

SYMPOSIUM REPORT

Calcium events in smooth muscles and their interstitial cells; physiological roles of sparks

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The observation of spontaneous sporadic releases of packets of stored calcium made 20 years ago has opened up a number of new concepts in smooth muscle physiology: (1) the calcium release sites are ryanodine and inositol 1,4,5-trisphosphate (IP₃) receptor channels which contribute to cell-wide increases in [Ca²⁺]_i in response to cell depolarization, activation of IP₃-generating receptors, or other stimuli; (2) changes in [Ca²⁺]_i act back on the cell membrane to activate or modulate K⁺, Cl⁻ and cation channel activity so affecting contraction, in arterial smooth muscle for example affecting blood pressure; (3) IP₃ production is voltage dependent and is believed to contribute to pacemaker potentials and to refractory periods which control the rhythmical motility of many hollow organs. Most smooth muscle tissues contain interstitial cells (ICs) in addition to contractile smooth muscle cells (SMCs). The interactions of these internal mechanisms, and in turn the interactions of SMCs and ICs in various smooth muscle tissues, are major factors in determining the unique physiological profiles of individual smooth muscles.

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Some 20 years ago it was discovered that calcium is spontaneously and sporadically released in packets from stores within smooth muscle cells leading to transient increases in the internal ionized calcium concentration, [Ca²⁺]_i, and to bursts of openings of calcium-sensitive potassium channels (BK_{Ca} channels); each burst gives rise to a spontaneous transient outward current or STOC (Benham & Bolton, 1986). These observations were made on single isolated visceral and vascular smooth muscle cells (SMCs) separated by enzyme treatment and since then it has been found that these discharges also occur *in vivo* and are of physiological relevance (e.g. see review of results in vascular muscle by Wier & Morgan, 2003). There is now a large literature on the physiological relevance of these spontaneous and evoked calcium transients in skeletal (review: Baylor, 2005), mammalian cardiac (review: Guatimosim *et al.* 2002) and mammalian smooth muscle cells (recent reviews: Kotlikoff, 2003; Wellman & Nelson, 2003; Macrez & Mironneau, 2004; McCarron *et al.* 2004). Following their initial discovery, the development of fluorescent calcium indicator dyes (such as fluo3), which

change their emission upon binding calcium (Tsien, 1992), has allowed an exhaustive study of these calcium transients. The brief flash of fluorescence which is evoked by the transient rise in [Ca²⁺]_i in cardiac myocytes if fluo3 or fluo4 are present in the cytoplasm was termed a 'spark' (Cheng *et al.* 1993). The spark in SMCs commonly evokes a STOC (Nelson *et al.* 1995).

A number of important conclusions were established about transient spontaneous intracellular calcium releases from experiments on STOC discharges of SMCs which were summarized by Bolton & Imaizumi (1996):

(1) Sporadic transient intracellular releases of calcium come from calcium stores in the cell rather than from entry of calcium through the plasmalemma.

(2) However, maintained depolarization of the cell generally accelerates their rate of release probably by increasing calcium influx. This effect presumably operates by indirectly increasing [Ca²⁺]_i within the store but the exact mechanism is not known. It could involve a voltage-dependent increase in the activation of phospholipase C (Best & Bolton, 1986). Other processes acting to alter the amount of stored calcium also affect their frequency and size.

(3) When agents (such as ryanodine and caffeine) activate ryanodine receptors (RyRs) or when inositol 1,4,5-trisphosphate receptors (IP₃Rs) are activated by IP₃

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or by activation of excitatory G-protein-coupled receptors (e.g. muscarinic or adrenergic receptors) the rate of calcium release(s) may accelerate. At high concentrations these agents empty the stores abolishing transient calcium releases. Direct activation of G-proteins by GTP γ S produces similar effects.

(4) Release occurs from store sites close to the plasmalemma because following depletion of stored calcium by a brief caffeine application, STOCs recover more quickly than the response to caffeine. Presumably restoration of a full caffeine response depends on deeper calcium stores being fully refilled.

As has been pointed out (Macrez & Mironneau, 2004) the response of calcium indicator dyes, which have a relatively high affinity for calcium, are unlikely to report the time course of the high calcium concentrations (> 10 μ M) likely to be achieved in the microdomain close to the open mouth of a calcium channel. This is because the small volume consequently contains few molecules of fluo3 and those that are present will virtually all be bound to calcium which is present at high concentration. Such calcium indicators simply follow the more extensive and attenuated rise in $[Ca^{2+}]_i$ which occurs as calcium ions diffuse away from the mouth of the channel. The latter is visualized as a spark of fluorescence. Calcium indicators also provide some extra buffering of the change in calcium concentration. For these reasons, the response of BK_{Ca} channels, which have a low affinity for calcium, and which seem to be situated close to the SR (sarco/endoplasmic reticulum) calcium channels (i.e. IP₃R and RyR channels), has advantages as an indicator of the rise in $[Ca^{2+}]_i$. Indeed, the duration of the burst of openings of BK_{Ca} channels that occurs is shorter than the spark (e.g. Perez *et al.* 1999). This arises presumably because the BK_{Ca} channels at physiological potentials respond only to the higher concentrations of calcium achieved (Benham *et al.* 1986), and so close while the spark is still declining (ZhuGe *et al.* 2002).

In addition to BK_{Ca} channels, there are a number of other calcium-sensitive channels in SMCs which potentially may open during a spark. These include small conductance calcium-activated potassium channels (Bayguinov *et al.* 2000; Kong *et al.* 2000) cation channels (Pacaud & Bolton, 1993) and particularly calcium-activated chloride channels (Large & Wang, 1996). Intracellular calcium transients can cause bursts of openings of calcium-activated chloride (Cl_{Ca}) channels which give rise to spontaneous transient depolarizations (STDs) or spontaneous transient inward currents (STICs) in voltage-clamped cells (van Helden, 1991; Wang *et al.* 1992). In some smooth muscles both Cl_{Ca} channels and BK_{Ca} channels open although the latter seem invariably to open first during the calcium transient. The time course of STICs is a little slower than STOCs and more closely reflects the time course of associated sparks (Henmi *et al.* 1996;

ZhuGe *et al.* 1998; Karkanis *et al.* 2003). The implication is that BK_{Ca} channels are clustered in a plasmalemmal region close to the RyR calcium SR release channels and so are exposed to a high concentration of store-released calcium early after SR calcium channel opening. Cl_{Ca} channels are more evenly distributed in the cell, more sensitive to ionized calcium, and so can respond to calcium transients at later times, at lower concentrations of calcium, and at more distant sites from the SR calcium-release point. In rabbit urethra STICs seem to result from a more widespread rise in $[Ca^{2+}]_i$ than occurs during a spark as inhibitors of IP₃Rs blocked STICs but not STOCs (Sergeant *et al.* 2001). This implies that the release of IP₃ was necessary to promote more widely the spread of the calcium transient. A facilitatory action of IP₃ receptor activation on calcium release through RyR channels due to recruitment of adjacent RyR domains has been suggested from work on portal vein (Boittin *et al.* 1999; Gordienko & Bolton, 2002). However, in vas deferens 2APB, an agent often used to block IP₃Rs, increased spark size probably by increasing the levels of stored calcium (White & McGeown, 2003). There are three types of RyR and three types of IP₃R in various smooth muscles and their roles are under investigation (e.g. Tasker *et al.* 1999; Boittin *et al.* 2000; Coussin *et al.* 2000; Yang *et al.* 2005). In mouse colonic myocytes, ryanodine (at 10 μ M) did not block spontaneous calcium transients but these were inhibited by IP₃R blockers. This suggests that, in this tissue, calcium transients are 'puffs' rather than 'sparks' and that IP₃Rs are arranged in clusters or domains (Bayguinov *et al.* 2000) since a single IP₃R would seem to be insufficient to generate a puff (Boittin *et al.* 1998). Thus, as with the RyRs, some calcium-induced calcium-release (CICR) mechanism which synchronizes the opening of IP₃Rs in a small domain would be required. However, in this tissue ryanodine (as well as IP₃R blockers) reduced the stimulation of calcium events by ATP emphasizing the importance of ryanodine receptors for calcium transients in this tissue. In view of this it would seem important to test the effect of higher concentrations of ryanodine than those applied during spontaneous calcium transients to decide whether they result from the opening of domains of RyRs rather than of IP₃Rs. With the exception of these experiments on mouse colonic myocytes, the results in other SMCs suggest that calcium events result primarily from the opening of clusters or domains of RyRs which are activated to open together by CICR. They further suggest that the role of IP₃Rs is to facilitate CICR between RyRs in a domain and between RyR domains, so giving rise to calcium waves or to a cell-wide increase in $[Ca^{2+}]_i$ leading to cell contraction. In different smooth muscles the relative importance of RyRs and IP₃Rs for $[Ca^{2+}]_i$ changes will certainly vary.

SMCs are normally interconnected as a three-dimensional electrical syncytium and this would be

expected to smooth and to integrate the effects of multiple random STOC discharges in a smooth muscle tissue, producing a hyperpolarizing effect and promoting relaxation of tension (Jaggar *et al.* 2000). However, in certain thin smooth muscle tissues, where the smoothing effect of the electrical interconnections seems weak, discrete spontaneous depolarizations due to bursts of Cl_{Ca} channel openings have been recorded by van Helden and his associates (van Helden, 1991, mesenteric veins; van Helden, 1993, mesenteric lymphatics; Hashitani *et al.* 1996, urethral smooth muscle). These important observations presaged the concept that pacemaker potentials in gastrointestinal and other smooth muscles may arise due to such spontaneous releases from calcium stores leading to depolarization as calcium-activated channels open (Suzuki & Hirst, 1999; van Helden *et al.* 2000; van Helden & Imtiaz, 2003; Kito & Suzuki, 2003). Production of IP_3 is believed to be voltage dependent (Best & Bolton, 1986; Itoh *et al.* 1992; Ganitkevich & Isenberg, 1993) so the depolarization initiates a positive feedback loop leading to a slow wave or pacemaker potential. In stomach smooth muscle spontaneous transient releases of calcium through the action of IP_3 are believed to give rise to 'unitary potentials' due to the opening of bursts of calcium-activated chloride channels. Unitary potentials summate to produce the pacemaker component of the slow wave (Edwards *et al.* 1999; Hirst & Ward, 2003; Hennig *et al.* 2004). However, other mechanisms of slow wave generation have been suggested such as the switching off of a calcium-inhibitable cation current (Koh *et al.* 2002) and voltage-dependent activation of the release of stored calcium leading to the generation of a cation current (Goto *et al.* 2004). Mitochondria have been suggested to play an important role in rhythmical calcium releases. Manipulation of mitochondrial function affected sparks in cerebral arterial myocytes (Cheranov & Jaggar, 2004) and calcium transients in mitochondria are also observed during pacemaker oscillations of $[Ca^{2+}]_i$ in interstitial cells of Cajal (Ward *et al.* 2000).

Thus the initial observations in smooth muscle of membrane events consequential on the sporadic release of calcium packets from the SR, has led to several important discoveries in cellular and tissue physiology. These releases, either spontaneous or evoked (and in the former case there may be precipitating factors as yet unknown) are now known to alter smooth muscle tension. They are also the building blocks of the larger global calcium increases associated with contraction and/or shortening of amphibian (and probably mammalian, Baylor, 2005) striated, cardiac (Cheng *et al.* 1996; Guatimosim *et al.* 2002) and smooth muscles. They appear to be involved in the important pacemaking potentials of gut (van Helden *et al.* 2000; Hirst & Ward, 2003; Imtiaz & van Helden, 2003) and of other (Sergeant *et al.* 2001) smooth muscles. They seem to be important for the regulation

of blood pressure through a hyperpolarizing influence on membrane potential through BK_{Ca} channels in systemic vascular myocytes (Jaggar *et al.* 2000; Wier & Morgan, 2003). They also exert a depolarizing influence through Cl_{Ca} channels in other smooth muscles such as pulmonary vascular myocytes (Remillard *et al.* 2002). Loading of the SR during the prolonged action potential of the ureter results in a subsequent discharge of sparks or calcium events which hyperpolarize the membrane inhibiting further action potentials for several tens of seconds until this discharge has subsided. In this way the refractory period of the ureter is created (Burdyga & Wray, 2005).

The store sites releasing packets of calcium spontaneously are also the sites from which calcium is first released upon depolarization of the cell (Imaizumi *et al.* 1998; Jaggar *et al.* 1998; Ohi *et al.* 2001; Kotlikoff, 2003), i.e. in SMCs which normally discharge action potentials the process of CICR takes place at these sites triggered by entering calcium. Depolarization (Arnaudeau *et al.* 1997; Herrera *et al.* 2001) or stretch (Ji *et al.* 2002) of single cells also increased spark discharge so these sites may be activated also during contractions of smooth muscles. In arterial segments increased intraluminal pressure depolarizes the SMCs and this leads to calcium entry through voltage-dependent calcium channels and accelerates spark discharge (Jaggar *et al.* 1998; Jaggar, 2001). Calcium channel blockers such as diltiazem block the increase in spark discharge frequency upon depolarization and dilate the artery due to reduction in STOCs (Jaggar *et al.* 1998). Forskolin or membrane-permeable cAMP analogues accelerate spark discharge and associated STOCs in rabbit portal vein slightly (Komori & Bolton, 1989) and in cerebral arteries appreciably (Porter *et al.* 1998). If phospholamban was absent in genetically deficient ('knock-out') mice then forskolin was without effect and basal spark discharge frequency increased. This suggests that protein kinase A by phosphorylating phospholamban promotes SR calcium storage (Wellman *et al.* 2001). Protein kinase C activation inhibits spark discharge (Kitamura *et al.* 1992; Bonev *et al.* 1997).

The origin and mechanism of pacemaker potentials in the gut has intrigued investigators for several decades. The present consensus is that the pacemaker function lies in specialized cells found in several locations within the wall of the stomach and large and small intestines. These cells, the interstitial cells of Cajal (ICCs, Thuneberg, 1982) are poorly or not contractile and have processes which distinguish them from the adjacent SMCs. Also, they generally express the tyrosine kinase antigen, CD117 also known as c-Kit (Sanders, 1996; Huizinga, 1999). However, the relationship between the expression of this antigen and their pacemaker function is entirely obscure (Canonica *et al.* 2001); c-Kit is particularly expressed on cells of haemopoietic lineage and certain other cell lines

(Ashman, 1999; Linnekin, 1999) and its function in ICCs is unknown, so its use as a marker for such cells needs justification. There are other types of interstitial cells in the gut (Horiguchi & Komuro, 2000; Vanderwinden *et al.* 2002; Goto *et al.* 2004) which carry the antigen C34. Their morphology is different from ICCs and they have been described as having different electrophysiological properties. ICCs often show regular depolarizations or slow waves which are associated with a rise in $[Ca^{2+}]_i$ believed to cause the opening of calcium-sensitive cation channels whereas C34-positive cells show transient hyperpolarizations caused by the opening of BK_{Ca} channels in response to increases in $[Ca^{2+}]_i$ (Goto *et al.* 2004).

ICC-like cells have been identified in other smooth muscle tissues such as bladder, rabbit urethra (Sergeant *et al.* 2001), renal calyx and proximal pelvis (Klemm *et al.* 1999), guinea-pig urinary bladder (McCloskey & Gurney, 2002), rat, mouse and guinea pig vas deferens (Burton *et al.* 2000), sheep mesenteric lymphatic vessels (McCloskey *et al.* 2002) and uterus (Duquette *et al.* 2005). We have identified cells with extensive processes in rabbit portal vein which we call interstitial cells (ICs) in two layers within the media. These cells are c-Kit positive and do not contract in response to high potassium solution, caffeine, or noradrenaline, although these agents produce a cell-wide increase in $[Ca^{2+}]_i$ (Povstyan *et al.* 2003; Pucovsky *et al.* 2003). As the portal vein is a spontaneously contractile organ it is possible that these cells play a pacemaker role as in the gut. However, we have also found cells with a similar morphology in guinea-pig mesenteric artery (Pucovsky *et al.* 2003) and in other arteries. In the mouse mesenteric artery, ICs with processes are c-Kit positive (Povstyan *et al.* 2005). The nature and function of these cells is at present uncertain, as is the significance of c-Kit as a marker. Its presence in some of these ICs and not others may indicate that there are several subtypes. Perhaps a more important question is their relationship developmentally to SMCs; do they arise from, or give rise to, SMCs?

We have examined ICs and SMCs for the presence of various proteins as well as surface antigens such as c-Kit. Many of the smooth muscle proteins are present in ICs which suggests that they are related to, or form part of, a SMC lineage. Most of our work so far has been done in small guinea-pig mesenteric arteries where vimentin, desmin, smooth muscle myosin heavy chain, α -smooth muscle actin, β -actin, and smoothelin were all detected immunohistologically in both SMCs and ICs, although the last mentioned was expressed significantly less in ICs. Nevertheless, both were c-Kit negative and lacked the neuronal marker PGP9.5 (Pucovsky *et al.* 2003, 2005). Inward and outward currents in single ICs were greater than in SMCs from the same artery and transient calcium events were much longer (nearly 3 s), spread further through the cell and were associated with

a corresponding longer outward current, compared to SMCs where they were much briefer (about 50 ms) and much more localized. In rabbit portal vein longer-lasting calcium transients were observed in ICCs, in this case associated with depolarizations of the membrane (Harhun *et al.* 2004).

The numerous processes of some ICs are notable. Presumably they served some function in the tissue which is at present unknown. We have studied the growth of these processes in short-term culture and found that they grew at an average rate of about $0.15 \mu\text{m min}^{-1}$. This growth depended on actin polymerization as it was inhibited by latrunculin B (Pucovsky *et al.* 2003). The processes have also been seen to exhibit calcium sparks at least in their portions closest to the cell (Povstyan *et al.* 2003) and to contact smooth muscle cells in partially dissociated preparations treated with enzymes (Harhun *et al.* 2004). Depolarizing an isolated IC by passing current through a patch clamp pipette while recording in the whole cell mode could in some cases elicit a depolarization and a rise in $[Ca^{2+}]_i$ in an adjacent SMC (Harhun *et al.* 2004). This presumably arises due to the release of some substance from the IC. However, since processes of ICs also contact SMCs, the nature of the mechanism(s) which allow(s) ICs and SMCs to communicate are not understood at present.

Spontaneous releases of packets of calcium from stores has proved to be an observation which has led to several important advances over the last 20 years in our understanding of smooth muscle physiology. It has led to a better understanding of the regulation of vascular tension, intestinal rhythmicity and responses to receptor activation. The realization that there are feedback loops whereby changes in $[Ca^{2+}]_i$ affect membrane potential through calcium-sensitive K^+ , Cl^- and cation channels has been a significant step in our understanding of mechanisms which control smooth muscle tension and contractile behaviour. In addition, it is also apparent that smooth muscle tissues commonly include other cells which represent a specialization of the basic smooth muscle contractile phenotype. The interaction of these SMC types, utilizing the basic intracellular mechanisms of calcium control described, is a major contributor to the unique physiologies of the various smooth muscles.

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