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CALCIUM SPARKLETS IN ARTERIAL SMOOTH MUSCLE

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SUMMARY

1. Voltage-dependent, L-type Ca^{2+} channels (LTCC) play an essential role in arterial smooth muscle contraction and, consequently, the regulation of arterial diameter, tissue perfusion and blood pressure. However, the spatial organization of functional LTCC in arterial myocytes is incompletely understood.
2. Total internal reflection fluorescence and swept-field confocal microscopy revealed that the opening of a single or a cluster of LTCC produces local elevations in $[\text{Ca}^{2+}]_i$ called Ca^{2+} sparklets. In arterial myocytes, Ca^{2+} sparklets are produced by the opening of Cav1.2 channels.
3. The Ca^{2+} sparklet activity is bimodal. In low activity mode, rare stochastic openings of solitary LTCC produce limited Ca^{2+} influx ('low activity Ca^{2+} sparklets'). In contrast, discrete clusters of LTCC associated with protein kinase C α (PKC α) operate in a sustained, high-activity mode resulting in substantial Ca^{2+} influx ('persistent Ca^{2+} sparklets').
4. The Ca^{2+} sparklet activity varies regionally within a myocyte depending on the relative activities of nearby PKC α and opposing protein phosphates 2A and 2B.
5. Low- and high-activity persistent Ca^{2+} sparklets modulate local and global $[\text{Ca}^{2+}]_i$ in arterial smooth muscle, suggesting that this Ca^{2+} signal may play an important role in the regulation of vascular function.

Keywords

arterial myocytes; Ca^{2+} sparklets; myogenic tone; protein kinase C α ; total internal reflection fluorescence microscopy

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INTRODUCTION

Myogenic tone determines arterial diameter and, consequently, tissue perfusion and blood pressure.¹ This vital response is achieved by the coordinated contraction or relaxation of the smooth muscle cells located in the outer layer of the arterial wall. Because arterial myocytes run perpendicular to the longitudinal axis of the arterial wall, their contraction directly translates into changes in arterial diameter.

The mechanisms linking changes in intravascular pressure and myogenic contraction have been the subject of intense investigation. The generally accepted model suggests that increasing luminal pressure causes gradual depolarization of arterial smooth muscle. Elegant work by the laboratories of Brayden^{2–4} and Gill⁵ suggests that the transient receptor potential (TRP) channels TRPC6 and TRPM4 play a crucial role in translating increases in luminal pressure into membrane depolarization. Voltage-gated, dihydropyridine-sensitive L-type Ca^{2+} channels (LTCC) then transduce these changes in membrane potential into changes in global intracellular calcium ($[\text{Ca}^{2+}]_i$).^{6,7} On the basis of these findings, a general model has been proposed where increased intraluminal pressure depolarizes arterial smooth muscle, which increases the open probability of LTCC in the sarcolemma of arterial myocytes. This increases Ca^{2+} influx and, consequently, global $[\text{Ca}^{2+}]_i$. Increasing $[\text{Ca}^{2+}]_i$ initiates a signalling cascade where myosin light chain kinase activates the contractile apparatus, resulting in smooth muscle contraction and arterial constriction.^{7–10} Decreasing intraluminal pressure has the opposite effect.

Here, we present new data and review recent work from our group, as well as others, on the spatial and temporal organization of functional LTCC in arterial myocytes. We propose a new model of Ca^{2+} influx via LTCC that incorporates these new findings and discuss some of the implications of this new model on vascular smooth muscle function under physiological conditions.

L-TYPE CALCIUM CHANNELS IN ARTERIAL MYOCYTES

The LTCC are composed of pore-forming α_1 -subunits and four additional accessory subunits (α_2 , β , γ , δ).¹¹ Of the four α_1 -subunits identified to date, Cav1.2 is the predominant isoform expressed in murine arterial smooth muscle.^{12–14} Although expression of Cav1.3 channels has been reported in canine basilar artery,¹⁵ the functional role of these channels in arterial myocytes is unclear.

Patch-clamp and immunofluorescence experiments indicate that LTCC are expressed broadly throughout the surface sarcolemma of smooth muscle cells.^{16–18} It has been estimated that the typical arterial myocyte expresses approximately 5000–10 000 functional LTCC.¹⁶ Thus, a cell with a surface area of approximately $1000 \mu\text{m}^2$ would, on average, have approximately five to 10 functional LTCC per μm^2 . Interestingly, ventricular myocytes have a similar LTCC density (i.e. three to five channels per μm^2) in their sarcolemma.^{19,20}

Unlike cardiac myocytes, arterial smooth muscle cells do not develop action potentials under physiological conditions. Rather, increases in intravascular pressure cause graded membrane depolarization of arterial myocytes from approximately -60 to -35 mV. Because the

threshold for activation of whole-cell L-type Ca^{2+} currents (I_{Ca}) is approximately -60 mV,^{16,21–23} these channels play a crucial role in matching Ca^{2+} influx to the needs of arterial myocytes. Over the range of physiological membrane potentials experienced by arterial myocytes, the open probability (P_o) of LTCC increases exponentially, changing e-fold in approximately 7–9 mV, from approximately 10^{-7} to 10^{-3} . These observations suggest that although the change in LTCC function is large (approximately 10 000-fold), the absolute activity (P_o) of these channels in the physiological range of membrane potentials is relatively low (maximum P_o approximately 0.001). Thus, a relatively low level of LTCC activity is responsible for the regulation of Ca^{2+} influx during the development of myogenic tone.

On the basis of these findings, a general model was proposed for Ca^{2+} entry via LTCC during the development of myogenic tone. In this model, increases in intravascular pressure depolarize arterial smooth muscle, which increases the P_o of LTCC. The stochastic activation of these channels throughout the sarcolemma results in a global increase in $[\text{Ca}^{2+}]_i$. A central assumption of this model is that all functional LTCC have a similar open probability.

CALCIUM SPARKLETS IN ARTERIAL MYOCYTES

Examination of the spatial and temporal organization of functional LTCC has been difficult owing to technical limitations. The patch-clamp technique, which has been used extensively to investigate the biophysical properties of LTCC in arterial myocytes, cannot be used reliably to determine the spatial distribution of these, or any other, ion channel. To circumvent these limitations, we recently used a swept-field confocal and evanescent field total internal reflection fluorescence (TIRF) microscopy to image Ca^{2+} influx events (' Ca^{2+} sparklets') through individual LTCC in arterial myocytes with high temporal and spatial resolution^{14,17,24,25} (Fig. 1). Using these approaches, in combination with patch-clamp electrophysiology, we tested the generally accepted view that stochastic activation of a broadly distributed population of LTCC is the primary means of Ca^{2+} entry in arterial smooth muscle cells.^{14,17,24,25}

The average area of Ca^{2+} sparklets was small at approximately $0.8 \mu\text{m}^2$ or approximately 0.08% of the surface membrane of a typical arterial myocyte. Importantly, and contrary to expectation, we found that Ca^{2+} sparklet activity was not purely stochastic. Analysis of the spatial distribution of Ca^{2+} sparklets revealed that although the specific location of Ca^{2+} sparklets varied between cells, there was a significant difference between the spatial distribution of Ca^{2+} sparklets and the predicted Poisson distribution in each cell. Rather, Ca^{2+} sparklet activity predominantly occurred in persistent, specific regions of the sarcolemma of arterial myocytes.

As shown in the representative records in Fig. 1, Ca^{2+} sparklet amplitude is variable. Using an approach similar to one implemented by del Castillo and Katz,²⁶ we fitted an all-points histogram generated from representative Ca^{2+} sparklet traces with a multicomponent Gaussian function with a quantal unit of Ca^{2+} elevation of approximately 35 nmol/L. This analysis suggests that Ca^{2+} entry through Ca^{2+} sparklet sites is quantal in nature and that the size of the Ca^{2+} sparklets depends on the number of quanta activated. Furthermore, it

suggests that multiquantal Ca^{2+} sparklets likely arise from overlapping openings of adjacent LTCC.

Multiple lines of evidence indicate that Ca^{2+} sparklets are produced by a Ca^{2+} influx event via sarcolemmal LTCC.^{14,17,24,25} First, Ca^{2+} sparklets are insensitive to the sarcoplasmic reticulum (SR) Ca^{2+} ATPase pump inhibitor thapsigargin. Second, Ca^{2+} sparklets are rapidly eliminated by perfusion with a Ca^{2+} -free solution. Third, the amplitude of quantal Ca^{2+} sparklets varied in size according to the electrochemical gradient for Ca^{2+} across the membrane.

To analyse Ca^{2+} sparklet activity, we developed an analytical approach analogous to that used on single-channel recordings whereby Ca^{2+} sparklet activity is measured as nP_s , where n is the number of quantal levels and P_s is the probability of Ca^{2+} sparklet occurrence.²⁴ Using this analysis, we observed that Ca^{2+} sparklet activity was modal.^{17,24} By default, silent sites had no activity ($nP_s = 0$). Note that a silent Ca^{2+} sparklet site represents a site that is ordinarily inactive, but can be activated by an agonist. Low-activity Ca^{2+} sparklet sites had a relatively low probability of activation (nP_s between 0.0 and 0.2). High-activity ('persistent') Ca^{2+} sparklet sites ($nP_s > 0.2$) were characterized by nearly continuous Ca^{2+} influx. Interestingly, protein kinase C α (PKC α) activity is necessary for persistent Ca^{2+} sparklet activity.^{17,24,25}

MOLECULAR IDENTITY OF CALCIUM CHANNELS UNDERLYING CALCIUM SPARKLETS IN ARTERIAL MYOCYTES

The channels underlying Ca^{2+} sparklets in arterial smooth muscle meet the general criteria used to identify LTCC.²⁷ The Ca^{2+} sparklets are activated by the dihydropyridine agonist Bay-K 8644, are eliminated by the dihydropyridine antagonist nifedipine and have similar voltage dependencies (activity and amplitude) as LTCC. Consistent with this, Ca^{2+} sparklets in tsA-201 cells expressing Cav1.2 channels and PKC α reproduce all the basic features of native Ca^{2+} sparklets.²⁴ Accordingly, Cav1.2 channels are the predominant LTCC expressed in arterial myocytes.^{12–14}

A striking feature of persistent Ca^{2+} sparklets in arterial myocytes is that they are observed at hyperpolarized membrane potentials (e.g. -70 mV), a voltage where the average P_o of Cav1.2 channels is very low (approximately 10^{-6} to 10^{-8}).^{16,21} This raised the intriguing possibility that another LTCC with a voltage dependence of activation more negative than Cav1.2 channels underlies Ca^{2+} sparklets in arterial myocytes. Cav1.3 LTCC meet this condition, because their threshold for activation is 10–20 mV more negative than for Cav1.2 channels.^{28,29} Interestingly, Cav1.3 transcript and protein have been detected in canine basilar artery.¹⁵

Table 1 summarizes the basic features of Cav1.2, Cav1.3 and native Ca^{2+} sparklets. Note that Cav1.3 channels are capable of producing Ca^{2+} sparklets similar (amplitude, duration and modal activity) to those in murine cerebral arterial myocytes and Cav1.2 channels. However, two findings rule out a potential role for Cav1.3 channels in the generation of Ca^{2+} sparklets in these cells. First, arterial myocytes expressing Cav1.2 channels that have a

single point mutation (Thr¹⁰⁶⁶Tyr), which renders them insensitive to inhibition by dihydropyridines without otherwise altering their functional properties,^{28,30} produced dihydropyridine-insensitive Ca²⁺ sparklets.¹⁴ This finding is of particular importance because it eliminates the possibility that Ca²⁺ sparklets are produced by a dihydropyridine-insensitive, non-Cav1.X channel (e.g. TRP channel) in arterial myocytes. Second, Cav1.3 transcript and protein were not detected in mouse cerebral arterial myocytes.¹⁴ These observations provided the first direct demonstration that Cav1.2 channels underlie persistent Ca²⁺ sparklets in mouse arterial myocytes.

Note, however, that expression of PKC α is required for persistent Ca²⁺ sparklet activity in tsA-201 cells expressing Cav1.2 or Cav1.3 and arterial myocytes.^{14,24} Taken together, these observations indicate that persistent Ca²⁺ sparklet activity is a fundamental feature of LTCC in association with PKC α . Furthermore, given the ubiquity of Cav1.2, Cav1.3 and PKC α expression, our data suggest the intriguing possibility that persistent Ca²⁺ sparklets may be a general mechanism underlying steady state Ca²⁺ entry in numerous excitable cells.

The importance of Cav1.2 with respect to I_{Ca} and myogenic tone has been examined recently in small mesenteric arteries.¹³ Although the authors of that study did not measure Ca²⁺ sparklets and arterial wall [Ca²⁺]_i, they found that Cav1.2 channels play a predominant role in I_{Ca} and myogenic tone in small mesenteric arteries. Together with the observations reviewed above, these data suggest that Cav1.2 channels are crucial in the regulation of Ca²⁺ influx in smooth muscle from multiple arterial beds.

Even though Cav1.3 channels do not contribute to Ca²⁺ sparklet activity and I_{Ca} in mouse cerebral¹⁴ and mesenteric¹³ artery smooth muscle, these channels could, at least in principle, modulate Ca²⁺ influx in arterial smooth muscle from other species. Indeed, Cav1.3 transcript and protein has been detected in canine basilar artery.¹⁵ Future studies should examine Ca²⁺ sparklets in smooth muscle cells from arteries of different species to assess the contribution of Cav1.3 channels to these events.

The Cav1.2 and Cav1.3 channels are expressed in multiple excitable cells, including neuronal, cardiac, smooth muscle, endocrine and outer hair cells.^{15,31–35} Thus, the observation that Cav1.2 and Cav1.3 channels in association with PKC could produce persistent Ca²⁺ sparklet activity has broad physiological implications. For example, recent work from our laboratory²⁵ indicates that persistent Cav1.2 sparklets regulate local and global [Ca²⁺]_i in murine arterial smooth muscle. Future studies, should examine if, as in arterial myocytes, persistent Ca²⁺ sparklets modulate physiological processes in which Cav1.2 and/or Cav1.3 channels and PKC have been shown to be involved. These processes include synaptic plasticity,^{36,37} gene expression and excitability,^{38,39} hormone release,⁴⁰ cardiac excitation–contraction coupling⁴¹ and cardiac pacemaker activity.⁴²

MECHANISMS FOR REGIONAL VARIATIONS IN CALCIUM SPARKLETS

Multiple studies have suggested that LTCC function is modulated by PKC^{43–45} by diverse mechanisms. For example, PKC can enhance LTCC activity via direct phosphorylation of the pore-forming Cav1.2 subunit of the channel.⁴⁶ Furthermore, PKC activation inhibits

voltage-gated, delayed-rectifier K^+ channels⁴⁷ and large-conductance, Ca^{2+} -activated K^+ channels,^{48–50} which would lead to arterial smooth muscle depolarization. As demonstrated by Amberg *et al.*,²⁵ membrane depolarization increases low- and high-activity persistent Ca^{2+} sparklets in arterial myocytes. Thus, PKC could increase Ca^{2+} sparklet activity by voltage-dependent and voltage-independent mechanisms. Consistent with this, two recent studies have suggested that PKC activity plays an important role in the regulation of membrane potential, Ca^{2+} influx via LTCC and the development of myogenic tone in coronary⁴³ and cerebral arteries.³

A striking observation using TIRF and swept-field confocal microscopy is that PKC α -dependent persistent Ca^{2+} sparklet activity in arterial myocytes is confined to specific foci within the sarcolemma of these cells. As noted above, electrophysiological and immunofluorescence data suggest that Cav1.2 channels are broadly expressed throughout the sarcolemma of arterial myocytes.^{17,18} Thus, what are the mechanisms underlying regional variations in Ca^{2+} sparklet activity?

The diagram in Fig. 2 summarizes our proposed model for heterogeneous Ca^{2+} sparklet function in arterial myocytes. First, note that PKC α activity is required for persistent Ca^{2+} sparklet activity. Thus, it is reasonable to speculate that membrane targeting of PKC α to specific regions of the surface membrane where this kinase could phosphorylate nearby LTCC promotes persistent Ca^{2+} sparklet activity. The observation that PKC expression is restricted to specific regions at or near the sarcolemma of arterial myocytes supports this view.^{17,51} The effects of PKC α on Ca^{2+} sparklet activity are opposed by the Ca^{2+} -sensitive protein phosphatase calcineurin (also known as protein phosphatase 2B). Inhibition of calcineurin increases persistent Ca^{2+} sparklet activity by recruiting silent sites and by increasing the activity of previously active sites. The effects of calcineurin inhibition on Ca^{2+} sparklets requires the function of PKC α . Thus, calcineurin and PKC α have opposing actions on Ca^{2+} sparklets. Furthermore, these observations suggest that the level of Ca^{2+} sparklet activity varies regionally depending on the relative activities of PKC α and calcineurin.

The model in Fig. 2 proposes a feedback mechanism that explains why the LTCC agonist Bay-K 8644 increases persistent Ca^{2+} sparklet activity in arterial myocytes and tsA-201 cells expressing Cav1.2 channels and PKC α , but not in tsA-201 cells expressing only Cav1.2 channels. In this model, application of Bay-K 8644 (or any other Ca^{2+} channel agonist) to arterial myocytes causes an increase in Ca^{2+} influx (i.e. by increasing the mean open time and/or open probability of LTCC), which activates nearby Ca^{2+} -sensitive PKC α . Once activated, this kinase can induce LTCC to operate in a persistent gating mode, causing further Ca^{2+} influx, which could presumably maintain high PKC α activity and hence persistent Ca^{2+} sparklet activity. Because PKC expression in arterial myocytes is punctate,^{17,51} PKC α and persistent Ca^{2+} sparklet activity does not propagate throughout the cell. As noted above, protein phosphatases or negative regulators of the $G_{q/11}$ signalling pathway (i.e. regulators of G-protein signalling (RGS) proteins) could provide additional negative control to this system. An interesting implication of this model is that although Ca^{2+} sparklets can be evoked by voltage or pharmacological means wherever Cav1.2 channels are expressed, persistent Ca^{2+} channel activity will only occur in regions of the cell membrane where both

Cav1.2 and PKC α are expressed. Future experiments should investigate the spatial and temporal relationship between Ca²⁺ sparklets and PKC α in arterial myocytes.

A MODEL FOR CALCIUM SPARKLET-CONTROLLED CALCIUM INFLUX

Recent work has examined the physiological role of Ca²⁺ sparklets in arterial myocytes. Amberg *et al.*²⁵ demonstrated that Ca²⁺ sparklet activity regulates local and global [Ca²⁺]_i in a voltage-dependent manner. Interestingly, it was observed that although the amount of Ca²⁺ entry associated with a Ca²⁺ sparklet from a high *nP_s* site is larger than that from a low *nP_s* site at -70 and -40 mV, membrane depolarization increases Ca²⁺ influx via Ca²⁺ sparklets from both low and high *nP_s* sites. Because the driving force for Ca²⁺ entry decreases with depolarization, this voltage-evoked increase in Ca²⁺ entry is likely due to an increase in the activity of LTCC. The greater Ca²⁺ influx associated with high *nP_s* sites at -70 and -40 mV results from longer event duration times than those of low *nP_s* sites. Membrane depolarization increases Ca²⁺ influx by increasing the duration of low and high *nP_s* Ca²⁺ sparklets.

On average, it was calculated that persistent Ca²⁺ sparklet activity contributes to approximately 50% of the dihydropyridine-sensitive Ca²⁺ influx required for maintenance of steady state [Ca²⁺]_i under physiological conditions (i.e. 2 mmol/L Ca²⁺ and -40 mV).²⁵ On the basis of these data, a new model for steady state Ca²⁺ influx in arterial myocytes has been proposed. In this model, membrane depolarization increases Ca²⁺ influx at least in part by increasing the duration of low- and high-activity Ca²⁺ sparklets. Thus, by regulating persistent Ca²⁺ sparklet, PKC α plays an important role in the regulation of local and global [Ca²⁺]_i in arterial myocytes.

CONCLUSIONS

L-Type Ca²⁺ channels play a crucial role in the regulation of gene expression, excitability and contraction in arterial myocytes. The application of novel imaging approaches, including TIRF and swept-field confocal microscopy, has revealed unexpected aspects of LTCC function in arterial myocytes. Particularly interesting is the observation of single or small clusters of Ca²⁺ channels operating in a high probability mode that creates sites of nearly continual Ca²⁺ influx (persistent Ca²⁺ sparklets). Persistent Ca²⁺ sparklets contributes to steady state local and global calcium in arterial myocytes under physiological conditions. A growing body of evidence now suggests that persistent Ca²⁺ sparklet activity is a fundamental property of LTCC when associated with PKC. The activity of persistent Ca²⁺ sparklets varies regionally within the cell depending on the relative activity of PKC α and opposing protein phosphatases. Thus, the available evidence suggests that persistent Ca²⁺ sparklets could be a dynamic Ca²⁺ signal capable of regulating multiple cellular processes during physiological, and likely pathophysiological, conditions. Future studies should examine these important issues.

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Fig. 1.

Ca²⁺ sparklets in arterial myocytes. (a) Total internal reflection fluorescence (TIRF) and swept-field confocal images of Ca²⁺ sparklets from representative arterial myocytes (MF Navedo *et al.*, unpubl. data, 2007). Images were collected while cells were held at the membrane potential of -70 mV with 20 mmol/L external Ca²⁺. The traces to the right of each image show the time-course of $[Ca^{2+}]_i$ in the sites marked in the images. (b) Representative $[Ca^{2+}]_i$ records from Ca²⁺ sparklet sites with one (top), two and three (bottom) quanta events. Dotted lines mark the quantal levels. (c) All-points histogram generated from 25 representative Ca²⁺ sparklet traces. Solid lines represent a fit of the data using the multi-Gaussian function with a quantal unit of Ca²⁺ release of 34 nmol/L.²⁴

Fig. 2.

Proposed mechanism by which PKCα regulates Ca²⁺ sparklet activity in arterial myocytes. Binding of angiotensin II to AT₁ receptors activates heterotrimeric G_{q/11}-proteins. Activation of G_{q/11} increases phospholipase C (PLC) activity and diacylglycerol (DAG) production, which, in turn, activates protein kinase Ca (PKCα). G_{q/11} activity can negatively regulated by regulators of G-protein signalling (RGS) 2. Protein kinase Ca, and presumably calcineurin, are targeted to specific regions of the sarcolemma. Those sites in which L-type Ca²⁺ channels (LTCC) are associated with the PKCα/calcineurin complex can develop persistent Ca²⁺ sparklet activity. Interestingly, LTCC agonists, such as Bay-K 8644, and activators of the PKCα signalling pathway could increase Ca²⁺ influx by promoting additional clusters of LTCC to operate in persistent Ca²⁺ influx mode and by increasing the probability of solitary LTCC. PP2B, protein phosphatase 2B.

Table 1Properties of Ca²⁺ sparklets

Parameter	Calcium sparklets			
	Rat arterial myocyte	Mouse arterial myocyte	Cav1.2 channels (tsA-201 cells)	Cav1.3 channels (tsA-201 cells)
Modal gating	Yes [†]	Yes [‡]	Yes [‡]	Yes [¶]
Activity (nP_s)	Low $nP_s = 0.07$	Low $nP_s = 0.08$	Low $nP_s = 0.05$	Low $nP_s = 0.07$
	High $nP_s = 1.25^{\dagger}$	High $nP_s = 0.80^{\ddagger}$	High $nP_s = 0.79^{\ddagger}$	High $nP_s = 0.46^{\parallel}$
Voltage dependency	Yes [†]	ND	Yes [‡]	Yes [¶]
Inhibition by nifedipine (10 μ mol/L)	Yes [†]	Yes [‡]	Yes [‡]	Partial [¶]
Activation by Bay-K 8644 (500 nmol/L)	Yes [†]	Yes [‡]	Yes [‡]	Yes [¶]
Quantal level (nmol/L)	38 [†]	34 ^{‡§}	38 [‡]	34 [¶]
Signal mass †† (fC)	Low $nP_s = 122$	Low $nP_s = 292$	ND	Low $nP_s = 273$
	High $nP_s = 270$	High $nP_s = 452$		High $nP_s = 917^{\parallel}$
Duration (msec)	Low nP_s	Low nP_s	ND	Low nP_s
	$\tau = 23$	$\tau = 41$		$\tau = 31$
	High nP_s	High nP_s		High nP_s
	$\tau_{\text{fast}} = 42$	$\tau_{\text{fast}} = 35$		$\tau_{\text{fast}} = 37$
	$\tau_{\text{slow}} = 104^{\S \dagger\dagger}$	$\tau_{\text{slow}} = 200^{\S}$		$\tau_{\text{slow}} = 126^{\parallel}$

Data are from [†]Navedo *et al.*,¹⁷ [‡]Navedo *et al.*,²⁴ [§]Amberg *et al.*²⁵ and [¶]Navedo *et al.*¹⁴^{††}The signal mass and duration of the Ca²⁺ sparklets were recorded at -40 mV and 2 mmol/L extracellular Ca²⁺, as described previously.²⁵

ND, not determined.