Ca\textsubscript{\textit{v},1.3} channels produce persistent calcium sparklets, but Ca\textsubscript{\textit{v},1.2} channels are responsible for sparklets in mouse arterial smooth muscle

Manuel F. Navedo,\textsuperscript{1} Gregory C. Amberg,\textsuperscript{1} Ruth E. Westenbroek,\textsuperscript{2} Martina J. Sinnegger-Brauns,\textsuperscript{3} William A. Catterall,\textsuperscript{2} Jörg Striessnig,\textsuperscript{3} and Luis F. Santana\textsuperscript{1}

\textsuperscript{1}Departments of Physiology and Biophysics and \textsuperscript{2}Pharmacology, University of Washington, Seattle, Washington; and \textsuperscript{3}Department of Pharmacology and Toxicology, Institute of Pharmacy, University of Innsbruck, Innsbruck, Austria

Submitted 12 April 2007; accepted in final form 21 May 2007

Ca\textsubscript{\textit{v},1.3} channels produce persistent calcium sparklets, but Ca\textsubscript{\textit{v},1.2} channels are responsible for sparklets in mouse arterial smooth muscle. Am J Physiol Heart Circ Physiol 293: H1359–H1370, 2007. First published May 25, 2007; doi:10.1152/ajpheart.00450.2007.—Ca\textsuperscript{2+} channels operating in a high-activity mode are also observed. These “high-activity, persistent Ca\textsubscript{2+} sparklets,” which are protein kinase C (PKC) dependent, arise from high L-type Ca\textsubscript{2+} channel activity and result in substantial Ca\textsubscript{2+} influx. Subsequently, we demonstrated that low- and high-activity Ca\textsubscript{2+} sparklets modulate local and global Ca\textsubscript{2+} in arterial smooth muscle (2).

The channels underlying Ca\textsubscript{2+} sparklets in arterial smooth muscle meet the general criteria used to identify L-type Ca\textsubscript{2+} channels (22). Ca\textsubscript{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) to L-type Ca\textsubscript{2+} channels. Consistent with this, voltage-dependent Ca\textsubscript{1,2} L-type Ca\textsubscript{2+} channels are expressed in arterial smooth muscle (26). We recently suggested that Ca\textsubscript{2+} sparklets produced by heterologously expressed Ca\textsubscript{1,2} channels resembled native Ca\textsubscript{2+} sparklets in arterial myocytes (35). Thus, according to an indirect comparative approach, Ca\textsubscript{1,2} channels could, at least in principle, underlie Ca\textsubscript{2+} sparklets in arterial myocytes. At present, however, whether or not Ca\textsubscript{1,2} channels underlie Ca\textsubscript{2+} sparklets in arterial myocytes remains to be tested directly.

A striking feature of persistent Ca\textsubscript{2+} sparklets in arterial myocytes is that they are observed at hyperpolarized membrane potentials (e.g., −70 mV), a voltage where the open probability (\textit{P}\textsubscript{o}) of L-type Ca\textsubscript{2+} channels is very low (≈10\textsuperscript{−6}–10\textsuperscript{−8}) (41, 43). This raises the intriguing possibility that another L-type Ca\textsubscript{2+} channel with a voltage dependence of activation more negative than Ca\textsubscript{1,2} channels underlies Ca\textsubscript{2+} sparklets in arterial myocytes. Ca\textsubscript{1,3} L-type Ca\textsubscript{2+} channels meet this condition, because their threshold for activation is 10–20 mV more negative than that of Ca\textsubscript{1,2} channels (27, 52). Furthermore, Ca\textsubscript{1,3} transcript and protein have been detected in canine basilar artery (37). These observations raise four important questions: 1) What are the biophysical properties of single Ca\textsubscript{1,3} channels? 2) Are Ca\textsubscript{1,3} channels capable of producing Ca\textsubscript{2+} sparklet activity? 3) If so, are Ca\textsubscript{1,3} sparklets similar to Ca\textsubscript{2+} sparklets in mouse cerebral arterial myocytes? 4) Are Ca\textsubscript{1,3} channels expressed in mouse cerebral arterial myocytes, and do they contribute to Ca\textsubscript{2+} influx in these cells?

The goal of this study was to address these four fundamental questions. Beyond the obvious relevance of determining whether Ca\textsubscript{1,3} channels contribute to Ca\textsubscript{2+} influx in mouse
arterial myocytes (question 4), by addressing questions 1, 2, and 3 we provide what to our knowledge is the first single-channel analysis of heterologously expressed Ca$_{1.3}$ channels and their ability to transport Ca$^{2+}$. Given the importance of Ca$_{1.3}$ channels in nerve (5) and muscle (37, 55), addressing these issues would impact multiple fields.

**METHODS**

*Isolation of arterial myocytes.* We used wild-type mice (C57BL/6J) and mice genetically engineered to express Ca$_{1.2}$ channels that are insensitive to dihydropyridines (DHP$^{-/-}$; C57BL/6J background) (44). Mice were euthanized with a lethal dose of sodium pentobarbital (250 mg/kg ip) as approved by the University of Washington Institutional Animal Care and Use Committee. Myocytes were dissociated from cerebral arteries by using standard enzymatic techniques described elsewhere (4). After dissociation, cells were maintained in a nominally Ca$^{2+}$-free Ringer solution until they were used. Thapsigargin (1 mM) was included in all solutions used to record Ca$^{2+}$ sparks to eliminate Ca$^{2+}$. Resting potential to 0 mV. The pipette solution contained (in mM) 110 KCl, 2 MgCl$_2$, 10 HEPES and 10 glucose adjusted to pH 7.3 with KOH to set the membrane potential (E$_{test}$) of 0 mV as previously described (34, 35). Briefly, F is fluorescence, concentration units by using the “Fmax” equation (32)

\[
[Ca^{2+}] = K_d \frac{F/F_{max} - 1/R_f}{1 - F/F_{max}}
\]

as described in detail previously (13, 35). Briefly, F is fluorescence, F$_{max}$ is the fluorescence intensity of fluo-4/5F or rhod-2 in the presence of saturating free Ca$^{2+}$, K$_d$ is the dissociation constant (fluor-5F = 1,280 nM; fluo-4 = 800 nM; rhod-2 = 700 nM), and R$_f$ is the indicator’s F$_{max}$/F$_{min}$ ratio (fluor-5F = 286; fluo-4 = 150; rhod-2 = 125). F$_{max}$ is the fluorescence intensity of the indicator in a solution where the Ca$^{2+}$ concentration is 0. K$_d$ and R$_f$ values were determined in vitro by using standard methods and are similar to those reported by others (49). F$_{max}$ was determined at the end of each experiment by exposing cells or arteries to the Ca$^{2+}$ ionophore ionomycin (10 µM) and 20 mM external Ca$^{2+}$.

**Total internal reflection fluorescence microscopy.** Ca$^{2+}$ sparks were recorded at ~70 mV as previously described (34, 35). Briefly, we used a through-the-lens total internal reflection fluorescence (TIRF) microscope built around an inverted Olympus IX-70 microscope equipped with an Olympus PlanApo (×60, numerical aperture = 1.45) oil-immersion lens and an Andor iXON charge-coupled device camera (South Windsor, CT). To monitor [Ca$^{2+}$], cells were loaded with the calcium indicator fluo-5F or rhod-2. Rhod-2 was used in all experiments in which the EGFP was expressed. Excitation of fluo-5F or rhod-2 was achieved with the 488 or 568 nm line of an argon or krypton laser, respectively (Dynamic Lasers). Images were acquired at 100–300 Hz. As before (34, 35), we determined the activity of Ca$^{2+}$ sparks by calculating the n$_P$ of each Ca$^{2+}$-sparklet site, where n is the number of quanta levels and P$_s$ is the

AJP-Heart Circ Physiol • VOL 293 • SEPTEMBER 2007 • www.ajpheart.org
probability that a quantal Ca\(^{2+}\)-sparklet event is active. Using this analysis, we have grouped Ca\(^{2+}\)-sparklet sites into three categories: silent (by default has an \(n_P\) of 0), low (\(n_P\) between 0 and 0.2), and high (\(n_P > 0.2\)). A detailed description of this analysis can be found in Navedo et al. (35).

CA\(^{2+}\)-sparklet signal mass and duration analysis. We used the “signal mass” approach developed by Zou et al. (56, 57) to determine the amount of Ca\(^{2+}\) flux (\(\Delta Q_{Ca}\), measured in coulombs) associated with Ca\(^{2+}\) sparklets at -70 mV as described in Amberg et al. (2). Briefly, for this analysis the total fluorescence intensity (\(F_{total}\)) associated with a Ca\(^{2+}\) sparklet is determined from raw images by summing the fluorescence from all the pixels within an area of the image larger than the entire fluorescence signal produced by a Ca\(^{2+}\) sparklet. The change in \(F_{total}\) (\(\Delta F_{total}\)) is then determined by subtracting the total fluorescence before the channel(s) open from the total fluorescence at each time point during the opening. Signal mass was obtained by determining the peak of the integral of \(\Delta F_{total}\) trace over time (\(\Delta F_{total} dt\)) for each Ca\(^{2+}\) sparklet. The relationship between sparklet signal mass and \(\Delta Q_{Ca}\) is linear (2, 47, 56, 57). Thus the slope (555 peak \(\Delta F_{total} dt\) units/\(\Delta Q_{Ca}\)), where \(\Delta Q_{Ca}\) is inverse femtocoulomb) of a previously obtained signal mass-\(\Delta Q_{Ca}\) relationship for a L-type Ca\(^{2+}\) channel (2) was used to calculate the \(\Delta Q_{Ca}\) of Ca\(^{2+}\) sparklets from \(\Delta F_{total} dt\) values.

CA\(^{2+}\)-sparklet event-duration times at the membrane potential of -70 mV were obtained from fits to CA\(^{2+}\)-sparklet records by using pClamp 9 “threshold detection analysis” as described in detail elsewhere (2). This analysis is similar to the one implemented by the Parker group for the study of CA\(^{2+}\) signals via single N-type Ca\(^{2+}\) channels (11) and acetylcholine receptors (12).

RNA isolation and RT-PCR. Total RNA was isolated from -60 arterial myocytes by using the RNaseasy Micro kit (Qiagen, Valencia, CA) as instructed by the manufacturer. We designed primers specific for the Cav\(_{1.2}\) subunit (GenBank accession no. NM_009781; sense nt 5292–5316 and antisense nt 5884–5910; amplicon = 618 bp) and Cav\(_{1.3}\) subunit (GenBank accession no. NM_028981; sense nt 4589–4613 and antisense nt 5065–5090; amplicon = 510 bp) of this channel. We used \(\beta\)-actin (GenBank accession no. V01217; sense nt 2384–2404 and antisense nt 3071–3091; amplicon = 496 bp) transcripts as an internal control for these experiments. \(\beta\)-Actin primers amplify a region between exons 4 and 6 such that genomic contamination within the RNA preparation is identified by the presence of a 708-bp band in addition to the 496-bp band. Reverse transcription and amplification was performed by using the OneStep RT-PCR kit from Qiagen following the manufacturer’s instructions. To do this, we used a Eppendorf thermal cycler running the following program: 35 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min/cycle, with a final extension step of 72°C for 10 min. Amplons were separated by using 2% agarose gel electrophoresis.

Immunofluorescence. Isolated arterial myocytes were plated on laminin-coated coverslips and were allowed to settle for 2 h. Cells were then fixed with 4% paraformaldehyde for 45 min, rinsed in 0.1 M phosphate buffer, rinsed in 0.1 M Tris buffer (TB) for 15 min, rinsed in 0.1 M Tris-buffered saline (TBS) for 15 min, blocked in 2% avidin in TBS for 30 min, rinsed in TBS for 30 min, blocked in 2% biotin for 30 min, and then rinsed in TBS for 30 min. Cells were then incubated in the anti-Cav\(_{1.2}\) (chicken, diluted 1:50) or anti-Cav\(_{1.3}\) (rabbit, diluted 1:50) antibodies overnight at 4°C (48). Cells were rinsed and incubated in biotinylated goat anti-chicken IgG (diluted 1:300; Vector) or biotinylated goat anti-rabbit IgG for 2 h at room temperature, rinsed, and then incubated in avidin D fluorescein (diluted 1:300; Vector) or 2 h at room temperature, rinsed, and then incubated in avidin D fluorescein (diluted 1:300; Vector) or 2 h at room temperature, rinsed, and then incubated in avidin D fluorescein (diluted 1:300; Vector) or 2 h at room temperature, rinsed, and then incubated in avidin D fluorescein (diluted 1:300; Vector) or 2 h at room temperature, rinsed, and then incubated in avidin D fluorescein (diluted 1:300; Vector) or 2 h at room temperature, rinsed, and then incubated in avidin D fluorescein (diluted 1:300; Vector) or 2 h at room temperature. Cells were then immunostained with the primary antibody followed by a secondary antibody conjugated to a fluorochrome.

Chemicals and statistics. Gö-6976 and ionomycin were from Calbiochem (San Diego, CA); Dulbecco’s modified Eagle’s medium was from Gibco. Lipofectamine 2000 was from Invitrogen. All other chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Normally distributed data are presented as means ± SE. Two-sample comparisons were made by using a Student’s t-test; multigroup comparisons were made by ANOVA, which, if necessary, was followed by Tukey’s multicomparison test. Nonparametric statistical analyses (Mann-Whitney test) were used for nonnormally distributed data. P values <0.05 were considered significant. Asterisks used in the figures indicate a significant difference between groups.

RESULTS

Properties of single Cav\(_{1.3}\) channels. We recorded single Cav\(_{1.3}\)-channel activity in tSA201 cells (Fig. 1). For these experiments, the pore-forming Cav\(_{1.3}\) subunit was coexpressed with accessory Ca\(_{\text{v}3}\) and Ca\(_{\text{v}2.6}\) subunits and EGFP. Transfected cells were identified on the basis of EGFP fluorescence. Cav\(_{1.3}\)-channel currents were recorded in cell-attached patches, with 110 mM Ba\(^{2+}\) in the pipette solution used as the charge carrier.

Figure 1A shows a representative family of single Cav\(_{1.3}\)-channel sweeps during step depolarization (1 s) from a holding potential of -80 mV to voltages ranging from -40 to +20 mV. Note that membrane depolarization increased the \(P_o\) of Cav\(_{1.3}\) channels and that the threshold for the activation of these channels was -40 mV. Similar findings were obtained in four additional independent experiments. Indeed, the \(P_o\) of Cav\(_{1.3}\) channels was 0.003 ± 0.001 and 0.686 ± 0.006 at -40 and +20 mV, respectively (\(n = 5\); Fig. 1B). The voltage dependence of Cav\(_{1.3}\) channels was fitted (\(R^2 = 0.99\)) with a Boltzmann function with a maximum \(P_o\) of 0.69 ± 0.006 at +20 mV, the voltage at which 50% of the maximum \(P_o\) was observed (\(V_{1/2}\)) of -2.4 ± 0.2 mV, and a slope factor of 6.4 ± 0.2.

The voltage dependence of unitary Cav\(_{1.3}\)-current amplitudes is shown in Fig. 1C. At -40, -10, and +20 mV, the amplitude of single Cav\(_{1.3}\)-channel currents was 1.65 ± 0.04, 1.07 ± 0.02, and 0.63 ± 0.04 pA, respectively (\(n = 6\)). These data were fit with a linear function that revealed a slope conductance of 20 ± 1 pS (\(n = 6\); \(R^2 = 0.99\)).

We also analyzed the open times of single Cav\(_{1.3}\)-channel openings at -10 mV (Fig. 1D). The Cav\(_{1.3}\)-channel open-times histograms at this voltage was fit with the sum of two log-normal probability density functions (PDFs) with a short (\(\tau_s\)) and long (\(\tau_l\)) time constant of 1.6 (standard deviation, \(\sigma_s = 0.7\) ms; 67%) and 9.5 ms (standard deviation, \(\sigma_l = 0.6\) ms; 33%), respectively (\(\chi^2 = 1.7; n = 2,806\) events). This analysis was validated by using Akaike’s Information Criterion, which determines the probability that a data set could be described by a particular set of competing models (1). Indeed, this test revealed that the probability that the Cav\(_{1.3}\) open-time data described above could be fit by the sum of two log-normal PDFs was >99.99% but was <0.01% with a single log-normal PDF. Together, these data suggest that Cav\(_{1.3}\) channels, like Cav\(_{1.2}\) channels (21), could operate in two gating modes with short and long open times.

Cav\(_{1.3}\) channels produce Ca\(^{2+}\) sparklets. Having examined the function of single Cav\(_{1.3}\) channels, we investigated the mechanisms of Ca\(^{2+}\) influx through Cav\(_{1.3}\) channels by imaging near-membrane Ca\(^{2+}\) in tSA201 cells expressing Cav\(_{1.3}\) channels with the use of TIRF microscopy. We and others (11, 12, 34, 35) have used this approach to image Ca\(^{2+}\) influx via single Ca\(^{2+}\) channels with high temporal and spatial resolution.
Because PKCα was necessary for high-activity persistent Ca\(^{2+}\) sparklets with Cav1.2 channels (35), we coexpressed PKCα-EGFP (PKCα) with Cav1.3 for these experiments. Expression of functional Cav1.3 channels in tsA201 cells was verified by the application of a brief (200 ms) voltage step from the holding potential of -70 mV to +10 mV. As illustrated in Fig. 2A, application of this voltage protocol evoked robust whole cell \(I_{\text{Ca}}\). \(I_{\text{Ca}}\) was never observed in tsA201 cells expressing PKCα-EGFP only.

Figure 2B shows a TIRF image of a representative tsA201 cell expressing functional Cav1.3 channels. Note that at -70 mV most of the imaged area was devoid of Ca\(^{2+}\)-signaling activity. However, local [Ca\(^{2+}\)]\(_t\), transients were detected, as shown in the [Ca\(^{2+}\)]\(_t\), time course for the region enclosed by the green circle in Fig. 2B. Because the local, submembranous [Ca\(^{2+}\)], signals produced by Cav1.3 channels were similar to Cav1.2 sparklets (i.e., transient, spatially restricted, repetitive Ca\(^{2+}\)-entry events via L-type Ca\(^{2+}\) channels), we concluded that these Ca\(^{2+}\)-influx events were indeed Cav1.3 sparklets (34, 47).

Accordingly, we performed analyses on Cav1.3 sparklets developed for detecting and quantifying the activity, amplitude, and duration of Ca\(^{2+}\) sparklets in arterial myocytes. Detailed descriptions of these analyses can be found in METHODS and elsewhere (2, 34, 35). Ca\(^{2+}\)-sparklet activity was quantified by determining the nP\(_s\) of Cav1.3-sparklet sites under control conditions and after the application of a Ca\(^{2+}\)-free solution or a solution containing 20 mM Ca\(^{2+}\) and 10 \(\mu\)M nifedipine (Fig. 2, B–D). As expected, Cav1.3 sparklets were completely eliminated by the application of a Ca\(^{2+}\)-free external solution, confirming that they resulted from Ca\(^{2+}\) entry, presumably via Cav1.3 channels (Fig. 2, B and D).

Next, we recorded Ca\(^{2+}\) sparklets at -70 mV before and after application of 10 \(\mu\)M nifedipine. Previous studies suggest that Cav1.3 channels are less sensitive (≈9-fold) to dihydropyridine blockers than Cav1.2 channels (20, 27, 33, 52). Accordingly, we found that 10 \(\mu\)M nifedipine decreased Cav1.3-sparklet activity (i.e., nP\(_s\)) by 34 ± 9% (\(n = 8\) sparklet sites from 4 cells; \(P < 0.05\); Fig. 2, C and D). Although 10 \(\mu\)M nifedipine partially inhibited Cav1.3-sparklet activity at -70 mV, this concentration of nifedipine completely eliminated Cav1.2 sparklets in tsA201 cells at the same membrane potential (35). It is important to note that the 34 ± 9% decrease in Cav1.3-sparklet activity by 10 \(\mu\)M nifedipine observed here is consistent with a recent study (33) suggesting that a similar concentration of this dihydropyridine decreased Cav1.3 currents in outer hair cells by ~36% (holding potential = -90 mV).
Figure 2E shows an amplitude histogram of Cav1.3 sparklets at −70 mV with 20 mM external Ca2+ concentration ([Ca2+]o). As for Cav1.2 channels and native arterial myocyte sparklets under similar experimental conditions (34, 35), this histogram was fit with a multi-Gaussian function with a quantal unit of Ca2+ influx of 34 nM (n = 339 events; χ² = 1.5). This value is similar to that of Cav1.2 (36 nM) and arterial myocyte Ca2+ sparklets (38 nM) under similar experimental conditions (34, 35). Thus, as in arterial myocytes and tsA201 cells expressing Cav1.2 channels, our analysis indicates that Ca2+ entry via Cav1.3 channels is quantal in nature and that multi-amplitude Cav1.3 sparklets result from coincidental activation of multiple quantal units.

As measured the voltage dependence of the amplitude of quantal Cav1.3 sparklets (Fig. 2F). As expected from the single Cav1.3-channel data described above, the amplitude of Cav1.3 sparklets decreased with membrane depolarization as the driving force for Ca2+ influx decreased. Indeed, the Cav1.3 sparklet amplitude-voltage relationship was fit with a linear function with a slope of 0.44 nM/mV (R² = 0.99).

As with Cav1.2 and arterial smooth muscle Ca2+ sparklets under similar conditions, Cav1.3-sparklet activity was bimodal (Fig. 3, A and B). Cav1.3 sparklets could be divided into low and high nPq sites. On average, the nPq for low and high Cav1.3-sparklet sites were 0.07 ± 0.01 (n = 37) and 0.46 ± 0.1 (n = 20), respectively (Fig. 3B). Interestingly, these nPq values are similar to those for Cav1.2 and arterial myocyte Ca2+ sparklets (34, 35). Application of the dihydropyridine agonist BAY K 8644 (500 nM) evoked Ca2+-sparklet activity in previously silent sites and increased the activity of low nPq sites from 0.06 ± 0.01 (n = 45) to 0.31 ± 0.1 (n = 35), a value similar to that of high nPq sites. BAY K 8644 did not increase the activity of high nPq Cav1.3-sparklet sites, indicating that these channels were maximally activated (Fig. 3, A and B).

Duration and signal-mass analysis of Cav1.3 sparklets. We used the signal-mass approach to estimate the ΔQCa associated with Cav1.3 sparklets at −70 mV (56, 57) (Fig. 4A). As described in METHODS, Ca2+-sparklet signal-mass values were obtained by determining the peak ΔFtotal dr for each Cav1.3 sparklet. We then used the slope (555 peak ΔFtotal dr units·fC⁻¹) of a previously obtained signal-mass-ΔQCa relationship for an L-type Ca2+ channel under similar experimental conditions (2) to calculate the ΔQCa of Cav1.3 sparklets from their ΔFtotal dr values. Using this analysis, we found that the signal mass of Cav1.3 sparklets (at −70 mV) had a broad...
distribution, ranging from 32 to 2,810 pC for low nP$_s$ and 52 to 9,150 pC for high nP$_s$ sites. The median signal mass for low and high nP$_s$, Cav1.3 sparklets was 273 and 917 pC, respectively.

Next, we tested the hypothesis that Ca$^{2+}$ entry via high nP$_s$ Cav1.3-sparklet sites is greater than via low nP$_s$ sites because the duration of Cav1.3 sparklets in high nP$_s$ sites is longer than that of low nP$_s$ sites. To do this, we constructed event-duration histograms of Cav1.3 sparklets from low and high nP$_s$ Ca$^{2+}$-sparklet sites (Fig. 4, B and C). The histogram from low nP$_s$, Cav1.3-sparklet sites could be fit with a single exponential function with a $\tau$ of 31 ms ($R^2 = 0.93; \chi^2 = 0.01$). The duration histogram of high nP$_s$ Cav1.3 sparklets could be fit with the sum of two exponential functions with short ($\tau_{\text{fast}}$) and long ($\tau_{\text{slow}}$) durations of 37 and 126 ms, respectively ($R^2 = 0.98; \chi^2 = 0.01$). As done for the open-time analysis of single Cav1.3 channels (see above), we validated our choice of models to fit the data by determining the Akaike Information Criterion for a single vs. a two-exponential function. This analysis revealed that the probability that the duration histogram of Cav1.3 sparklets in low and high nP$_s$ could be fit by a single or two-exponential function was >99.99% each. Thus our data suggest that the relatively small Ca$^{2+}$ entry via low nP$_s$ Cav1.3-sparklet sites is, at least in part, due to the relatively short duration of Cav1.3 sparklets in these sites. In high nP$_s$ Cav1.3-sparklet sites, Cav1.3 sparklet events with $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ contribute to small and larger Ca$^{2+}$ influx events.

Cav1.2 channels underlie $I_{Ca}$ in mouse arterial myocytes. After demonstrating that Cav1.3 channels can produce Ca$^{2+}$ sparklets similar in amplitude, activity, and duration to those observed for Cav1.2 and arterial myocyte Ca$^{2+}$ sparklets, we investigated whether these channels contribute to $I_{Ca}$ in cerebral arterial myocytes. We recorded Ca$^{2+}$ currents with 20 mM external Ca$^{2+}$ from cerebral arterial myocytes and tsA201 cells.
expressing Cav1.2 or Cav1.3 channels (Fig. 5). Cav2+ currents were evoked by 200-ms voltage pulses from −70 mV to potentials ranging from −70 to +70 mV. As reported by others (27, 52), Cav1.3 currents activated at more negative potentials than Cav1.2 currents (Fig. 5, A and B). Similar to that of Cav1.2 currents, the current-voltage relationship of ICa in cerebral arterial myocytes was shifted toward more depolarized potentials. The voltage at which 50% of ICa was observed (V1/2) in cerebral arterial myocytes and tsA201 cells expressing Cav1.2 was +33.8 ± 1.2 mV (n = 7) and +31.9 ± 1.2 mV (n = 7), respectively, whereas the V1/2 for tsA201 cells expressing Cav1.3 was +9.7 ± 2.9 mV (n = 8; P < 0.05).

We also determined the voltage dependence of steady-state inactivation of ICa in cerebral arterial myocytes and tsA201 cells expressing Cav1.2 or Cav1.3. Our data indicate that the V1/2 of steady-state inactivation was −4.9 ± 1.5 mV (n = 8), −2.7 ± 1.0 mV (n = 6; P > 0.05), and −18.7 ± 0.7 mV (n = 6; P < 0.05) in cerebral arterial myocytes, Cav1.2-expressing cells, and Cav1.3-expressing cells, respectively. Thus the voltage dependencies of conductance and steady-state inactivation of ICa in cerebral arterial myocytes are similar to that of tsA201 cells expressing Cav1.2 channels but not Cav1.3 channels. These results suggest that Cav1.2 channels are the predominant voltage-gated, dihydropyridine-sensitive Ca2+ channels in cerebral arterial myocytes.

Cav1.2 channels, not Cav1.3 channels, underlie Ca2+ sparklets and control dihydropyridine-sensitive [Ca2+]i spikes in arterial myocytes. With the data presented above, we cannot rule out that low-level expression of Cav1.3 channels in arterial myocytes is sufficient to contribute to Ca2+-sparklet activity without significantly influencing ICa. To address this issue, we recorded Ca2+ sparklets in arterial myocytes from wild-type and DHP−/− mice. These DHP−/− mice have a single-point mutation (Thr1066Tyr) that renders them insensitive to inhibition by dihydropyridines without otherwise altering their functional properties (27, 44). DHP−/− mice are useful because dihydropyridine L-type Ca2+ channel antagonists can be used to selectively block Cav1.3 (relative to Cav1.2) channels and to determine their contribution to Ca2+-sparklet activity. If, as our data suggest, Cav1.2 and not Cav1.3 channels underlie Ca2+ sparklets in arterial myocytes, then we
expect that nifedipine would eliminate Ca$^{2+}$ sparklets in wild-type but not in DHP$^{-/-}$ cells.

Figure 6A shows Ca$^{2+}$-sparklet records (at −70 mV) from wild-type and DHP$^{-/-}$ arterial myocytes before and after the application of 10 μM nifedipine. Note that nifedipine eliminated Ca$^{2+}$ sparklets in wild-type but not in DHP$^{-/-}$ cells (Fig. 6, A–C). This is consistent with the hypothesis that Ca$_{1.2}$ and not Ca$_{1.3}$ channels underlie Ca$^{2+}$ sparklets in mouse arterial myocytes. To provide further support to this hypothesis, we examined arterial wall [Ca$^{2+}$]$\text{i}$ in pressurized (80 mmHg) intact mesenteric arterial segments from wild-type and DHP$^{-/-}$ mice. As expected (25), application of 10 μM nifedipine decreased wild-type arterial wall [Ca$^{2+}$]$\text{i}$ from ∼210 to ∼112 nM [Ca$^{2+}$]$\text{i}$. Application of a Ca$^{2+}$-free solution did not produce any further decrease in [Ca$^{2+}$]$\text{i}$. Consistent with our Ca$^{2+}$-sparklet data, and unlike in wild-type arteries, we found that 10 μM nifedipine did not decrease [Ca$^{2+}$]$\text{i}$ in pressurized DHP$^{-/-}$ arteries. Application of a Ca$^{2+}$-free solution, however, decreased arterial wall [Ca$^{2+}$]$\text{i}$ in DHP$^{-/-}$ arteries to a similar extent (100 ± 5 nM; Fig. 6D) to the decrease caused by nifedipine in wild-type arteries (∼210 to 107 nM; Fig. 6, D and E).

Finally, we used RT-PCR and immunofluorescence approaches to examine transcript and protein levels of Ca$_{1.2}$ and Ca$_{1.3}$ in mouse arterial myocytes. For the RT-PCR experiments, we measured Ca$_{1.2}$, Ca$_{1.3}$, and β-actin transcript in brain and dissociated, spindle-shaped arterial myocytes (∼60 cells; Fig. 7A). As expected, Ca$_{1.2}$ transcript was detected in brain and arterial myocytes. Note, however, that Ca$_{1.3}$ transcript was detected in brain but not in arterial myocytes. Similar results were obtained in four independent experiments. These data suggest that Ca$_{1.3}$ transcript is not expressed in arterial myocytes.

Consistent with this observation, as well as the $I_{Ca,Ca^{2+}}$–sparklet, and arterial wall [Ca$^{2+}$]$\text{i}$ data described above, we detected Ca$_{1.2}$, but not Ca$_{1.3}$-associated fluorescence in dissociated mouse arterial myocytes (Fig. 7, B and C; n = 25). It is important to note that the Ca$_{1.2}$ and Ca$_{1.3}$ antibodies used for these immunofluorescence experiments have been extensively characterized and found to be specific for these channels (19, 48). Collectively, our data indicate that although Ca$_{1.2}$ and Ca$_{1.3}$ channels have similar conductances and gating modalities and are capable of producing persistent Ca$^{2+}$ sparklets, only Ca$_{1.2}$ channels underlie $I_{Ca,Ca^{2+}}$–sparklets and hence dihydropyridine-sensitive Ca$^{2+}$ influx into mouse arterial myocytes.

**DISCUSSION**

In this study, we report three fundamental observations. First, we provide the first examination of single Ca$_{1.3}$-channel function. Second, we demonstrate that as with Ca$_{1.2}$ channels,
Ca_{1.3} channels can produce persistent Ca^{2+} sparklets. Third, we found that Ca_{1.2} channels, not Ca_{1.3} channels or any other Ca^{2+}-permeable channel, underlie I_{Ca}, Ca^{2+} sparklets and hence dihydropyridine-sensitive Ca^{2+} influx in mouse arterial myocytes. These findings and their physiological implications are discussed below.

There are four known members of the Ca_{1.X} family of L-type Ca^{2+} channels (1.1, 1.2, 1.3, and 1.4) (7, 29). A hallmark of Ca_{1.X} channels is that they are voltage gated and are sensitive, although to differing degrees, to dihydropyridines. Ca_{1.3} channels have a single-channel conductance of \( \approx 21 \) pS with 110 mM Ba^{2+}, which is similar to that of Ca_{1.1} (\( \approx 16 \) pS) (8) and Ca_{1.2} (\( \approx 19 \) pS) (9, 15) channels but larger than Ca_{1.4} channels (\( \approx 4 \) pS) (15) under similar ionic conditions. Our data also suggest that like Ca_{1.1} and Ca_{1.2} channels, Ca_{1.3} channels could operate in two gating modes with short (\( \approx 1.6 \) ms) and long (\( \approx 9.5 \) ms) open times (8–10, 14, 21, 39). Interestingly, Ca_{1.4} channels apparently do not have this bimodal gating behavior (15). Together, these data indicate that Ca_{1.1–3} channels form part of a subgroup within the Ca_{1.X} family of channels with similar conductance and gating modalities.

The hyperpolarized voltage dependence of activation of Ca_{1.3} channels suggests that these channels contribute to Ca^{2+} influx at relatively negative potentials (27, 52). Our single Ca_{1.3}-channel data provide important quantitative insight into mechanisms of Ca^{2+} entry via these channels. For example, recording Ca_{1.3} channel activity at \(-70\) mV with conventional patch-clamp techniques is difficult because of the low \( P_o \) of these channels at this voltage. However, the Boltzmann function used to fit our Ca_{1.3}-channel \( P_o \)-voltage relationship can be used to predict the \( P_o \) of these channels at \(-70\) mV. Under this approach, the estimated \( P_o \) of a typical Ca_{1.3} channel is calculated to be \( \approx 10^{-5} \) at \(-70\) mV, a value that is one to three orders of magnitude higher than the average \( P_o \) for Ca_{1.2} channels (\( P_o \approx 10^{-8} \)–\( 10^{-9} \)) under similar experimental conditions (42, 50).

Our Ca_{1.3}-sparklet data suggest that these channels could contribute to Ca^{2+} influx at hyperpolarized potentials. As with Ca_{1.2} channels (35), heterologously expressed Ca_{1.3} channels are capable of producing persistent Ca^{2+} sparklets at hyperpolarized potentials. As expected from our single Ca_{1.3}-channel analysis (see above), we found many similarities between Ca_{1.2} and Ca_{1.3} sparklets. Ca_{1.3} sparklets have a quantal amplitude of \( \approx 34 \) nM, which is similar to that of Ca_{1.2} (\( \approx 36 \) nM) and native smooth muscle (\( \approx 38 \) nM) Ca^{2+} sparklets (34, 35). This is consistent with our observation that Ca_{1.2} and Ca_{1.3} channels have similar single-channel conductances. Another similarity between Ca_{1.2}, Ca_{1.3}, and smooth muscle cell Ca^{2+} sparklets is that their duration and activity are bimodal. This feature of Ca^{2+} sparklets likely reflects the ability of Ca_{1.2} and Ca_{1.3} channels to operate in two gating modes with either a brief (i.e., mode 1) or long (i.e., mode 2) open time. It is important to note, however, that the total \( nP_o \) of Ca_{1.3} sparklets is significantly higher (\( P < 0.05 \); see the supplemental figure for this article, available online at the American Journal of Physiology-Heart and Circulatory Physiology website) than that of Ca_{1.2} sparklets at \(-70\) mV. This is consistent with greater Ca_{1.3} activity than Ca_{1.2} activity at this negative potential.

Although Ca_{1.2} and Ca_{1.3} channels produce Ca^{2+} sparklets with similar properties, we provide compelling evidence supporting the hypothesis that Ca_{1.2} channels, not Ca_{1.3} channels, underlie Ca^{2+} sparklets and \( I_{Ca} \) in mouse arterial
myocytes. First, the voltage dependencies of activation and inactivation of $I_{Ca}$ in arterial myocytes are similar to those of Ca,1.2 but not of Ca,1.3 currents. Second, nifedipine eliminated Ca$^{2+}$ sparklets in wild-type myocytes but was without effect in Ca,1.2 DHP/- arterial myocytes. This finding is of particular importance because it eliminates the possibility that Ca$^{2+}$ sparklets are produced by a dihydropyridine-sensitive, non-Ca,1.2X channel (e.g., a member of the transient receptor potential superfamily of channels) in arterial myocytes. Third, Ca,1.2, but not Ca,1.3, transcript and protein were detected in isolated arterial myocytes. Thus, although Ca,1.3 channels are capable of producing persistent Ca$^{2+}$ sparklets, Ca,1.2 channels underlie $I_{Ca}$, Ca$^{2+}$ sparklets and hence dihydropyridine-sensitive Ca$^{2+}$ influx in mouse cerebral arterial myocytes.

The importance of Ca,1.2 with respect to $I_{Ca}$ and myogenic tone was recently examined in small mesenteric arteries (54). Although the authors of this study did not measure Ca$^{2+}$ sparklets and arterial wall [Ca$^{2+}$], they found that Ca,1.2 channels play a predominant role in $I_{Ca}$ and myogenic tone in small mesenteric arteries. Together with the data presented here, these data suggest that Ca,1.2 channels are crucial in the regulation of Ca$^{2+}$ influx in smooth muscle from multiple arterial beds.

Although Ca,1.3 channels do not contribute to Ca$^{2+}$-sparklet activity and $I_{Ca}$ in mouse cerebral (this study) and mesenteric (54) artery smooth muscle, we cannot rule out that these channels modulate Ca$^{2+}$ influx in arterial smooth muscle from other species. Indeed, Ca,1.3 transcript and protein has been detected in canine basilar artery (37). Future studies should examine Ca$^{2+}$ sparklets in smooth muscle cells from basilar arteries from dog and other species and should assess the contribution of Ca,1.3 channels to these events.

Ca,1.2 and Ca,1.3 channels, as well as PKC, are expressed in multiple excitable cells, including neuronal, cardiac, smooth muscle, endocrine, and outer hair cells (7, 30, 33, 37, 38, 40). Thus our observation that Ca,1.2 and Ca,1.3 channels in association with PKC could produce persistent Ca$^{2+}$-sparklet activity has broad physiological implications. For example, the findings in this study, in combination with recent work from our laboratory (2), indicate that persistent Ca,1.2 sparklets regulate local and global [Ca$^{2+}$]i in murine arterial smooth muscle. Future studies should examine whether, as in arterial myocytes, persistent Ca$^{2+}$ sparklets modulate physiological processes in which Ca,1.2 and/or Ca,1.3 channels and PKC have been shown to be involved. These include synaptic plasticity (28, 51), gene expression and excitability (3, 16), hormone release (53), cardiac excitation-contraction coupling (6), and cardiac pacemaking activity (31).

The Ca,1.3-sparklet data presented here add to a growing body of evidence suggesting that the activity of dihydropyridine-sensitive, voltage-gated Ca$^{2+}$ channels is heterogeneous dispersed along the plasma membrane (2, 34, 35). In arterial myocytes, Ca$^{2+}$-sparklet activity varies regionally depending on the relative activities of nearby PKCα and protein phosphatases 2A and 2B (35). Although the specific mechanisms underlying regional variations in Ca$^{2+}$-sparklet activity (or lack thereof) may vary in different cell types, the findings underscore the power of TIRF microscopy to optically record Ca$^{2+}$ influx via single Ca$^{2+}$ channels from a relatively large surface area (11, 12).

The current-voltage relationship in Fig. 5B shows that, under our experimental conditions (i.e., [Ca$^{2+}$]o = 20 mM), Ca$^{2+}$ currents produced by Ca,1.3, Ca,1.2, and native smooth muscle L-type calcium channels peaked at +20 mV, +40 mV, and +40 mV, respectively. This represents a +30- to +40-mV shift in the current-voltage relationships of these currents relative to those recorded in physiological 2 mM [Ca$^{2+}$]o (20, 24, 43). Examination of the surface-potential model (17, 23) suggests a potential mechanism for the differences between the voltage dependencies of activation of Ca$^{2+}$ currents at 2 and 20 mM external Ca$^{2+}$. In this model, negatively charged particles (phospholipids and sugars) on the surface membrane of the cell produce a surface potential. This surface potential determines the intramembrane voltage, which is the voltage modulating the voltage sensor of the Ca$^{2+}$ channels. According to the surface-potential model, increasing external Ca$^{2+}$ from 2 to 10 mM would neutralize negatively charged particles associated with the outer leaflet of the plasma membrane, which would reduce the magnitude of the surface potential and thus increase the intramembrane potential. Consequently, a greater membrane depolarization will have to be applied for the Ca$^{2+}$ channels to “feel” the same trans-bilayer field at 20 as at 2 mM [Ca$^{2+}$], which would result in a rightward shift in the voltage dependencies of these channels.

To conclude, we have provided the first examination of single Ca,1.3 channels. Our data demonstrate, for the first time, that Ca,1.3 channels have similar conductance and gating modalities to Ca,1.2. Accordingly, Ca,1.3 sparklets resemble most of the features of Ca$^{2+}$ sparklets in arterial myocytes and in tsA201 cells expressing Ca,1.2 channels. Our data indicate that although Ca,1.3 channels can produce persistent Ca$^{2+}$ sparklets, Ca,1.2 channels underlie $I_{Ca}$, Ca$^{2+}$ sparklets, and hence dihydropyridine-sensitive Ca$^{2+}$ influx in mouse cerebral arterial myocytes.

ACKNOWLEDGMENTS

We thank V. Scott Votaw for help with image analysis, Jennifer Cabarrus for technical assistance, and Drs. Carmen A. Ufret-Vincenty and Madeline Nieves-Cintrón for reading this manuscript. Drs. Dianne Lipscombe and John Eton kindly provided Ca,1.3 and PKCα2,δ clones, respectively.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-85870, HL-77115, HL-07828, and HL-44948 and by grants from the American Heart Association (0635118N) and the Austrian Science Fund (P17159).

REFERENCES


49. Zhang J, Berra-Romani R, Sinnegger-Brauns MJ, Striessnig J, Blaustein MP, Matsesse DR. Role of Ca,1.2 L-type Ca2+ channels in...

