The technique of simultaneous recording calcium transients and spontaneous transient outward currents in arterial smooth muscle cells

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Abstract: Laser scanning confocal microscopy (LSCM) and whole-cell perforated patch-clamp techniques were combined to study simultaneously the changes of intracellular signal molecules and membrane currents. Intracellular calcium transients and spontaneous transient outward currents (STOCs) were recorded simultaneously in freshly isolated mouse cerebral artery smooth muscle cells. The cells loaded with fluo-4/AM were scanned with the confocal line-scan mode. Triggering voltage pulses derived from an EPC-10 patch clamp amplifier triggered the confocal line scan. The results showed that STOCs and intracellular calcium transients could be simultaneously recorded in the same cell. This technique will be useful in studies of diseases caused by impairments of intracellular Ca$^{2+}$ signaling and related ionic channel activities, or vice versa.

Key words: laser scanning confocal microscopy; patch clamp technique; spontaneous transient outward current; Ca$^{2+}$ transients

An increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) is a key signaling in the initiation and maintenance of numerous cellular responses. The discovery of local Ca$^{2+}$ transients (“Ca$^{2+}$ sparks”) radically altered the view that a spatially homogeneous elevation in cytoplasmic Ca$^{2+}$ is necessary for effective Ca$^{2+}$ signaling. The Ca$^{2+}$ sparks are caused by the opening of ryanodine-sensitive Ca$^{2+}$-release (RyR) channels in the sarcoplasmic reticulum (SR). Single Ca$^{2+}$ spark causes a very high (10-100 μmol/L) local (~1% of the cell volume) increase in [Ca$^{2+}$] while increasing the global [Ca$^{2+}$] only by <2 nmol/L. A Ca$^{2+}$ spark, by virtue of its high local [Ca$^{2+}$] elevation, has the potential to modulate Ca$^{2+}$-dependent processes that are not responsive to global increases in [Ca$^{2+}$]. It has been demonstrated that Ca$^{2+}$ sparks activate neighboring clusters of large-conductance Ca$^{2+}$-activated K$^+$ channels (BK channels) to generate spontaneous transient outward currents (STOCs) in virtually all of smooth muscles. The enhanced BK channels...
channel activity causes membrane hyperpolarization and reduces Ca\(^{2+}\) influx by decreasing the open-state probability of dihydropyridine-sensitive L-type calcium channels (voltage-dependent Ca\(^{2+}\) channels)[4].

Patch-clamp, developed by Erwin Neher and Bert Sakmann[5], is a versatile and powerful tool to measure directly ion currents across biological membranes. More recently, laser scanning confocal microscopy (LSCM) has become an advanced technology for molecular and cellular biology. Based on the fluorescent microscopy, the laser scanning equipment and digital image analysis, confocal microscopy has become a primary method in many laboratories, and it has been applied to collect the fluorescence labeled images of the tissues and cells, analyze the changes of ions at subcellular level, and visualize the correlations among physiology, morphology and the cellular mobility in combination with electrophysiology. Some researchers have tried to study the relationship between intracellular Ca\(^{2+}\) changes and membrane currents by applying LSCM and patch-clamp, but most of these studies were independently and not real time. The aim of this study was to construct an experimental technology platform by combining LSCM and patch-clamp, therefore allowing the studies of the relationship between intracellular Ca\(^{2+}\) and STOCs changes simultaneously.

1 MATERIALS AND METHODS

1.1 Preparation of mouse brain artery smooth muscle cells

Animal procedures used in the present work were approved by the Animal Care and Use Committee at Luzhou Medical College. Mice (20±2 g) of either sex were euthanized, and the brains were removed and maintained in ice-cold HEPES-buffered physiological salt solution (PSS). Posterior cerebral, cerebellar, and middle cerebral arteries were harvested and used for cell isolation. The arteries were digested in a low Ca\(^{2+}\) (0.1 mM/L) PSS containing 0.8 mg/mL papain, 0.2 mg/mL dithoerythritol (DTE) and 1 mg/mL bovine serum albumin (BSA) for 9 min at 37 °C, and further digested in a low Ca\(^{2+}\) (0.1 mM/L) PSS containing 1 mg/mL collagenase Type II, 0.8 mg/mL collagenase Type F, 1 mg/mL dithoerythritol (DTT) and 1 mg/mL BSA for 9 min at 37 °C. Then, the arteries were subsequently washed in ice-cold low Ca\(^{2+}\) PSS and triturated using a polished glass pasteur pipette to yield single smooth muscle cells. Cells were allowed to adhere to the bottom of a chamber for 10 min at 4 °C prior to experimentation.

1.2 Patch-clamp recording

The chamber with cells was mounted on the stage of a Leica inverted microscope. Amphotericin-perforated configuration of patch-clamp technique was used to measure STOCs. Glass pipettes were back-filled with a pipette solution and had a resistance of 2-4 MΩ. EPC-10 patch clamp amplifier and PULSE software (HEKA Electronic, Lambrecht, Germany) were used for the current recordings. Currents were filtered at 1 kHz and digitized at 10 kHz. Series resistance was compensated; leak current and capacitive transients were digitally subtracted. Access resistances of the perforated-patch configuration were ≤10 MΩ following series resistance compensation.

Amplitude and frequency of STOCs were detected and analyzed by MiniAnalysis program (Synaptosoft Software, Leonia, NJ), and the events were then checked by visual inspection to eliminate anomalies such as multiple events overlapping in time or excessively noisy traces.

1.3 Imaging and measurement of Ca\(^{2+}\) sparks

The cells were incubated in HEPES-buffered PSS containing calcium indicator fluo-4/AM (5 μmol/L) for 25 min at room temperature, followed by a 30 min wash. Fluo-4 fluorescence was imaged with a Leica TCS SP2 scanning confocal microscope (Leica Microsystems, Germany) equipped with an inverted Leica DMIRE2 microscope and a PL APO 63x oil immersion objective (numerical aperture=1.3). The 488 nm line of a multiline argon laser provided fluorescence excitation by illuminating with 488 nm light and collecting emitted light >500 nm. Images were collected at 8-bit with 1 024×1 024 pixels per frame. Sparks images were acquired in the line scan mode, which is described in detail elsewhere[6].

For Ca\(^{2+}\) sparks, the scan line was set to cross the subsurface just beneath the patch site (Fig. 1). Each line scan image consisted of 2 000 scans obtained at 5 s, and each line comprised 512 pixels (spaced at 0.028 μm intervals). Simultaneous currents and fluorescence measurements were synchronized with an optical trigger control panel that was connected to the confocal system. External trigger signal from EPC-10 patch clamp amplifier was delivered to the confocal system via trigger control panel using BNC cable. Simultaneous recording protocols were set in Pulse software with stimulating voltage (3 V) and length (300 ns). In the line scan mode, each cell was imaged for at least 5 s under each condition. The site of a Ca\(^{2+}\) spark was determined as the centre of the spark at the time of its initiation. Baseline fluorescence (F0) was background fluorescence intensity, and Ca\(^{2+}\) sparks were defined as local
fractional fluorescence \((F/F_0)\) increases greater than 1.2\(^{[7]}\). Ca\(^{2+}\) sparks were detected and analyzed with the use of Custom software (written with Interactive Data Language 6.3; Research Systems Inc.).

1.4 Solutions and reagents

Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich. Fluo-4/AM was purchased from Invitrogen Molecular Probes. PSS contained (in mmol/L): 130 NaCl, 5.4 KCl, 1 MgSO\(_4\), 10 HEPES, 1.8 CaCl\(_2\), and 10 glucose, pH adjusted to 7.4 with NaOH. The extracellular solution contained (in mmol/L): 137 NaCl, 5.9 KCl, 1.2 MgCl\(_2\), 1.8 CaCl\(_2\), 10 HEPES and 14 Glucose, pH 7.4 (NaOH). The pipette solution contained (in mmol/L): 110 K-aspartate, 30 KCl, 10 NaCl, 1 MgCl\(_2\), 10 HEPES, 0.05 EGTA and 200 \(\mu\)g/mL amphotericin B, pH 7.2 (KOH).

1.5 Data analysis and statistics

All values were given as mean±SEM. Data were statistically compared using Student’s \(t\)-test. \(P<0.05\) is considered as statistically significant.

2 RESULTS

2.1 Localization of the membrane patch and the line scan image

Patch clamp experiments were carried out on the cells that exhibited morphological features characteristic of vascular smooth muscle with uniform fluo-4/AM loading. As shown in Fig. 1, the \(x\) and \(y\) coordinates of the pipette tip were located from the transmitted light image. The \(z\) coordinate of the pipette tip was determined by observing

![Confocal microscopy](image1.png)

![Transmission microscopy](image2.png)

![Overlay](image3.png)

![Line scan image and the fractional fluorescence change \((F/F_0)\) of sparks](image4.png)

Fig. 1. Images of the Ca\(^{2+}\) sparks recorded in freshly isolated mouse cerebral artery myocyte. A-C: Fluorescence image of whole-cell patch clamping in freshly isolated mouse cerebral artery myocyte loaded with fluo-4/AM. Red line represents the position of line scan recorded (Scale bar, 5 \(\mu\)m). D: Line scan image and the fractional fluorescence change \((F/F_0)\) of sparks. The horizontal dimension represents 5 s, and the vertical scale bar represents 5 \(\mu\)m. Fluorescence signals \((F)\) of each confocal image were normalized in terms of \(F/F_0\), where \(F\) is the fluorescence intensity at any given time point, and \(F_0\) is the baseline fluorescence in a region of the image without Ca\(^{2+}\) sparks.
changes in fluo-4 fluorescence at the x position in line scan images during changes in the focal plane. When the line scan intersected the pipette, the resultant image had a focal decrease in fluorescence at x, since the pipette did not contain fluo-4/AM. For each experiment, “xyr” frame scan mode was first used to locate the cell, “xr” line scan mode was then applied to position the scan line along the longitudinal axis of the cell (avoiding nuclei). The horizontal dimension represented scan time and vertical dimension represented line length. An example of line scan image of Ca²⁺ sparks was shown in Fig. 1D.

2.2 Simultaneous STOCs and Ca²⁺ sparks recordings
STOCs were measured, as previously described[8], at steady-state holding potentials between -50 mV and 0 mV using the amphotericin B perforated-patch configuration. As shown in Fig. 2, the shape of single STOC was like asymmetrical bell with rapid upstroke and slow descent. In myocytes voltage clamped to a physiological membrane potential of -40 mV, the observed average amplitude and frequency of STOCs were (8.82±0.95) pA and (5.57±0.77) Hz (n=15 cells). Under these conditions, the average rise time and decay time of STOCs were (11.11±0.82) ms and (15.93±1.54) ms, respectively. At potentials positive to -20 mV, it was often observed that individual STOCs were overlapping and formed complex STOCs with different shapes. Iberiotoxin (IbTX, 200 nmol/L), a specific BKCa channel blocker, completely blocked STOC activity at a membrane potential of -40 mV (n = 5 cells, P<0.05), indicating that STOCs in mouse cerebral artery

Fig. 2. Representative STOCs recorded at a test potential of -40 mV and IbTX abolished STOCs in mouse cerebral artery myocytes. Insert: expanded scale showing amplified STOCs. IbTX (200 nmol/L) completely blocked STOCs within 20 min.

Fig. 3. Simultaneous measurements of Ca²⁺ sparks and STOCs in the same cell of mouse cerebral artery at different test potentials. The data illustrated include line-scan image (top panel), the fractional fluorescence (F/F₀) changes for this panel and simultaneously recorded STOCs at a Vm of -10 mV (left) and at a Vm of -40 mV (right).
myocytes were generated by opening of BK<sub>Ca</sub> channels.

To explore the relationship between Ca<sup>2+</sup> sparks and BK<sub>Ca</sub> channel openings in mouse cerebral artery smooth muscle, patch-clamp was combined with LSCM to measure simultaneously Ca<sup>2+</sup> sparks and STOCs attributable to BK<sub>Ca</sub> channel activation. As shown in Fig. 3, Ca<sup>2+</sup> sparks and STOCs were successfully simultaneously recorded by the patch clamp and confocal simultaneous recording system. Each line-scan image is a plot of fluorescence along a scanned line (on the ordinate) versus time (on the abscissa). The dynamical changing of single normalized Ca<sup>2+</sup> sparks was similar to that of STOCs. On average, the peak [Ca<sup>2+</sup>]i amplitude of these local Ca<sup>2+</sup> transients (Ca<sup>2+</sup> sparks) (measured as F/F<sub>0</sub>) was 1.52±0.15 at -40 mV. The duration of the local Ca<sup>2+</sup> transients at half-maximal amplitude was (44.52±2.79) ms and the average rise time and decay time of sparks were (11.75±0.86) ms and (23.40±4.18) ms, respectively (n=20 sparks).

Consistent with others’ reports, it was observed that both STOCs and sparks were voltage-dependent. Figure 3 illustrated simultaneous recordings of Ca<sup>2+</sup> sparks and STOCs in mouse cerebral artery myocytes at different test potentials. Membrane potential depolarization (from -40 mV to -10 mV) significantly increased Ca<sup>2+</sup> sparks activity (F/F<sub>0</sub>=2.74±0.20, at -10 mV, P<0.05, n=7 cells) in the same cell. Similarly, STOCs amplitude and frequency also increased with increasing membrane potential. Compared with the currents recorded at -40 mV (above shown), the average amplitude [(21.94±4.16) pA] and frequency [(9.38±1.70) Hz] of STOCs were significantly augmented at -10 mV (P<0.05, n=15 cells). Nevertheless, it was found that excessive laser exposure or higher clamp voltage would induce a significant global [Ca<sup>2+</sup>] increase. Although the majority of Ca<sup>2+</sup> sparks (82%±5%) was associated with BK<sub>Ca</sub> channel currents, a small number of these intracellular Ca<sup>2+</sup> releases events were uncoupled with STOCs (Fig. 4).

### 3 DISCUSSION

In this study, we have designed and implemented a novel experimental setup, which combines LSCM with whole-cell patch-clamp techniques for simultaneous recordings of membrane currents and intracellular Ca<sup>2+</sup> imaging in cerebral artery smooth muscle cell. In nearly 80% of the cells Ca<sup>2+</sup> sparks and STOCs can be simultaneously recorded. Recently, more and more researchers have trying to study the relationship between intracellular signal molecules and membrane ion channels. Since the simultaneous recording of currents and imaging is not easy, the technique is only available in few laboratories. Compared with the loose-patch method (combining loose-seal patch-clamp with confocal microscopic Ca<sup>2+</sup> imaging) setup by the National Laboratory of Biomembrane and Membrane Biotechnology in Peking University, we try to realize the simultaneous recording at the traditional whole-cell patch configuration, to avoid the possible impact of leak current through the greatly reduced seal with loose-patch method on currents recording.

Our data showed that the basic properties of Ca<sup>2+</sup> sparks and STOCs in freshly isolated mouse cerebral artery smooth muscle cells were similar to those measured in a number of other smooth muscle preparations. As illustrated in Fig. 3, Ca<sup>2+</sup> sparks and STOCs exhibited similar temporal characteristics. Each spark was associated with a STOC in this sequence, with both spark and STOC rising simultaneously. Consistent with others’ report, it was also observed that the activities of STOCs and sparks significantly increased with membrane potential depolarization (from -40 mV to -10 mV). However, it could be observed that Ca<sup>2+</sup> fluorescence intensity increased greatly (global [Ca<sup>2+</sup>] increased) in myocytes exposed to excessive laser or voltage clamped to a more positive membrane potential. So specimen preparation is the most important aspect of collecting meaningful results.

In this study, it was also observed that STOCs and sparks...
were uncoupled occasionally. As an example in Fig. 4, not all STOCs were associated with sparks. This result is consistent with the idea that line scan has its limitations. Since whole-cell patch recording registers all STOCs in the cell, whereas the image captures only a portion of the events in the cell. Traditional confocal line scanning achieves excellent temporal spark resolution but at the cost of spatial information in the perpendicular dimension\[^{[3]}\]. However, as image scans are too slow to detect Ca\(^{2+}\) sparks, line scans are usually used to detect dynamical changes in intracellular Ca\(^{2+}\). In addition, the duration of Ca\(^{2+}\) spark is so short and its spatial scale so small that fast line scans could better determine the spatial and temporal characteristics of Ca\(^{2+}\) sparks.

At present, patch-clamp and LSCM technology have been widely used in the study of membrane ion channel function and cell morphology. The patch-clamp technique provides a powerful method for ion channel studies, rather than a real-time detection tool to study the dynamic changes of intracellular ion and quantitative analysis of ion concentration, whereas LSCM can make up for this deficiency. This knowledge is expected to promote a better understanding of the relationship between internal signaling changes and ion channel function by combing these two independent techniques. The patch-clamp and LSCM synchronous real-time control system is becoming a new method in studying the morphology and function of ion channels and can be useful for various biological studies in other fields.

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