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**REVIEW**

# Novel $Ca^{2+}$ signalling mechanisms in vascular myocytes: symposium overview*

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**Abstract**

This commentary presents the proceedings of the symposium sponsored by Cardiovascular Section of American Physiological Society in San Diego, CA on 12 April 2003. The major focus of this symposium was on the actions and physiological relevance of several novel $Ca^{2+}$ signalling mechanisms in vascular smooth muscle (VSM) cells. Five important topics were presented in this symposium including the discovery and roles of cyclic ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) in mediating $Ca^{2+}$ release, $Ca^{2+}$ sparks and activation of plasma membrane $K_{Ca}$ channels in VSM cells, the role of cADPR-mediated activation of ryanodine receptors in the control of vascular tone, the role of $[Ca^{2+}]_i$ in mechanotransduction in the arterioles, and interactions of mitochondrial $Ca^{2+}$ release and SR $Ca^{2+}$ mobilization. The purpose of this symposium was to promote discussions and exchange of ideas between scientists with interests in $Ca^{2+}$ signalling mechanisms and those with interests in vascular physiology and pharmacology. The cross-fertilization of ideas is expected to greatly advance our understanding of the physiological and pharmacological relevance of these new $Ca^{2+}$ signalling mechanisms.

*Keywords* artery, integrin, mitochondria, ryanodine receptor, sarcoplasmic reticulum, signal transduction, smooth muscle.

It is well known that changes in cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) play a critical role in activation of vascular smooth muscle (VSM) (Nelson *et al.* 1990, Berridge 1994, Aarhus *et al.* 1995, Himpens *et al.* 1995). Previous studies have shown that these $Ca^{2+}$ signals are initiated by influx of extracellular $Ca^{2+}$ through $Ca^{2+}$ channels in the plasma membrane (PM) and release of $Ca^{2+}$ from the sarcoplasmic reticulum (SR) through $IP_3$ receptors ($IP_3R$) and ryanodine receptors (RyR) (Nelson *et al.* 1990, Aarhus *et al.* 1995). A great deal of heterogeneity exists with respect to the participation of the various $Ca^{2+}$ influx and release channels dependent on which specific blood vessel and agonist are involved. Thus, noradrenaline, angiotensin II (Ang II), vasopressin (ADH), endothelin (ET), thromboxane $A_2$ appear to induce $Ca^{2+}$ release mainly by the opening of $IP_3R$ (Nelson *et al.* 1990, Fasolato *et al.* 1994, Petersen 1996), while acetylcholine (Ach), prostaglandin $F_{2a}$ and $Ca^{2+}$ activate predominantly RyR. Caffeine opens RyR and inhibits $IP_3R$ (Galione & Sethi 1996). The novel $Ca^{2+}$ mobilizing second messenger cyclic ADP-ribose (cADPR) appears to play an important role in the control of vascular tone through the activation of RyR (Kannan *et al.* 1997, Yu *et al.* 2000, Li *et al.* 2001). Most interestingly, the spatio-temporal characteristics of the $Ca^{2+}$ signals determine their

 

functional outcomes. The sparks induced by spontaneous opening of clusters of RyR lead to relaxation, while $Ca^{2+}$ waves are mediated by regenerative activation or $IP_3R$ signal vasoconstriction. One of the unsolved mysteries is how resistance arteries sense pressure and respond to it with myogenic tone. Recent studies have demonstrated the critical role of integrin receptors in mediating mechanotransduction in the arterioles (Nelson *et al.* 1995, Zou *et al.* 2001, Lee *et al.* 2002a). All these mechanisms, which are emerging as fundamental to the regulation of vasomotion were the focus of this symposium.

## Discovery and $Ca^{2+}$ release action of cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (H. C. Lee, University of Minnesota)

### *Discovery of cADPR and NAADP*

Cyclic ADP-ribose and nicotinic acid adenine dinucleotide phosphate (NAADP) were first identified in sea urchin eggs, which has long been a favourite model for investigating $Ca^{2+}$ signalling mechanisms in cells (Clapper *et al.* 1987, Lee *et al.* 1989, Lee & Aarhus 1995). A rather dramatic $Ca^{2+}$ wave occurs at fertilization. Immediately after sperm–egg fusion, a small and localized flush of $Ca^{2+}$ occurs in the cortical region of the egg, which is followed shortly by a spark of $Ca^{2+}$ starting at the site of sperm–egg fusion and propagating as a $Ca^{2+}$ wave across the entire egg. The consequence of this $Ca^{2+}$ wave is the activation of cortical exocytosis, resulting in the formation of the fertilization membrane that begins at the fusion site and eventually surrounds the whole egg. The source of $Ca^{2+}$ for the wave is mainly from internal stores, suggesting that sperm–egg fusion at the egg surface activates the production of a $Ca^{2+}$ messenger, which in turn triggers $Ca^{2+}$ release from internal stores (Lee 1996).

To identify this $Ca^{2+}$ messenger, the strategy used was to isolate the internal $Ca^{2+}$ stores and use them as a cell-free assay for $Ca^{2+}$ release. The isolated egg microsomes contain an endogenous $Ca^{2+}$ pump, which, in the presence of ATP, can catalyse their own accumulation of $Ca^{2+}$. Release of $Ca^{2+}$ from the microsomes can easily be measured using a fluorescent $Ca^{2+}$ indicator. Moreover, the isolated microsomes are extremely stable and can be stored frozen for several years without loss of $Ca^{2+}$ release activity (Clapper *et al.* 1987).

At the time, it was fully expected that the $Ca^{2+}$ messenger generated during fertilization was $IP_3$. This was because $IP_3$ was the only substance that was known to be capable of releasing $Ca^{2+}$ from internal stores. Indeed, addition of $IP_3$ to the isolated egg microsomes elicited $Ca^{2+}$ release. In addition to $IP_3$, NAD was also found to be able to release as much $Ca^{2+}$. The kinetics of the release were, however, quite different. With $IP_3$, the release was immediate, while a prominent delay was seen with NAD. This initial delay suggested that NAD may be enzymatically converted to an active form, accounting for the delay. This turned out to be the case and provided a way to produce enough active metabolite for identification (Clapper *et al.* 1987, Lee *et al.* 1989).

The active metabolite from NAD was eventually purified and crystallized. X-ray diffraction studies showed that the active metabolite is a cyclic compound formed by linking the adenine ring of NAD to the terminal ribose (Lee *et al.* 1994). The nicotinamide group, which normally attached to the terminal ribose is released during the enzymatic synthesis. The site of cyclization is at N1 of the adenine ring. This cyclization bond can be broken either chemically by heat or hydrolysed enzymatically. The hydrolysis product is ADPR. Because the active metabolite is cyclic and upon hydrolysis produces ADPR, it was named cyclic ADP-ribose (Lee *et al.* 1989).

Using egg microsomes as an assay, it was found that, additionally, a derivative of NADP, with a nicotinic acid group replacing the nicotinamide group, was also able to release $Ca^{2+}$ from the microsomes. This compound was subsequently identified as nicotinic acid adenine dinucleotide phosphate (NAADP), which differs from NADP by only one mass unit (Lee & Aarhus 1995). This small structural change gives it potent $Ca^{2+}$ releasing activity. Three critical determinants of $Ca^{2+}$ releasing activity of this molecule have been identified. They are the carboxyl group of the nicotinic acid moiety, the 2′-phosphate and the amino group of the adenine ring. Any modifications of these determinants greatly reduce its calcium release activity (Lee & Aarhus 1997).

Cyclic ADP-ribose and NAADP can release $Ca^{2+}$ not only *in vitro* but are fully active inside live cells. This was shown convincingly using caged analogues of cADPR and NAADP. A UV-sensitive cage group is attached onto the 2′-phosphate of NAADP (Lee *et al.* 1997). As indicated above, the phosphate group is critical for the $Ca^{2+}$ releasing activity. Attaching the cage group makes the analogue biologically inactive. Exposure of the cage group to UV-light photolyses it and regenerates the biologically active NAADP. In the case of cADPR, the cage group was attached to one of the two phosphates (Aarhus *et al.* 1995). Again it is biologically inactive but can regenerate cADPR after exposure to UV. Sea urchin eggs loaded with caged cADPR and fluo 3 by microinjection produced elevated intracellular $Ca^{2+}$ when exposed to UV. Similar results were obtained with caged NAADP.

 

As a control, eggs were injected with caged ATP and prolong UV exposure did not produce any $Ca^{2+}$ release. It is thus clear that both cADPR and NAADP are effective in mobilizing calcium stores in live cells.

Work in the past decade has shown that both cADRP and NAADP are active in a wide variety of cells. In the case of cADPR, more than 48 different responsive cell types have so far been reported. They include more than 20 different species from three biological kingdoms, from protist to plant, and to animal (Lee 1997). Cells responsive to NAADP are equally widespread and include plant, marine invertebrates such as sea urchin eggs, ascidian oocytes, starfish oocytes and *Aplysia* neurones. Amphibian neurones are also responsive. So are a variety of mammalian cells, including various cells from rat and mouse, as well as several types of cultured cell lines (Lee 2001) Finally, human lymphocytes and human pancreatic beta cells have also been shown to be responsive to NAADP (Berg *et al.* 2000, Johnson & Misler 2002). It is therefore clear that both cADPR- and NAADP-induced $Ca^{2+}$ release should be of fundamental relevance as signalling mechanisms in cells. More information about physiology, pharmacology and enzymology of the cADPR and NAADP signalling systems can be found in a recently published book (Lee 2002).

### *Mechanisms mediating cADPR- or NAADP-induced $Ca^{2+}$ release*

The calcium release mechanisms activated by cADPR, NAADP and $IP_3$ are distinct and can be pharmacologically separated. In sea urchin egg homogenates, heparin effectively blocks $IP_3$ from releasing $Ca^{2+}$. Subsequent addition of NAADP to the same homogenate can still release $Ca^{2+}$ (Lee & Aarhus 1995). A specific antagonist of cADPR, 8-amino-cADPR, blocks the releasing effect of cADPR, but not NAADP (Walseth & Lee 1993, Lee & Aarhus 1995). NAADP was found to induce desensitization in the egg microsomes, and under this desensitized condition cADPR still remains active (Aarhus *et al.* 1996, Genazzani *et al.* 1996). These results indicate that three $Ca^{2+}$ release mechanisms are totally independent of each other. Accumulating evidence has indicated that the target of cADPR is the RyR. It has been demonstrated that the cADPR-sensitive channels reconstituted into lipid bilayers exhibit the characteristics similar to the RyRs (Lokuta *et al.* 1998, Perez *et al.* 1998, Li *et al.* 2001). The cADPR-sensitive release mechanism, however, appears to be more complicated and involves other associated proteins. Calmodulin (Lee *et al.* 1994, 1995) and FK506-binding protein (Noguchi *et al.* 1997) are such factors. Using a photoaffinity labelling technique, a protein of 140 kDa is also shown to be involved (Walseth *et al.* 1993). The specific type of channel that NAADP activates is currently unknown. The intracellular stores that it acts on are, however, different and separable from the endoplasmic reticulum (Lee & Aarhus 1995, 2000). A recent report has provided evidence that the NAADP-sensitive stores in sea urchin eggs are acidic organelles similar to lysosomes (Churchill *et al.* 2002).

### *The enzymatic production of cADPR and NAADP*

Much is now known about the enzymology of cADPR and NAADP production. The enzyme that produces cADPR is called ADP-ribosyl cyclase, which cyclizes NAD to produce cADPR (Lee & Aarhus 1991). In the process, nicotinamide, which is linked to the terminal ribose of NAD is released. The cyclase is a ubiquitous enzyme and three homologues have been identified. In *Aplysia*, it is a soluble protein of 30 kDa and contains 285 amino acids (Glick *et al.* 1991). A membrane-bound lymphocyte antigen called CD38 has been identified as a mammalian homologue (States *et al.* 1992, Howard *et al.* 1993). Another homologue is an antigen called BST-1, which is a GPI-anchored protein (Itoh *et al.* 1994). In the middle of all three proteins, there is a highly conserved sequence. Additionally, the 10 cysteines in the soluble cyclase can be perfectly aligned with the other two proteins. Overall, there is about 30% sequence identity among the three proteins (Lee 2000).

It is well characterized that cADPR is derived from NAD, while NAADP is from NADP. Surprisingly, ADP-ribosyl cyclase is responsible for the synthesis of both cADPR and NAADP (Aarhus *et al.* 1995). The reaction that leads to NAADP synthesis is a base-exchange reaction. In the presence of nicotinic acid, the cyclase or its homologue, CD38, catalyses the exchange of the nicotinamide group of NADP with nicotinic acid and produces NAADP. An important factor that controls the multiple reactions of this enzyme is pH. Under acidic conditions, the base-exchange reaction is in fact the dominant reaction catalysed by the soluble cyclase as well as CD38 (Lee 2000).

The *Aplysia* cyclase has been recombinantly produced in yeast and crystallized (Prasad *et al.* 1996, Munshi & Lee 1997). This cyclase has also been co-crystallized with one of its substrates, nicotinamide. X-ray diffraction studies show that the nicotinamide binding site is in a pocket near the central cleft (Munshi *et al.* 1999). The highly conserved sequence in the middle of the cyclase, in fact, forms part of the active site pocket. The critical residues at the active site have been identified by site-directed mutagenesis. The glutamate 179 situated deep inside the pocket has been shown to be the catalytic

 

residue. Any modification, even highly conservative substitution, renders the cyclase inactive. The nicotinamide is bound to the rim of pocket very close to tryptophan 140. There is another critical tryptophan that lines the other side of the rim of the pocket (Munshi *et al.* 1999).

A unified catalytic model for the *Aplysia* cyclase and CD38 has been proposed (Lee 1999). NAD is a long linear molecule. In order to cyclize it into cADPR, the enzyme must bind it and fold it into a ring such that the two ends are close enough to react. The two tryptophans at the active site pocket can serve such a function. The catalytic residue, glutamate 179 in the soluble cyclase or glutamate 226 in CD38 (Munshi *et al.* 1999, 2000) can then attack and release the nicotinamide group, forming an intermediate. Apparently, both the cyclase and CD38 can bind either NAD or NADP in this folded conformation. This suggests that the active site does not recognize the 2′-position. That is, the presence of a phosphate group at this position, as in the case of NADP, does not interfere with the binding of the substrate. What happens next depends on what is present. If no other nucleophiles are present, the N1 of the adenine ring reacts with the terminal ribose and form the cyclic product cADPR. If nicotinic acid is present, nucleophilic attack of the intermediate results in an exchange reaction and NAADP is produced. This reaction is most prominent at acidic pH.

Cyclic ADP-ribose has now been shown to be involved in the regulation of various cell functions in a variety of tissues or organs (Lee 1997, 2001). In regard to muscle contraction, it is found that this cyclic nucleotide increases the $Ca^{2+}$ sensitivity of the cardiac RyRs reconstituted in bilayers (Meszaros *et al.* 1993). A similar sensitizing effect of cADPR is also seen in the type 3 RyRs (Sonnleitner *et al.* 1998) as well as RyRs from smooth muscle (Li *et al.* 2001). Functionally, cADPR has been shown to potentiate the calcium spark frequency in cardiac myocytes (Cui *et al.* 1999), regulate Ach-induced calcium oscillation in tracheal smooth muscles (Prakash *et al.* 1998), and modulate the spontaneous transient outward currents in VSMs (Cheung 2003). In smooth muscle, cADPR has also been reported not only to mediate contraction but also modulate relaxation (Dipp & Evans 2001, Barone *et al.* 2002). In coronary arteries, cADPR is found to be involved in controlling vascular tone (Geiger *et al.* 2000). The physiological role of NAADP in muscles has just begun to be investigated. It has been shown to be effective in mediating $Ca^{2+}$ signals and contraction in arterial smooth muscles via a novel two-pool mechanism (Boittin *et al.* 2002). Whether it does function as a second messenger in the process requires further investigation.

## Cyclic ADP-ribose-mediated activation of RyRs in the control of vascular tone (P.-L. Li, Medical College of Wisconsin)

### *Cyclic ADP-ribose-induced $Ca^{2+}$ release in VSM cells*

As discussed above, the role of cADPR in the intracellular $Ca^{2+}$ mobilization has been extensively studied in a variety of tissues and cells. In VSM cells, this nucleotide also stimulates $Ca^{2+}$ release from intracellular $Ca^{2+}$ stores. In this regard, Kannan *et al.* (1996) reported that cADPR induces the SR $Ca^{2+}$ release in $\beta$-escin-permeabilized smooth muscle cells freshly isolated from porcine coronary arteries. In studies using $\alpha$-toxin permeabilized cells, we found that cADPR produces $Ca^{2+}$ release in both cultured and freshly dissociated cow coronary and rat renal VSM cells. This cADPR-induced $Ca^{2+}$ release from the SR can be completely blocked by cADPR antagonist, 8-bromo-cADPR, but not by $IP_3R$ blockers. It is concluded that cADPR mobilizes intracellular $Ca^{2+}$ through a mechanism independent of $IP_3$ in VSM cells (Yu *et al.* 2000). In the experiments using KCl to depolarize the cell membrane and consequently produce $Ca^{2+}$ influx-induced $Ca^{2+}$ release, inhibition of ADP-ribosylcyclase by nicotinamide, blockade of cADPR action by cell-permeable antagonist 8-bromo-cADPR and RyR blockade by ryanodine all suppress KCl-induced $Ca^{2+}$ release in VSM cells. These results suggest that endogenous cADPR participates in the production of $Ca^{2+}$-induced $Ca^{2+}$ release (CICR) response and thereby plays an important role in the control of intracellular $[Ca^{2+}]$ in VSM cells (Geiger *et al.* 2000, Yu *et al.* 2000).

### *Cyclic ADP-ribose signalling in control of vascular tone*

In the isolated, perfused and pressurized small coronary arteries, we found that basic vascular tone or spontaneous tension can be developed during a 1.5-h equilibration period. Under this condition, CICR blocker, tetracaine and cADPR antagonist, 8-bromo-cADPR dilated these arteries, suggesting that $[Ca^{2+}]$ associated with cADPR-RyR signalling pathway and CICR is one of the determinants of the basic vascular tone (Zhang *et al.* 2002).

In addition to the role of cADPR in the maintenance of basic vascular tone, we have found that this signalling pathway also participates in response to several vasoconstrictors. First, cADPR has been confirmed to play a role in mediating the amplification of $Ca^{2+}$ signal or $Ca^{2+}$ propagation within VSM cells related to $Ca^{2+}$ influx and thereby involves in the vasoconstriction in response to membrane depolarization and activation of membrane $Ca^{2+}$ channels. Using CICR blocker tetracaine and cADPR antagonist,

 

8-bromo-cADPR, the vasoconstrictor response to KCl-induced membrane depolarization was significantly attenuated in small resistant coronary arteries. However, a cell permeable $IP_3$ blocker, xestospongin C at a concentration that attenuated U46619-induced contraction, has no effect on KCl-induced vasoconstriction in these coronary arteries. Moreover, the vasoconstrictor actions induced by a $Ca^{2+}$ channel activator, Bay K8644 or $CaCl_2$ are also attenuated by tetracaine or 8-bromo-cADPR. It is concluded that cADPR-mediated $Ca^{2+}$ signalling is of importance in amplification of intracellular $Ca^{2+}$ signal and vasoconstriction through CICR (Geiger *et al.* 2000, Li *et al.* 2000, Zhang *et al.* 2002).

Much effort has been spent to determine whether cADPR could mediate the vasoconstrictor response of the arteries to agonists, as $IP_3$ does. It has been reported that cADPR may serve as a second messenger to the action of Ach receptors in adrenal chromaffin cells, oestrogen receptors in uterus, 5-HT 2B receptor in arterial endothelial cells and retinoic acid in renal tubular cells and aortic smooth muscle. Recently, we have demonstrated that cADPR plays a role in mediating Ach-induced contraction in coronary arteries. In isolated, perfused and pressurized small coronary arteries, the inhibition of cADPR formation or blockade of cADPR action significantly attenuated the vasoconstrictor response to Ach or $M_1$ agonist, oxotremorine (OXO), suggesting that cADPR is probably linked to $M_1$ mAChRs in VSM cells. By measuring intracellular [$Ca^{2+}$], we found that OXO produced a rapid $Ca^{2+}$ release in single VMS cells bathed with $Ca^{2+}$-free solution, which was significantly attenuated by inhibition of cADPR production by nicotinamide, blockade of cADPR action by 8-bromo-cADPR and direct blockade of RyRs. These results strongly suggest that endogenous cADPR and subsequent RyR activation contribute to OXO-induced $Ca^{2+}$ mobilization in VSM cells. This view has been further supported by the finding that OXO markedly enhances the activity of ADP-ribosylcyclase and increases the production of cADPR in cultured coronary VSM cells. It seems that ADP-ribosylcylcase is directly coupled to $M_1$ mAChRs. When these receptors are activated, the production of cADPR increases, whereby $Ca^{2+}$ is released from the SR through RyR (Ge *et al.* 2003).

### *RyR activation by cADPR in VSM cells*

Despite controversial findings, most studies have indicated that cADPR can activate RyR, producing $Ca^{2+}$ release from the SR or ER in different tissues or cells (Lee *et al.* 1994, Sitsapesan *et al.* 1994, Lahouratate *et al.* 1997). Now, two mechanistic models have been proposed to elucidate the role of endogenous cADPR in mediating vascular activity. Considering cADPR as a mediator, it is proposed that the agonists or stimuli activate ADP-ribosylcyclase to produce cADPR, leading to activation of $Ca^{2+}$ release from the SR through the RyR. This activation of ADP-ribosylcyclase may also occur when intracellular $Ca^{2+}$ levels increases even slightly, thereby resulting in CICR. Another model considers cADPR as a modulator of CICR or RyR activity. In this way, cytosolic cADPR sensitizes the RyR, enhancing CICR activated by agonists or $Ca^{2+}$ influx. The relative contribution of these two mechanisms to the vascular reactivity may vary depending upon the concentrations of intracellular cADPR, [$Ca^{2+}$], and calmodulin and the functional status of RyR in different cells.

Three subtypes of RyR with specific tissue distributions are now recognized and named as RyR1, RyR2 and RyR3. Studies using ligand binding and molecular approaches demonstrate that RyR3 is a predominant form on the SR of VSM cells. Using electrophysiological methods, the RyR/$Ca^{2+}$ release channels can be reconstituted into a planar lipid bilayer and visualized with electrical stimulation or clamp. In coronary arterial smooth muscle, a calcium channel with 245 pS conductance is present on the SR membrane. cADPR increases the $NP_O$ of these RyR/$Ca^{2+}$ release channels in a concentration-dependent manner. In the presence of ryanodine (50 $\mu$M), cADPR-induced activation of these channels is completely abolished. These results provide direct evidence that cADPR activates RyR and therefore may serve as an endogenous activator or modulator of the RyR in these VSM cells (Li *et al.* 2001, Tang *et al.* 2002).

However, cADPR has been reported to bind to an accessory protein FK506 binding protein 12.6 (FKBP 12.6), rather than RyR itself. Recently, we have demonstrated that FKBP 12.6 is expressed in coronary arterial smooth muscle and cultured coronary arterial smooth muscle cells, as measured by Western blot analysis. In reconstituted lipid bilayer membrane, cADPR-induced activation of RyR/$Ca^{2+}$ release channels could be abolished by blockade, dissociation or removal of FKBP 12.6 protein from the RyR. It is suggested that dissociation of FKBP 12.6 from the RyR complex is activated by binding of this protein with cADPR. This dissociation of FKBP 12.6 protein plays a critical role in mediating cADPR-induced activation of RyR/$Ca^{2+}$ release channels in the SR of VSM cells (Tang *et al.* 2002).

## Role of $[Ca^{2+}]_i$ in mechanotransduction in the arterioles (G. A. Meininger, Texas A&M University Health Science Center)

### *Cellular mechanisms of mechanotransduction in VSM*

Numerous vascular functions depend on the responses of VSM cells to mechanical forces. Examples include

 

physiological regulation of tissue blood flow, capillary pressure and vascular resistance. These regulatory functions are dependent on the vascular myogenic mechanism, in which resistance vessels constrict in response to increases in intravascular pressure and dilate in response to pressure reductions. Vascular responses to mechanical forces are also important for growth, proliferation, migration and remodelling of VSM cells. Abnormalities of these responses are often observed in cardiovascular diseases like hypertension, diabetes and atherosclerosis. Thus, it is important to understand the cellular responses to mechanical forces. Intracellular signalling pathways of mechanotransduction in VSM cells have been extensively studied, and integrins have been implicated as the possible mechanosensor in these cells (Hein *et al.* 2001, Zou *et al.* 2001, Gloe *et al.* 2002, Platts *et al.* 2002). Several studies have shown that applying force to integrin-ligand bonds using paramagnetic beads coated with extracellular matrix (ECM) proteins causes increases in cytosolic $[Ca^{2+}]_i$, tyrosine phosphorylation and cell cortical stiffness. Consequently, integrins may act as the mechanosensor in VSM cells by linking to $Ca^{2+}$ channel function (Waitkus-Edwards *et al.* 2002), to play a central role in mechanotransduction. Our knowledge concerning how integrins sense and transduce physical forces into cellular signals and which integrins are involved is incomplete. The topic of this brief overview will be to focus on the relationship between integrins, myogenic phenomena and modulation of the L-type $Ca^{2+}$ channel (Meininger & Davis 1992, Davis *et al.* 2001, Zou *et al.* 2001).

### *Evidence for integrin involvement in the regulation of myogenic vascular tone*

The most attractive experimental evidence for integrins in regulation of vasomotor tone, comes from studies demonstrating that synthetic peptides or fragments of ECM proteins that contain integrin-binding amino acid sequences are strongly vasoactive (Martinez-Lemus *et al.* 2003). It is noteworthy that most integrins of VSM cells recognize the Asp-Gly-Asp (RGD) integrin-binding motif contained in many vascular wall ECM proteins, e.g. collagen, fibronectin and vitronectin. In previous work in our laboratory, we observed that isolated rat cremaster arterioles responded to RGD-containing peptides with a transient constriction followed by a sustained dilation, whereas, control peptides containing Arg-Gly-Glu (RGE) did not (Mogford *et al.* 1996, 1997, D'Angelo *et al.* 1997). We have also found that the dilation was endothelium-independent and that use of a cyclic form of RGD enhanced the vasodilatory potency, thereby implicating $\alpha_v\beta_3$ integrin involvement (Wu *et al.* 1998). In support of this, a function blocking anti-$\beta_3$ integrin antibody attenuated the vasodilation to cyclic-RGD. Proteolysed fragments of type 1 collagen, which contain multiple exposed RGD residues, were observed to induce a vasodilation that was inhibited with the anti-$\beta_3$ integrin antibody. These observations suggest that antagonism of integrin receptors may cause vasodilation by inhibiting spontaneous myogenic tone (D'Angelo & Meininger 1994, Davis *et al.* 2000). In addition to the RGD integrin recognition motif, two other integrin binding sequences may affect VSMC. These motifs include Asp-Gly-Glu-Ala (DGEA), which interacts with $\alpha_2\beta_1$, and Leu-Asp-Val (LDV), which interacts with $\alpha_4\beta_1$. To our knowledge, peptides containing the DGEA sequence have not been studied, but, we recently reported that LDV-containing peptides cause sustained constriction of rat cremaster arterioles. This constriction is endothelium-independent and blocked by an anti-$\alpha_4$ integrin function-blocking antibody. More recently, studies from our laboratory indicate that the myogenic response to step increases in intravascular pressure is also inhibited by RGD containing peptides. In summary, the evidence to date is convincing for the involvement of various integrins of VSM cells in the control of myogenic vascular tone and the myogenic response (Davis *et al.* 2001, Hill *et al.* 2001).

### *Integrin signalling through calcium entry and involvement of focal contact proteins*

Additional rationale to support integrin involvement in the myogenic response has been based on overlap between calcium-signalling and integrin-signalling pathways in VSM cells (Hill *et al.* 2001, Wu *et al.* 2001). It has been clearly established that calcium influx is essential for initiation and maintenance of myogenic tone in most vessels. For example, removal of extracellular calcium or the presence of antagonists of the L-type calcium channel, e.g. dihydropyridines, cause a loss of myogenic tone. More direct evidence comes from studies in our laboratories showing that ligands of the $\alpha_v\beta_3$ integrin cause arteriolar dilation that is preceded by a decrease in intracellular calcium ($[Ca^{2+}]_i$) of VSM cells, suggesting that the peptide acts by interfering with a calcium entry mechanism. In addition, ligands of the $\alpha_v\beta_3$ integrin inhibit L-type channel calcium current in VSM cells isolated from skeletal muscle arterioles. In contrast, ligands of the $\alpha_5\beta_1$ integrin cause a significant enhancement of L-type calcium current. The ability of $\alpha_5\beta_1$ to enhance current through the L-type calcium channel may explain how RGD peptides produced transient constrictions of skeletal muscle arterioles that were more pronounced following inhibition of $\alpha_v\beta_3$. In recently published work by our laboratory, we demonstrated that the vasoconstriction observed in response to

 

the $\alpha_4\beta_1$ ligand, LDV, is associated with increased $[Ca^{2+}]_i$ in VSM cells, that is dihydropyridine sensitive and is associated with enhanced L-type calcium current. Thus, there is convincing evidence to suggest that integrins and ECM proteins can exert control over the major calcium-ion channel in VSM cells and that regulation of calcium signalling by integrins can affect vascular tone (Davis *et al.* 2001, Wu *et al.* 2001, Zou *et al.* 2001, Waitkus-Edwards *et al.* 2002).

In arteriolar VSM cells, we have observed that engagement and clustering of $\alpha_5\beta_1$ integrins is required to produce the enhancement in L-type calcium current. We also observed that the enhancement of current was inhibited by PTK inhibitors and by a c-Src-specific inhibitory peptide or c-Src antibody implicating involvement of c-Src. Additional supportive observations have shown that enhancement of basal L-type current is obtained by intracellular application of constitutively active Src kinase or inhibition of tyrosine phosphatase. Antibodies to other components of the focal adhesion complex also interfere with $\alpha_5\beta_1$ regulation of calcium current (Wu *et al.* 1998, 2001, Hill *et al.* 2001). For example, anti-FAK, anti-paxillin and antibodies to other focal adhesion or CSK proteins containing SH3 or SH2 domains all inhibit enhancement of calcium current following $\alpha_5\beta_1$ stimulation. The enhancement of basal L-type current by $\alpha_4\beta_1$ was also prevented by inhibition of Src family kinases. Collectively, these studies strongly implicated the importance of c-Src, and/or other protein tyrosine kinases and cytoskeletal proteins, which are linked to integrin signalling, in the regulation of L-type calcium channels in VSM cells (Davis *et al.* 2001).

## $Ca^{2+}$ sparks and activation of plasma membrane $K_{Ca}$ channels in VSM cells (M. T. Nelson, University of Vermont Medical School)

### *$Ca^{2+}$ sparks*

The discovery of temporally and spatially modulated $Ca^{2+}$ signals, including $Ca^{2+}$ sparks and waves, in smooth muscle (Nelson *et al.* 1995, Jaggar *et al.* 2000) and in other cell types has radically altered the traditional view that the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) is homogeneously distributed within the cells (Fay *et al.* 1995, Nelson *et al.* 1995). There are a number of functional implications of this paradigm shift. (1) The type of $Ca^{2+}$ signal encodes the functional outcome. For example, in smooth muscle average cytosolic $Ca^{2+}$ is a potent signal for contraction, whereas the signal provided by $Ca^{2+}$ sparks promotes relaxation. $Ca^{2+}$ signalling plasticity is potentially subjected to regulatory control at multiple levels. (2) The proximity of the $Ca^{2+}$ source (e.g. voltage-dependent $Ca^{2+}$ channels (VDCCs), RyRs or $IP_3$Rs) to the target [e.g. large conductance potassium (BK) channels or small conductance potassium (SK) channels] determines whether the magnitude and duration of the $Ca^{2+}$ signal are sufficient to elicit a response. (3) The $Ca^{2+}$-sensitivity of a target protein (e.g. the BK channel) can be exquisitely matched or tuned to different $Ca^{2+}$ signals providing the capacity for differential target activation in the face of seemingly subtle differences in $Ca^{2+}$ signals. The modulation of $Ca^{2+}$ signalling to BK channels by membrane potential, cGMP-dependent protein kinase, and the $\beta_1$-subunit of the BK channel indicate that multiple mechanisms can contribute to the regulation of BK channel $Ca^{2+}$-sensitivity. (4) The same molecular components (e.g. VDCCs, RyRs and BK channels) can be organized differently within a cell to produce distinct, tissue-specific signalling properties. For example, in cardiac myocytes VDCCs deliver local $Ca^{2+}$ to the closely juxtaposed RyRs resulting in the rapid $Ca^{2+}$-activated release of SR $Ca^{2+}$. Summation of the resulting $Ca^{2+}$ sparks provides the $Ca^{2+}$ required for cardiac contraction. In VSM, RyRs are not in close proximity to VDCCs, but instead are closely apposed to BK channels. In this tissue, VDCC activation also increases spark frequency (although with slower kinetics), but here $Ca^{2+}$ sparks directly couple to $Ca^{2+}$-sensitive BK channels, resulting in an outward, hyperpolarizing current that relaxes the tissue. This modulation of $Ca^{2+}$ signalling to BK channels contributes to the regulation of arterial smooth muscle membrane potential and diameter (Nelson & Quayle 1995, Faraci & Heistad 1998), and is an important determinant of blood pressure (Brenner *et al.* 2000).

### *Global $[Ca^{2+}]_i$ is a key regulator of the contractile state of arteries*

Arteries exist in a partially constricted state from which they dilate or constrict depending on tissue perfusion requirements. The central stimulus for constriction is intravascular pressure which causes smooth muscle cells to depolarize, activating VDCCs and elevating $[Ca^{2+}]_i$. This pressure-induced constriction has been referred to as 'myogenic tone'. The relationship between global $[Ca^{2+}]_i$, intravascular pressure and diameter has been characterized, showing that the diameter of pressurized cerebral arteries is steeply dependent on membrane potential, with graded changes in membrane potential causing graded changes in arterial diameter. The entire dynamic range of global $[Ca^{2+}]_i$ is from 100 to 350 nM, which corresponds to an arterial diameter that spans the range from maximal dilation to almost maximal constriction. Arterial tone is dependent on $Ca^{2+}$ entry through VDCCs, as $Ca^{2+}$ channel antagonists dilate most small arteries.

 

$Ca^{2+}$ sparks were first identified in cardiac muscle (Cheng *et al.* 1993), and subsequently in skeletal (Klein *et al.* 1996) and smooth muscle (Nelson *et al.* 1995). $Ca^{2+}$ sparks are manifestations of $Ca^{2+}$ release events from a group of RyRs in the SR. In arterial smooth muscle, $Ca^{2+}$ sparks activate nearby BK channels in the sarcolemma to cause a hyperpolarizing $K^+$ current that leads to closure of VDCCs, a decrease in $Ca^{2+}$ entry and a corresponding decrease in global $[Ca^{2+}]_i$ (Nelson *et al.* 1995, Jaggar *et al.* 1998). The concept of local $Ca^{2+}$ opposing changes in global $Ca^{2+}$ motivated a paradigm shift in understanding $Ca^{2+}$ signalling (Fay *et al.* 1995, Nelson *et al.* 1995).

$Ca^{2+}$ sparks deliver high, local $Ca^{2+}$ to activate BK channels and can have a profound effect on membrane potential. $Ca^{2+}$ sparks and the associated transient BK channel currents have been measured simultaneously, demonstrating that the BK channel open probability ($P_o$) increases by $10^4$–$10^6$-fold during a spark event at −40 mV. Based on $Ca^{2+}$-sensitivity of BK channels, this increase in $P_o$ corresponds to an estimated increase in $Ca^{2+}$ from basal levels of 100 nM to 10–100 $\mu$M within a spark site. To provide additional support for this interpretation, the $[Ca^{2+}]_i$ required to elevate BK channel $P_o$ from $10^4$–$10^6$-fold was determined by changing $Ca^{2+}$ on the inside of BK channels in excised patches. An elevation of $[Ca^{2+}]_i$ to 10–50 $\mu$M was required to achieve the observed increase in channel $P_o$ in response to a $Ca^{2+}$ spark. These results are consistent with a very close apposition (~20 nm) of RyRs in the SR and BK channels in the surface membrane.

In cardiac muscle, the opening of VDCCs delivers high local $Ca^{2+}$ that activates nearby RyRs within milliseconds and causes a global $Ca^{2+}$ transient that leads to muscle contraction. This process is referred to as 'local control' of CICR (Cheng *et al.* 1993, Cannell *et al.* 1995). Although early measurements in urinary bladder smooth muscle (UBSM) by Ganitkevich & Isenberg (1992) showed that $Ca^{2+}$ currents activate ryanodine-sensitive $Ca^{2+}$ release, this process is remarkably different from that observed in cardiac muscle. CICR in UBSM is very slow, occurring over hundreds of milliseconds. Given that the spike of an action potential in UBSM is much shorter (about 20 ms) than this process, the contribution of CICR to muscle contraction in bladder is unclear, but likely provides, at best, only a fraction of the $Ca^{2+}$ for the transient. Recently, Collier *et al.* (2000) have provided compelling evidence that activation of VDCCs is not tightly coupled to activation of RyRs in UBSM. These investigators found that $Ca^{2+}$ spark activation by $Ca^{2+}$ influx through VDCCs occurred after a significant delay (about 30 ms at −30 mV) and was not related to the unitary $Ca^{2+}$ flux through a single VDCC. Furthermore, $Ca^{2+}$ influx stimulates only a small number of sparks (<3), whereas in cardiac muscle hundreds of sparks are activated summing to a global $Ca^{2+}$ transient.

### *Regulation of cerebral artery membrane potential and diameter by BK channels*

BK channels regulate the membrane potential of smooth muscle cells in pressurized cerebral arteries. These channels, which are activated by $[Ca^{2+}]_i$ and membrane potential depolarization, have an exceedingly low $P_o$ (approximately $10^{-6}$) at physiological membrane potentials (~−40 mV) and $[Ca^{2+}]_i$ (~200 nM), characteristic of intact pressurized arteries. This low $P_o$ under physiological conditions appears to be at odds with the clear contribution made by BK channels to the regulation of the membrane potential of smooth muscle cells in intact arteries. The discovery of $Ca^{2+}$ sparks in smooth muscle provided a resolution of this dilemma, i.e. a means to deliver sufficient $Ca^{2+}$ to elevate BK channel activity to a level, which would contribute to regulation of smooth muscle membrane potential. A $Ca^{2+}$ spark increases the $P_o$ of nearby BK channels $10^4$–$10^6$-fold, resulting in $K^+$ efflux substantial enough to hyperpolarize the membrane potential by 10–20 mV. Further evidence for the regulation of BK channels by sparks is provided by the observation that inhibitors of $Ca^{2+}$ sparks and BK channels depolarize arterial smooth muscle in a non-additive manner. $Ca^{2+}$ sparks, through activation of BK channels, thus, function as a negative feedback element to limit membrane depolarization and contraction.

### *Roles of the BK channel $\beta_1$-subunit*

The BK channel in smooth muscle is composed of the $\alpha$ pore and $\beta_1$-subunits. The $\beta_1$-subunit is highly expressed in smooth muscle, but not in other tissues. The $\beta_1$-subunit has been shown to increase the apparent $Ca^{2+}$-sensitivity of the pore-forming $\alpha$-subunit in heterologous expression systems. The important physiological role of the $\beta_1$-subunit in smooth muscle is just emerging.

*Roles of membrane potential and the $\beta_1$-subunit in regulating the coupling of local $Ca^{2+}$ events to BK channel activation.* Membrane potential depolarization and the $\beta_1$-subunit both increase the apparent $Ca^{2+}$-sensitivity of BK channels. The impact that this modulation of BK channel $Ca^{2+}$-sensitivity has on the efficacy of $Ca^{2+}$ spark and $Ca^{2+}$ wave activation of BK channels is largely unknown. The membrane potential modulates the coupling strength of $Ca^{2+}$ sparks to BK channels. Our recent studies also suggest that the coupling of $Ca^{2+}$ sparks to BK channels is decreased, and in some cases uncoupled, in smooth muscle cells from $\beta_1$-KO mice

 

(Brenner *et al.* 2000). Furthermore, cerebral arteries are more constricted, and the $\beta_1$-KO mouse exhibits elevated blood pressure and shows evidence of cardiac hypertrophy.

*Modulation of the expression of the $\beta_1$-subunit expression.* Ablation of the $\beta_1$-subunit gene of the BK channel leads to an elevation of blood pressure (Brenner *et al.* 2000). The role of the $\beta_1$-subunit has been also studied in the arteries from Ang II-induced hypertensive rats. The diameter of cerebral arteries was less sensitive to the BK channel blocker in Ang II animals, suggesting a decrease in the role of BK channels. Furthermore, the BK currents evoked by calcium sparks were about 2.5-fold smaller in hypertensive than in normotensive controls. Molecular investigations showed that the reduced coupling between BK channels and RyRs is produced by a down-regulation of the BK channel $\beta_1$-subunit, which results in BK channels with destabilized open conformations and decreased sensitivity to activation by calcium. These results support the concept that the molecular composition of the BK channel is altered in hypertension, and that this could contribute to vascular dysfunction.

Taken together, recent studies in our laboratory and by others have demonstrated that smooth muscle is selectively enriched in the $\beta_1$-subunit of the BK channel and deletion of the $\beta_1$-subunit gene leads to a significant decrease in the $Ca^{2+}$ sensitivity of BK channels and a marked reduction in functional coupling of $Ca^{2+}$ sparks to BK channel activation. Arterial tone and mean arterial blood pressure of $\beta_1$-KO mice are significantly increased, and Ang II-induced hypertension leads to a decrease in the apparent $Ca^{2+}$ sensitivity of BK channels, uncouples calcium sparks from BK channel, and decreases the ability of BK channels to regulate vascular tone. It is concluded that the molecular composition of the BK channels of VSM cells is modulated in hypertension, thereby decreasing the ability of the BK channel to decode calcium signals.

## $Ca^{2+}$ gradients and junctional membrane $Ca^{2+}$ transport in vascular smooth muscle (C. Van Breemen, University of British Columbia)

### *Transport of compartmentalized $Ca^{2+}$*

All fast processes in the vasculature, which include vasoconstriction, secretion of vasorelaxants and vasoconstrictors, and a number of slower ones including proliferation, migration and apoptosis, are regulated by intracellular $Ca^{2+}$. An important unresolved question is how fluctuations of the concentration of this inorganic ion can regulate a multitude of cellular processes (Chen & van Breemen 1992, Daniel *et al.* 1995, Lee *et al.* 2002a,b). This puzzle becomes even more daunting considering that several functions such as crossbridge cycling and myosin filamentogenesis in smooth muscle or NO and EETs secretion in the endothelium can be signalled simultaneously. The solution to this problem lies in determining how $Ca^{2+}$ transport is compartmentalized within the cell to create specifically timed and localized intracellular $Ca^{2+}$ gradients (van Breemen *et al.* 1997, Lee *et al.* 2002a,b). The necessity for compartmentalization becomes clear upon realizing that a number of cellular $Ca^{2+}$ sensors like mitochondrial dehydrogenases, voltage-gated $Ca^{2+}$ channels (VGCC), $IP_3R$ and DNAse are localized in different regions of the cell and even $Ca^{2+}$-calmodulin sensitive effector proteins are spatially segregated by tethering calmodulin to the effector complexes (van Breemen *et al.* 1995, 1997, Daniel *et al.* 1995, Lee *et al.* 2002a,b).

Recently, we analysed how the close interaction between $Ca^{2+}$-transport molecules located in the PM, the sarco-endoplasmic reticulum (SR/ER), the nuclear envelope and mitochondria (MT) can yield the spatial and temporal fluctuations in $[Ca^{2+}]_i$ that are essential for site-specific $Ca^{2+}$ signalling. This interaction takes place in two fundamentally different ways: (1) $Ca^{2+}$ supply to or removal from the $Ca^{2+}$ receptors controlling $Ca^{2+}$ transport and (2) $Ca^{2+}$ delivery from a transport site located in one membrane to a second $Ca^{2+}$ transport site located in an apposing membrane (Szado *et al.* 2003). An example of the first type is $Ca^{2+}$ inhibition of VGCC, which then alters the flux of $Ca^{2+}$, while the second type of interaction is exemplified by coupling of $Ca^{2+}$ entry through the $Na^+/Ca^{2+}$-exchanger (NCX) to the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA), during store refilling. The concept of one transporter delivering $Ca^{2+}$ preferentially to the next transporter, circumventing free diffusion throughout the cytoplasm is herein termed 'linked $Ca^{2+}$ transport'. Such linked transport is responsible for generating the $Ca^{2+}$ gradients between diffusion-restricted cytoplasmic domains, which then specifically regulate the ultrastructurally localized $Ca^{2+}$ receptors. There is growing evidence that the greater portion of linked $Ca^{2+}$ transport takes place at the junctions of cellular and organellar membranes (Duchen 1999, Gunter *et al.* 2000, Lee *et al.* 2002a, Szado *et al.* 2003).

#### *Plasmamembrane-SR junctions*

In the resting state, $Ca^{2+}$ enters the smooth muscle cells and in part cycles through the superficial SR without elevating the bulk cytoplasm. The basal rate of $Ca^{2+}$ influx into smooth muscle has been estimated to be $\sim$32 $\mu$mol of $Ca^{2+}$ per litre of cells per minute, which is more than 300 times the resting $[Ca^{2+}]_i$ per minute. This raises the following questions: (1) how does so much

 

$Ca^{2+}$ enters unstimulated cells, (2) how is this influx $Ca^{2+}$ distributed within the cell, and (3) how is it extruded to maintain resting levels of $[Ca^{2+}]_i$ and vascular tone? It is also unknown whether basal $Ca^{2+}$ influx via the leak pathway exhibits variations in magnitude in different regions of the PM.

It is becoming clear that the PM is made up of a patchwork of domains, some covered by dense bodies where the myofilaments are attached, some apposed by the superficial SR, some densely covered by caveolae, and the remainder simply facing the bulk cytoplasm. Resting $Ca^{2+}$ influx is attributed partly to a certain level of the open probability of excitable channels, including receptor-operated channel (ROC), store-operated channel (SOC), and VGCC. Some SOC may stay open due to the basal level of $IP_3$, whereas the resting membrane potential of VSM of resistance arteries allows for a certain degree of activation of the $Ca^{2+}$ window current. Additionally, a non-specific influx of $Ca^{2+}$, referred to as the calcium leak, may contribute to basal $Ca^{2+}$ influx; however, its precise mechanism remains elusive. The resting $[Ca^{2+}]_i$ is ~80 nM and is maintained at that low level despite the fact that every half second the equivalent of the total free cytosolic $Ca^{2+}$ enters the cells. Two mechanisms protect the cell from drastic changes in $[Ca^{2+}]_i$ as a result of $Ca^{2+}$ entry: (1) the presence of fixed and diffusible $Ca^{2+}$-binding sites in the cytoplasm and (2) sequestration by SERCA in the SR. It has been shown in several types of VSM that stimulated influx is more effective in raising $Ca^{2+}$ concentration near the myofilaments when $Ca^{2+}$ uptake in the SR is inhibited by either blockade of SERCA or opening of release channels. Thus, in the resting smooth muscle, the peripheral SR takes up $Ca^{2+}$ entering the cells before it can equilibrate with the deeper myoplasm (Daniel *et al.* 1995, Gurney *et al.* 2000, Lee *et al.* 2002a,b).

In the rabbit inferior vena cava (IVC), continuous unloading of the superficial SR to the extracellular space, involving coupling of SR release channels to the forward mode NCX, permits buffering of $Ca^{2+}$ influx to continue. A set of experiments involving sequential blocking of $Ca^{2+}$ entry, NCX, and SERCA revealed that the $Ca^{2+}$ release channels, probably RyR involved in the unloading of the superficial SR and located in the plasmamembrane-SR (PM-SR) junctional complex are closest to the NCX, next closest to SERCA and functionally furthest from the PMCA. The cyclical process of $Ca^{2+}$ buffering and unloading is known as the 'superficial buffer barrier'. Both the superficial SR and the perpendicular/radial SR traversing through the peripheral myosin-poor space function as the superficial buffer barrier, sequestering $Ca^{2+}$ before it reaches the deep myosin-rich myoplasm. The definition of the superficial SR thus includes the junctional SR and sheets of SR within ~200 nm of the PM (van Breemen *et al.* 1995).

During α-adrenergic activation the PM-SR junctional $Ca^{2+}$ transport is essentially reversed. First, $IP_3$-mediated SR $Ca^{2+}$ release, takes place at a frequent discharge site and is then spread by $Ca^{2+}$ activating $IP_3R$ in neighbouring SR elements. In conduit blood vessels the asynchronous repetitive $Ca^{2+}$ waves are initiated in a cyclical manner to produce $[Ca^{2+}]_i$ oscillations in the following manner: $IP_3$-mediated SR $Ca^{2+}$ release leads to activation of SOCs mediating influx of mainly $Na^+$, and also some $Ca^{2+}$. This causes depolarization and opening of VGCC. The $Na^+$ built up in the junctional space drives the NCX in the reverse direction, which supplies $Ca^{2+}$ of extracellular origin to SERCA in the process of refilling of the SR. The emptied SR ceases to release $Ca^{2+}$ due to lack of supply and inactivation of $IP_3R$. As the stores refill critical concentrations of luminal and cytoplasmic $[Ca^{2+}]$ are reached to reinitiate regenerative SR $Ca^{2+}$ release and commence the next $Ca^{2+}$ wave. The $[Ca^{2+}]_i$ oscillations have a fixed amplitude, consistent with their regenerative mechanism and regulate force through recruitment over the low agonist concentration range and through increasing frequency over the entire dose–response relationship. The frequency regulation of contraction allows for greater precision than achieved by changes in average $[Ca^{2+}]_i$. In addition, it is more efficient in both nuclear and mitochondrial $Ca^{2+}$ signalling (van Breemen *et al.* 1995, Daniel *et al.* 1995, Lee *et al.* 2002a,b).

### *SR-mitochondrial junctions*

Two-way communication of $Ca^{2+}$ signalling between SR/ER and mitochondria has been widely reported in numerous cells types including cardiomyocytes, smooth muscle cells, neurones, oligodendrocytes, adrenal chromaffin cells, hepatocytes and T-lymphocytes. It is now well established that the SR/ER communicates with the mitochondrial uniporter in so much as SR/ER $Ca^{2+}$ release: (1) sensitizes MT $Ca^{2+}$-uptake, (2) is taken up by MT elevating ATP production, or (3) alters bulk cytosolic $Ca^{2+}$ signalling. In cultured smooth muscle we observed that purinergic activation of the P2y receptors induced a large transient increase in $[Ca^{2+}]_{MT}$, which was dependent on functional SR $Ca^{2+}$ uptake by SERCA. In this preparation half of the mitochondrial $Ca^{2+}$ signal was lost, in a $Na^+$ dependent manner, in a $Ca^{2+}$-free medium. Removal of external $Na^+$ would prevent the decline in MT $Ca^{2+}$ signal indicating that half of the mitochondria communicated with the PM NCX through the peripheral SR. In addition, a fraction of the mitochondrial population was able to take up $Ca^{2+}$ delivered by the reverse mode NCX, upon sudden removal of external $Na^+$ (Hajnoczky *et al.* 1995, Gunter *et al.* 2000, Pozzan *et al.* 2000, Montero *et al.* 2001). Much of the current interest focuses on how mitochondrial $Ca^{2+}$ transport modulates

 

the cytoplasmic $Ca^{2+}$ signal. This is thought to occur by mitochondrial $Ca^{2+}$-uptake altering the local [$Ca^{2+}$] near the $Ca^{2+}$-sensitive RyR or $IP_3R$, or by MT $Ca^{2+}$-release directly supplying SERCA with $Ca^{2+}$. Moreover, it is possible that the enhancement of buffering capacity of the peripheral SR by mitochondria prevents $Ca^{2+}$ inhibition of $Ca^{2+}$ entry through VGCC and SOC (Gurney *et al.* 2000, Nassar & Simpson 2000, Dipp & Evans 2001, Szado *et al.* 2003).

In conclusion, we propose that junctional membrane complexes between the plasmamembrane, SR/ER and mitochondria play a crucial role in site-specific $Ca^{2+}$ signalling in the vasculature.

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