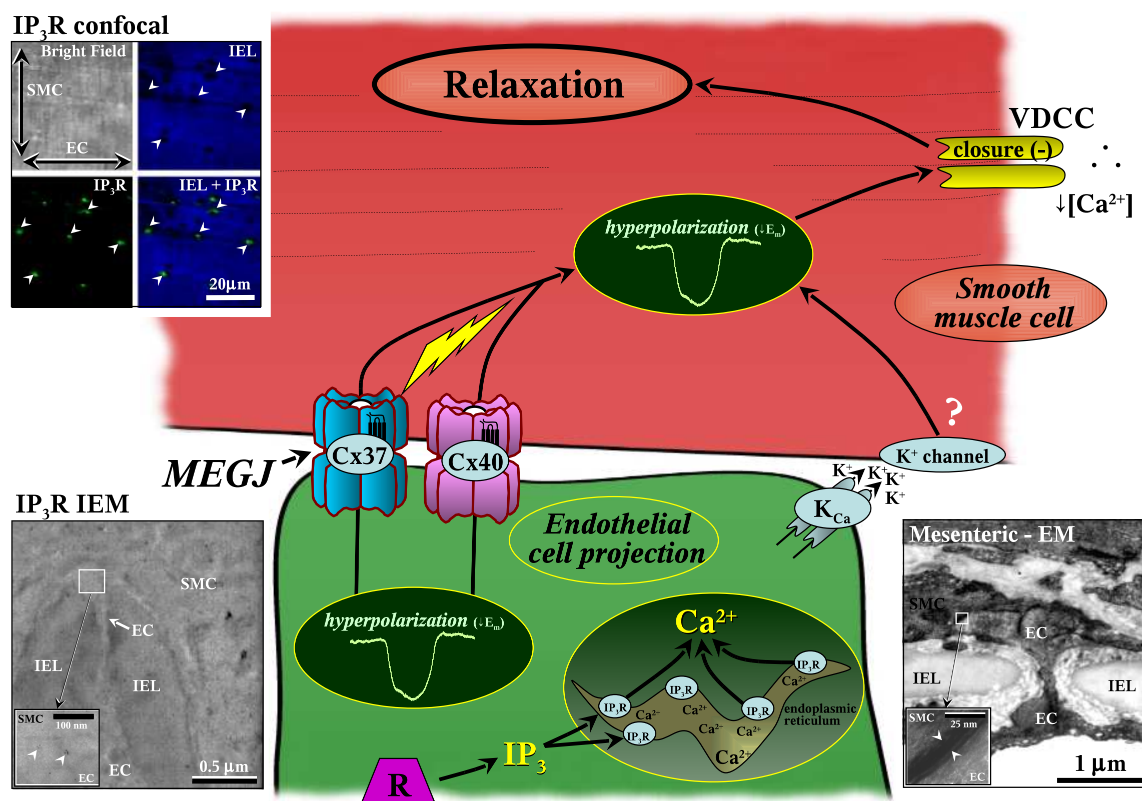
Localized vasodilator function due to endothelium-derived hyperpolarization (EDH), and conduction of vasodilator and vasoconstrictor responses over distance, are associated with heterocellular endothelial-smooth muscle myoendothelial gap junctions. Such junctions are critical for current and small molecule transfer to coordinate arterial function. In addition, the spatial and temporal modulation of calcium dynamics is critical for vascular function, and recent anatomical and functional studies suggest an association between gap junction connexins and sites of calcium release and action. In rat mesenteric arteries, adjacent endothelial cells are coupled by the gap junction connexins (Cxs)37, 40 and 43, which are associated with densities of small conductance calcium-activated potassium channels (SK_{Ca}); whilst myoendothelial gap junction Cxs37 and 40 are associated with densities of intermediate (I) K_{Ca}; corresponding to different facets of the functional EDH response. The aim of this study was to further examine the high resolution spatial association of Cxs and sites of calcium modulation. Segments of rat and mouse mesenteric arteries were high pressure frozen, freeze-substituted and low temperature embedded. Serial consecutive sections were incubated with multiple Abs to Cxs37, 40 and 43 and pan-IP_3R and secondary 5 and 10 nm Au conjugate. Confocal labeling of the same and other fixed flat tissue segments was also carried out using the same Abs and secondary Alexa conjugates. Confocal staining was overlayed with internal elastic lamina (IEL) autofluorescence to show the relationship between Cxs, IP_3R and IEL holes (as potential myoendothelial gap junction sites between endothelial and smooth muscle cells). In the mouse, in contrast to rat mesenteric artery, SK_{Ca} was localized to myoendothelial gap junctions. Intense IP_3R labeling was present at the intimal myoendothelial gap junction site in both rat and mouse mesenteric arteries (Figure). At the confocal level, the apparent K_{Ca} and gap junction relationship differed between species and vascular beds. The differential spatial localization of sites of calcium modulation and vascular Cxs suggests the potential for a causal relationship of their functional activation, in that these sites of current transfer and calcium modulation interact. Such interactions may represent a selective target for the control of vascular tone.



Rat and mouse mesenteric artery myoendothelial Cx, K_{Ca} and IP_3R relationship

Location of voltage dependent calcium channel subtypes controls different aspects of cerebrovascular function

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Vasomotion, or rhythmical vasoconstriction, is a common feature of the cerebral vasculature *in vivo* and *in vitro* (Fuji *et al.,* 1990; Haddock & Hill, 2002). The mechanism underlying vasomotion in cerebral vessels relies on a tonic depolarisation following release of calcium from intracellular stores and the activation of a membrane oscillation involving the alternate opening and closing of L-type voltage dependent calcium channels (VDCCs) and calcium activated potassium channels (Haddock & Hill, 2002). Vascular tone of cerebral arteries has also been reported to depend on calcium influx through L-type VDCCs (Alborch *et al*., 1995). The aim of the present study was therefore to investigate how VDCCs could participate in these two different vascular functions. Basilar arteries taken from juvenile Wistar rats, which had been deeply anaesthetised with ether and exsanguinated, were used in electrophysiological, anatomical and molecular biological studies to determine the expression and functional role of VDCC subtypes in vasomotion and vascular tone. Studies in which the membrane potential of the basilar artery was altered with current injection were conducted on short isopotential vessel segments. Blockade of L-type channels with nifedipine abolished vasomotion but had no effect on vascular tone, although hyperpolarisation of short arterial segments did produce immediate relaxation. This relaxation was still seen in the presence of nifedipine. In contrast, depolarisation of quiescent and relaxed vascular segments evoked constriction and vasomotion in control Krebs' solution, while in the presence of nifedipine, vasoconstriction but not vasomotion was evoked. Real time PCR showed that L- and T-type VDCCs were strongly expressed in the main basilar artery and side branches, with $Ca_V3.1$ and $Ca_V1.2$ the predominant subtypes. Confocal microscopy confirmed that these two channels were strongly expressed as protein and additionally demonstrated their differential distribution in the cell membrane of the vascular smooth muscle cells. The T-type VDCC blockers, mibefradil, pimozide and flunarizine, relaxed and hyperpolarised basilar arteries, while low concentrations of nickel chloride had no effect. When the IP_3 pathway was blocked with the phospholipase-C inhibitor, U73122, to produce relaxation, the addition of Krebs' solution containing 40 mmol/L KCl and nifedipine evoked depolarisation and constriction and this was significantly reduced by mibefradil. The results suggest that vasomotion in the rat basilar artery depends on calcium influx through L-type VDCCs, while vascular tone results from calcium influx through nifedipine-insensitive VDCCs with pharmacology consistent with $Ca_V3.1$, T-type channels. The ability of the calcium traversing these different VDCCs to participate in different vasomotor responses suggests that these channels and their immediate surroundings are physically separated in microdomains within the cell membrane.

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Imaging and modelling calcium microdomains around individual and clustered channels

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The Ca^{2+} microdomains generated around the mouth of open ion channels represent the basic building blocks from which cytosolic Ca^{2+} signals are constructed. Improvements in optical imaging techniques now allow these microdomains to be visualized as single channel calcium fluorescence transients (SCCaFTs). A key requirement is that fluorescence signals reported by a cytosolic Ca^{2+} indicator dye should be sampled from a very small imaging volume so as to maximize kinetic resolution, and modeling studies indicate that a volume of a few tens of attoliters represents an optimal compromise between temporal resolution and signal-to-noise ratio (Shuai & Parker, 2005). In this regard, total internal reflection fluorescence (TIRF) microscopy provides a near-ideal imaging methodology for visualizing Ca^{2+} transients in close proximity to the plasma membrane, because fluorescence excitation is restricted by the extremely thin (ca. 100 nm) evanescent wave formed by total internal reflection between the microscope cover glass and cells bathed in aqueous medium. In conjunction with fast (500 fps), highly sensitive imaging using an electron-multiplied ccd camera, TIRF imaging enables an "optical patch-clamp" technique that can provide information about channel properties previously accessible only by electrophysiological recording, but with further advantages of being massively parallel and mapping channel locations and motility (Demuro & Parker, 2006).

We have used the expression of Ca^{2+}-permeable nicotinic acetylcholine receptor channels (nAChR) in *Xenopus* oocytes as a model system with which to develop optical patch-clamping of individual plasmalemmal channels (Demuro & Parker, 2005). Oocytes (obtained after euthanasia of donor frogs) are loaded with the Ca^{2+} indicator fluo-4 dextran, bathed in Ringers solution with elevated (6 mM) [Ca^{2+}] containing low concentrations of ACh and are voltage clamped at hyperpolarized potentials to increase the driving force for Ca^{2+} influx. TIRF imaging then reveals SCCaFTs arising stochastically at as many as several hundred discrete sites within the imaging field. Fluorescence signals from individual sites are pulsatile and closely resemble electrophysiological single channel records. Consistent with their arising through openings of individual nicotinic channels, SCCaFTs are seen only when a nicotinic agonist is present in the bathing solution, are blocked by curare, increase in frequency as roughly the second power of [ACh], and have mean durations in good agreement with expected channel open lifetimes. The ability to record simultaneously from hundreds of channels reveals a surprisingly large variability in gating properties (e.g. P_{open}, τ_{open}) among nominally identical nAChR expressed from the same genes, and our preliminary data suggest that gating kinetics may be spatially modulated, as correlations between P_{open} are higher among closely neighbouring channels than among distant channels.

More recently, we have extended single channel image techniques to study the local calcium puffs generated by the concerted openings of several clustered IP_3R to liberate Ca^{2+} from ER stores. Questions remain regarding the numbers of IP_3R involved in a puff, and the mechanisms by which their activity is coordinated to initiate and terminate local calcium liberation. To address these issues, we utilize cultured SH-SY5Y neuroblastoma cells, in which a majority of puff sites are located adjacent to the plasma membrane, thereby permitting use of TIRF microscopy to monitor near-membrane (ca. 100 nm) calcium signals with high spatial (ca. 300 nm) and temporal (2 ms) resolution. In addition, intracellular loading with membrane–permeant EGTA-AM is used to buffer cytosolic Ca^{2+}, so that fluo-4 fluorescence signals more closely reflect instantaneous release flux rather than accumulation of Ca^{2+} in the cytosol. Flash photolysis of membrane-permeant caged IP_3 evokes persistent (minutes) activity at several sites per cell. Individual puff sites generally display a mix of 'square' quantal events that likely represent openings of a single IP_3R, together with larger puffs that often show abrupt step-wise transitions on their rising and falling phases. Measurements of event and step amplitudes follow a multi-modal distribution, suggesting a quantal composition of puffs as multiples (8-fold or greater) of single-IP_3R calcium flux.

In summary, we believe that functional imaging of calcium microdomains opens a new approach to study of Ca^{2+}-permeable ion channels, having particular advantages over electrophysiological patch-clamp recording in that it is massively parallel, provides high-resolution spatial as well as kinetic information, and is applicable to plasmalemmal and intracellular channels in intact cells with minimal perturbation.

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