SA15

Calcium Events in Interstitial Cells from the blood vessel wall and the Relationship of Smooth Muscle Cells to Interstitial Cells

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Cells which do not contract in response to high-K solution or to receptor activation and which have long thin processes can be found immediately after dispersion of vascular tissues. We have called these interstitial cells (ICs) and in some vessels these cells are positive for the tyrosine kinase c-kit which is usually used as a marker for Interstitial Cells of Cajal found especially in intestinal smooth muscle (Sanders et al, 199; Hirst & Ward, 2003). These ICs appear to be regulating their [Ca2]i after enzyme dispersion as imaging [Ca2]i using fluo-3 in single ICs revealed spontaneous transient events which were however, generally much longer lasting than calcium events in smooth muscle cells. They were associated with longer lasting outward currents probably caused by the opening of calcium-activated potassium channels. These calcium events also extended to the thin processes of ICs.After enzyme dispersion the processes of surviving ICs from guinea-pig mesenteric artery were observed to grow slowly. This growth was inhibited by latrunculin B which is an inhibitor of actin polymerisation. In enzyme dispersions of rabbit portal vein cells surviving contacts between the processes of single ICCs and the bodies of smooth muscle cells (SMCs) were observed in electron micrographs. These were also detected in living IC-SMC pairs under the optical microscope. Spontaneous rhythmical [Ca2]i oscillations were observed in ICs after fluo-3 loading and were associated with depolarisations of membrane recorded by a tight-seal perforated patch technique. To investigate the possibility of signal transmission from IC to SMC, the IC was stimulated under voltage-clamp using perforated patch technique, while changes in [Ca2]i in the stimulated cell as well as in an adjacent SMC were monitored using fast x-y confocal imaging of fluo-3 fluorescence. Following stimulation of an IC under voltage-clamp by depolarising steps similar in duration to depolarisations associated with spontaneous [Ca2]i oscillations, a transient elevation of [Ca2]i in adjacent SMC occurred with a substantial delay (up to 4 s). The role of these ICs in the walls of blood vessels is not clear but they show spontaneous transient changes in membrane potential and [Ca2]i which can trigger changes in [Ca2]i in smooth muscle cells.

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SA16

IP_3 receptors, mitochondria and Ca^{2+} waves in colonic smooth muscle.

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The cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) controls diverse cellular events via complex Ca^{2+} signalling patterns. Interactions between Ca^{2+} release channels on the SR and between the SR and other organelles such as mitochondria establish the characteristics of the Ca^{2+} signal. Here, in single voltage-clamped colonic smooth muscle cells, IP_3-generating agonists evoked either repetitive Ca^{2+} oscillations or propagated waves. The forward movement of the Ca^{2+} wave arose from Ca^{2+}-induced Ca^{2+} release (CICR) at the IP_3 receptor (IP_3R) without ryanodine receptor involvement. A functional compartmentalization of the SR store produced by an increase in $[Ca^{2+}]_c$, rendered the site of IP_3-mediated Ca^{2+} release, alone, refractory to the phosphoinositide and accounted for the decline in $[Ca^{2+}]_c$ at the back of the wave. Notwithstanding the unique recruitment of IP_3R in wave development, waves are modulated by mitochondrial activity. Collapsing the mitochondrial membrane potential (necessary for Ca^{2+} uptake by mitochondria) inhibited Ca^{2+} oscillations and waves. These inhibitory effects on oscillations and waves could be explained by mitochondrial regulation of IP_3-mediated Ca^{2+} release itself. Thus collapsing the mitochondrial membrane potential inhibited IP_3-mediated Ca^{2+} release. Mitochondria may accumulate Ca^{2+} to maintain a low local $[Ca^{2+}]$ near the IP_3R to prevent a Ca^{2+}-dependent negative feedback on the receptor and so sustaining IP_3-mediated Ca^{2+} release. These results raise the possibility that IP_3-mediated Ca^{2+} release may compromise ATP production as a result of mitochondrial depolarisation. However, neither Ca^{2+} influx (transient or sustained) or release of Ca^{2+} via IP_3R significantly altered the mitochondrial membrane potential. On the other hand, repetitive oscillations and waves transiently depolarised some mitochondria (<5%). These depolarisations were neither synchronised with the $[Ca^{2+}]_c$ changes or with events occurring in adjacent mitochondria. Thus repetitive Ca^{2+} changes appear necessary to evoke significant mitochondrial depolarisation and mitochondria themselves appear to be independent units in smooth muscle.

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SA17

Calcium signalling and imagining in smooth muscle of the urinogenital tract.

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While it is clear that both local and global Ca signals contribute to excitability and contraction in smooth muscles, the details of these mechanisms remain to be elucidated. It is however apparent that smooth muscle in different tissues may use different mechanisms to tailor its activity to each particular tissue. In this presentation we will compare and contrast two phasic smooth muscles of the urinogenital tract; uterus and ureter.It is important for the function of both these tissues that the muscular activity is phasic. In the ureter this allows for boluses of urine to pass from kidney to bladder, as urine is produced. In the uterus it allows for gradual expulsion of the fetus without hypoxic damage. From our studies it would appear that the mechanisms controlling this phasic activity are however very different. Both smooth muscles rely on action potentials being triggered to activate Ca entry and produce contraction. Both also have a substantial SR containing both IP3 and/or ryanodine receptors. In the intact uterus it has been shown that spontaneous activity does not depend upon the SR, and direct measurements of SR Ca in single cells have confirmed this. Imagining of uterine cells and intact strips reveal large global changes in Ca with spontaneous activity. However local SR release events and spontaneous transient outward currents (STOCs) are rarely seen. In contrast in guinea-pig ureter, in isolated cells and cells in situ, Ca sparks are readily seen. Ca sparks are generated by at least one frequent discharging site located close to the edge of the cell membrane several microns away from the nucleus. The temporal characteristics of Ca sparks are similar to those of STOCs. Ca sparks and STOCs are potentiated by low concentration of caffeine and inhibited by ryanodine and CPA. We will show that Ca sparks, by targeting BK channels, play an important role in control of the excitability of the guinea pig ureter, producing a refractory period of 20-40s. During the refractory period suprathreshold depolarising pulses produce regional Ca transient the amplitude of which is minimal at the beginning and maximal before the termination of the refractory period. The refractory period was eliminated by ryanodine, CPA or TEA. The data obtained suggest that in the guinea pig, Ca sparks are playing a key role in controlling the excitability of the ureteric muscle and protecting it from undergoing a tetanic contraction. This is in marked contrast to the uterine muscle, where the refractory period was not readily detected and an increase in the spike frequency resulted in stronger, prolonged contractions, suited to labour.

SA18

Confocal Ca Imaging in Intact Small Arteries Reveals Smooth Muscle Ca^{2+}Transients Attributable to Neurally Released ATP and NA

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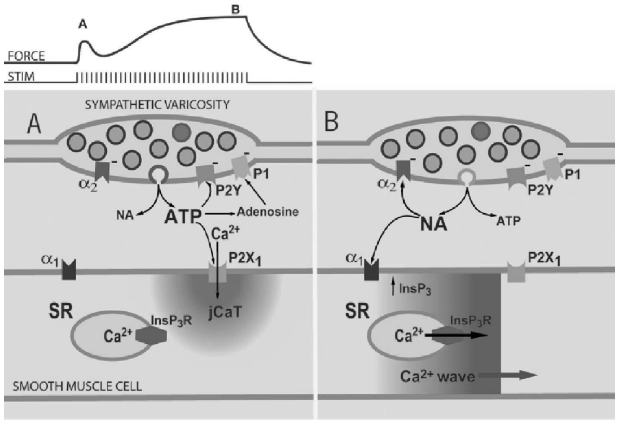
Small mesenteric arteries contribute significantly to the regulation of blood flow and to total peripheral resistance in the body. In these arteries, control of smooth muscle contraction by the sympathetic nervous system is of major importance. This control is complex, involving multiple neurotransmitters and receptors, as well as complex patterns of nerve fibre activity. For example, three different sympathetic neurotransmitters (ATP, NA and neuropeptide Y) are released at sympathetic neuroeffector junctions, and the effects of these different neurotransmitters are thought to be synergistic in activating smooth muscle Ca^{2+} signaling and contraction. Furthermore, the neural release of ATP and NA varies with the pattern of nerve fibre activity. In the face of such complexity, simple bath application of one neurotransmitter (e.g. NA) to isolated smooth muscle cells, as classically done, cannot be expected to reveal the events involved in the physiological control of arterial contraction by the sympathetic nervous system. Therefore, we and others have developed methods recently to visualize Ca^{2+} signaling in smooth muscle cells of intact arteries, during sympathetic nerve stimulation and development of force (isometric). Confocal myographs are an adaptation of classical wire myographs used to study contractions of blood vessels, which permit mounting the artery such that confocal imaging can be performed and perivascular nerves stimulated. Use of long working distance, high numerical aperture water objective lenses is necessary. As known from previous work, neurogenic contractions of small arteries consist of an early, relatively small and transient component that is activated mainly by ATP acting on P2X receptors (marked A on the force record Fig.1), and a later, larger, slowly developing component that is activated mainly by NA acting on alpha-1 adrenoceptors (marked B). Recent confocal Ca^{2+} imaging (fluo-4 fluorescence) with the confocal myograph reveals that early during a train of nerve fibre action potentials, smooth muscle contraction is activated mainly by post-junctional Ca^{2+} transients (jCaTs) (Lamont & Wier, 2002) induced by neurally released ATP. JCaTs are localized to the post-junctional region, and arise from Ca^{2+} that has entered via P2X receptors. At this time, sympathetic varicosities (neuroeffector junctions) may release mainly small vesicles that contain a relatively high concentration of ATP (viz. the relatively few big quanta proposed by Stjarne, 2001) (schematic A). Later during a train of nerve fibre action potentials, we found that jCaTs are rare (Lamont, Vainorius & Wier, 2003), and contraction is activated by Ca^{2+} waves that arise from sarcoplasmic reticulum (SR). These Ca waves appear identical to those activated by bath-applied NA and arise from Ca release from SR that is activated by inositol tris-phosphate. At this time, sympathetic varicosities may release small synaptic vesicles that contain a relatively high concentration of NA (schematic B). Notable differences in Ca^{2+} signaling during neurogenic contractions and those elicited by bath-applied neurotransmitters are: 1) the spike and plateau pattern of intracellular Ca^{2+} seen in isolated smooth muscle cells after application of NA is never seen in the smooth

muscle cells of intact arteries during nerve stimulation, and 2) neurally released ATP does not elicit Ca^{2+} waves in smooth muscle cells of intact arteries, as does bath-applied ATP in isolated smooth muscle cells. We suggest that these differences in Ca^{2+} signaling arise from the very different patterns in spatio-temporal patterns of transmitter concentration and receptor activation that occur with neural stimulation as opposed to bath-application of ATP and NA. The use of intact small arteries and the confocal myograph thus provides a useful new tool for revealing mechanisms involved in control of vascular resistance by the sympathetic nervous system.



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SA19

Spatial and temporal regulation of atrial cardiomyocyte contraction by calcium

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Cardiac excitation-contraction coupling (EC-coupling) is initiated by depolarisation of the sarcolemma as an action potential sweeps over a cell. The change in membrane potential activates voltage-operated Ca2+ channels (VOCs). This leads to a trigger Ca2+ signal that evokes a more substantial Ca2+ release from closely apposed RyR clusters on the SR. For ventricular myocytes, both channels are expressed with regular spacing throughout the cells. Consequently, action potential evoked Ca2+ signals in ventricular myocytes physiologically take the form of homogenenous global increases.The situation in atrial myocytes is different. Atrial cells lack the well-developed T-tubule invaginations of the sarcolemma found in ventricular myocytes, and therefore express VOCs only on the plasma membrane surrounding the cells. The distribution of RyRs in atrial cells is similar to that in ventricular myocytes, but with the important exception that only a small fraction of the RyRs (the 'junctional RyRs') are positioned to respond to the opening of the VOCs. Ca2+ signals in atrial myocytes therefore originate around the periphery of the cells and are locally amplified by the junctional RyRs. In many species, the subsarcolemmal Ca2+ signal does not propagate fully, or at all, into the centre of an atrial cell. This means that at the peak of the response, substantial Ca2+ gradients can be observed. This is surprising given that clusters of RyRs are expressed as a regular 3-dimensional lattice with a spacing of ~2 micrometres. It would be expected that RyRs would convey the subsarcolemmal Ca2+ signal deep into the cell via Ca2+-induced Ca2+ release (CICR). However, the non-junctional RyRs in the centre of atrial myocytes can be largely non-responsive. The mechanisms that inhibit inward propagation of the Ca2+ signal are unknown. Furthermore, the function of the non-junctional RyRs, which form the majority of intracellular Ca2+ release channels, is unclear.We examined how cellular organelles and Ca2+ transport mechanisms, such as SERCA pumps, ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (InsP3Rs), impact on the properties of physiological Ca2+ signals in adult rat atrial myocytes. The cellular ultrastructure of atrial myocytes is highly organised, providing an ideal system to examine the contribution of individual components of the Ca2+ signalling 'toolkit' in shaping the signals that occur during EC-coupling. We demonstrate that to stimulate significant contraction of atrial myocytes, Ca2+ signals need to reach the contractile machinery in the cell centre. Under control conditions, Ca2+ signals are limited to the periphery atrial cells by a functional 'firewall' formed by mitochondria and SERCA pumps. Consequently, the twitch of cells under control conditions is modest. An increase in cellular contraction is achieved by either reducing the potency of the firewall or boosting the triggers for CICR. The latter effect is utilised by physiological inotropic agents that modulate cardiac contractility. The non-junctional RyRs therefore constitute an inotropic reserve that is recruited under conditions where greater contractility is required.

SA20

Control and uncontrol of calcium in the heart

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Most of the calcium that activates cardiac contraction is released from the sarcoplasmic reticulum (SR). This release is triggered by the entry of calcium into the cell via the L-type Ca channel leading to opening of the ryanodine receptor (RyR). This process is known as Ca induced Ca release (CICR). The amount of Ca released depends on the Ca content of the SR; an increase of SR content increases the amount released. Stable contraction of the heart therefore depends on control of SR content (Eisner et al., 2000). We will review evidence showing that control of SR content depends on the effects of Ca release on surface membrane Ca fluxes. Specifically an increase of SR content increases SR Ca release and this (1) increases Ca efflux from the cell on Na-Ca exchange and (2) decreases Ca entry into the cell via the L-type Ca current. Both of these effects will decrease cell and thence SR Ca content (Trafford et al., 1997). Under some clinical conditions the cardiac output shows 'alternans'. At a cellular level this is seen as alternate large and small systolic Ca transients. Using confocal imaging, we find that the alternans can vary between different regions of the cell and is associated with propagation of 'mini waves' of Ca (Diaz et al., 2002). We are investigating the hypothesis that this alternation is due to breakdown of the homeostatic mechanism described above. We find that, under alternating conditions there is an increase in the steepness of the relationship between SR Ca content and Ca efflux from the cell. The factors responsible for this will be described.

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SA21

Ca release termination and refractoriness in cardiac muscle

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EC-coupling and calcium (Ca) signaling in cardiac muscle relies on Ca-induced Ca release (CICR) from the sarcoplasmic reticulum (SR), an amplification process exhibiting a high degree of positive feedback. To avoid instabilities of this feedback system, Ca release is highly localized to elementary Ca signaling events, Ca sparks. The amplitude of the cellular Ca transient can be modulated by recruiting fewer or more Ca sparks, each of which normally remains under local control by a L-type Ca channel located in the sarcolemma. For proper relaxation between cardiac beats and for the system to be stable, the Ca release process itself has to terminate. However, the processes limiting Ca release from the SR and terminating Ca sparks are still poorly understood. We used UV-laser flash photolysis and two-photon excitation photolysis of caged Ca (DM-nitrophen) to activate CICR in isolated cardiac myocytes with a paired pulse protocol, while simultaneously recording Na-Ca exchange currents with he patch-clamp technique or Ca concentration with fluo-3 and a confocal microscope. Using this combination of techniques, we observed a genuine refractoriness of global CICR that recovered slowly (within about 1 second (DelPrincipe et al. 1999)). Based on discrepancies between global refractoriness and fast recovery of local refractoriness we had proposed that the SR Ca content may be an important regulator of RyR Ca sensitivity and that recovery may be related to SR refilling after a release. To test our hypothesis that SR Ca refilling via the SR Ca pump (SERCA) may be rate limiting for recovery from refractoriness, we used pharmacological tools to stimulate or slow down the pump (isoproterenol, thapsigargin or CPA). Indeed, isoproterenol accelerated and CPA slowed recovery from refractoriness. From these results we conclude that refilling of the SR via the SERCA is the rate limiting step for recovery from refractoriness. Most likely, this refractoriness results from functional Ca depletion of the SR during release, leading to deactivation and termination of the Ca release by desensitization of the RyRs for trigger Ca on the cytosolic side of the channel (Sobie et al. 2002; Terentyev et al. 2003). This raises the question, how the SR Ca content or free SR Ca concentration can be detected by the EC-coupling machinery. A Ca receptor is required in the lumen of the SR, and a pathway signaling information about SR Ca content back to the RyR. This pathway might involve recently identified small accessory proteins located in the dyadic region.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

SA22

Simultaneous mapping of voltage and intracellular Ca^{2+}(Ca_i) transients in Heart: Ca_i triggers early afterdepolarizations and Torsade de Pointes in long QT Arrhythmias

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The role of intracellular Ca^{2+} (Ca_i) in triggering early (EADs) or delayers (DADs) afterdepolarizations (EADs), the origins of EADs and the mechanisms underlying Torsade de Pointes (TdP) were investigated in drug-induced models of the long QT syndrome (LQTS). LQTS types 1, 2, or 3 were elicited by perfusing the hearts with Anthopleurin-A (APA= 10-15 μM), HMR 1556 (0.1 or 1.0 μM) or E4031 (0.5 μM) which are know to suppress Na^{2+} channel inactivation (SCN5A), K currents involved in the repolarization of the action potential (AP) I_{Ks} or I_{Kr} respectively. EADs are frequently observed in LQTS and may precipitate TdP. Ca_i overload followed by spontaneous Ca^{2+} release from the sarcoplasmic reticulum has been proposed as a trigger of EADs by increasing Ca^{2+} dependent depolarizing currents. Ca_i overload may be due to enhanced Ca^{2+} influx during the long AP durations (APDs) typical of LQTS. While abnormal Ca_i handling, the firing of EADs and DADs was demonstrated in isolated cardiomyocytes, there is little data in whole hearts or evidence that Ca_i triggers EADs.To address these questions, we mapped APs and Ca_i optically from 256 sites of rabbit hearts that were perfused in a Langendorff apparatus. Hearts were loaded with Rhod-2/AM and RH237 to simultaneously map Ca_i and membrane potential (V_m) with 2 photodiode arrays. In some experiments we used a new potentiometric dye PGH1 because of its greater Stokes' shift, larger fractional fluorescence change per AP and stable recordings for >4 hours. LQTS 1: HMR 1556 caused a marked increase in APDs in male compared to female hearts, indicating a gender difference in the level of I_{Ks} and had a more pronounced at the base than the apex. Hence, there was no shift in the direction of repolarization (apex→ base). The I_{Ks} blocker failed to trigger EADs and TdP in either sex. The greater effects of HMR1556 on the APD of male than female hearts indicates that male hearts express a greater density of I_{Ks} than in female hearts. In long APs with HMR1556, Ca_i upstrokes followed the AP upstroke (10-12 ms) then gradually recovered to baseline value while V_m remained elevated during the AP plateau. LQTS 2: The I_{Kr} blocker, E4031 prolonged APDs in rabbits of both sexes from 230±17 ms to 2,364.4±112.9 ms (n=24 hearts), did not alter activation but reversed the repolarization sequence (apex→ base became base→ apex; n=24). There were marked sex-differences in LQTS2. Before puberty rabbits (>60 days), serum levels of sexual hormones (testosterone and estrogen) are very low. E4031 induced EADs and TdP in pre-pubertal male (n=9/10) but not female hearts (n=18). In adult rabbit hearts, females were highly vulnerable to E4031-induced EADs and TdP (n=10) whereas males were protected (n=10). In EADs induced by E4031, Ca_i rises after the AP upstroke, decays 100 ms later then oscillates during the plateau phase. In most experiments (n=5/6), Cai followed V_m resulting in phase maps with counterclockwise trajectories indicating that Ca_i follows V_m during salvos of EADs. To test if EADs fire preferentially from Purkinje and endocardial cell, the septum, right and left endocardium was cryoablated resulting in a thin layer (~ 1 mm) of surviving myocardium. In cryoablated hearts,

EADs originated at 2-3 sites on the anterior surface and at the origins of EADs, Ca_i preceded V_m. EADs fired out-of-phase from several sites, propagated and collided, consistent with the undulating EKGs of TdP. Phase maps of EADs had clockwise trajectories; that is Ca_i preceded V_m.LQTS 3: APA (10 μM) prolonged APDs (260 ± 30 ms to 870 ± 67 ms) in both sexes (n = 4/ each sex), elicited EADs and TdP. At EADs, Ca_i rose and peaked during the plateau before EADs fired. Phase maps had clockwise trajectories indicating that Ca^{2+} preceded V_m.Put together, the data show that in the LQTS, Ca_i elevation most likely due to spontaneous Ca^{2+} release from internal stores elicits salvos of EADs, a greater dispersion of repolarization and sustained TdP.