Regulation of L-type calcium channel sparklet activity by c-Src and PKC-α

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Gulia J, Navedo MF, Gui P, Chao JT, Mercado JL, Santana LF, Davis MJ. Regulation of L-type calcium channel sparklet activity by c-Src and PKC-α. Am J Physiol Cell Physiol 305: C568–C577, 2013. First published June 26, 2013; doi:10.1152/ajpcell.00381.2011.—The activity of persistent Ca2+- sparks, which are characterized by longer and more frequent channel open events than low-activity sparklets, contributes substantially to steady-state Ca2+ entry under physiological conditions. Here, we addressed two questions related to the regulation of Ca2+- sparks by PKC-α and c-Src, both of which increase whole cell Ca2+ current: 1) Does c-Src activation enhance persistent Ca2+ sparklet activity? 2) Does PKC-α activate c-Src to produce persistent Ca2+ sparks? With the use of total internal reflection fluorescence microscopy, Ca2+ sparks were recorded from voltage-clamped tsA-201 cells coexpressing wild-type (WT) or mutant Ca2+ channels (the neuronal isoform of CaV1.2α, 1b, 2b, and/or Cav1.2c) constructs tagged with GFP, using jetPEI (Polyplus transfection). In all protocols, 840 ng of Cav1.2α, Cav1.2b, and Cav1.2c cDNA were added to the transfection mixture. Successfully transfected cells were identified on the basis of GFP fluorescence. To


—The activity of persistent Ca2+ sparks, which are characterized by longer and more frequent channel open events than low-activity sparklets, contributes substantially to steady-state Ca2+ entry under physiological conditions. Here, we addressed two questions related to the regulation of Ca2+- sparks by PKC-α and c-Src, both of which increase whole cell Ca2+ current: 1) Does c-Src activation enhance persistent Ca2+ sparklet activity? 2) Does PKC-α activate c-Src to produce persistent Ca2+ sparks? With the use of total internal reflection fluorescence microscopy, Ca2+ sparks were recorded from voltage-clamped tsA-201 cells coexpressing wild-type (WT) or mutant Ca2+ channels (the neuronal isoform of CaV1.2α, 1b, 2b, and/or Cav1.2c) constructs tagged with GFP, using jetPEI (Polyplus transfection). In all protocols, 840 ng of Cav1.2α, Cav1.2b, and Cav1.2c cDNA were added to the transfection mixture. Successfully transfected cells were identified on the basis of GFP fluorescence. To

L-TYPE CALCIUM (CaV1.2) CHANNELS are a primary pathway for Ca2+ entry in smooth muscle cells (SMCs) and, therefore, play a key role in regulation of vascular tone (18, 29). Previous studies on CaV1.2 channels have revealed the occurrence of a high activity-gating mode characterized by frequent, prolonged channel openings, in addition to a more prevalent gating mode with brief and rare channel openings (13, 23, 24, 39). When recording CaV1.2 channel activity using total internal reflection fluorescence (TIRF) microscopy, these low and high activity-gating modes correspond to low activity and persistent Ca2+ sparklets, respectively (23, 24, 27, 34).

Persistent Ca2+ sparklets account for ~50% of the steady-state Ca2+ entry through CaV1.2 channels under physiological conditions, even though they constitute a very small population of the total CaV1.2 channels on the plasma membrane (1). Furthermore, an increase in persistent Ca2+ sparklet activity has been shown to underlie increased intracellular Ca2+ during angiotensin II-induced hypertension and hyperglycemia (27, 28). This evidence points to an important physiological role for the small population of CaV1.2 channels that conforms to persistent Ca2+ sparklets. In vascular smooth muscle, these events are thought to be highly regulated by the classical isoform of protein kinase C (PKC-α; Refs. 4, 5), and membrane targeting of PKC-α by A-kinase anchoring protein 150 (AKAP150) is considered essential in this process (27).

However, the mechanism of PKC-α action on CaV1.2 channels that results in the induction of persistent Ca2+ sparklet activity is not clear. Biochemical evidence points to residue S1928, the canonical PKA phosphorylation site on CaV1.2a, as several isoforms of PKC have been shown to phosphorylate the CaV1.2a subunit directly or acts via another kinase. While c-Src, another highly expressed kinase in SMCs, is known to directly phosphorylate the CaV1.2 channel subunits [wild-type (WT)/mutant αC, β3, and β3] along with either green fluorescent protein (GFP) or protein kinase constructs tagged with GFP, using jetPEI (Polyplus transfection). In all protocols, 840 ng of αC and 200 ng each of α2-δ and β1b were used for transfection in a 35-mm dish with ~70% cell confluence. When appropriate, 200 ng c-Src-GFP, PKC-α, and/or GFP cDNA were added to the transfection mixture. Successfully transfected cells were identified on the basis of GFP fluorescence. To

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 Materials and Methods

Culture and transfection of tsA-201 cells. The cells were maintained at 37°C in a 5% CO2 incubator in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin solution. tsA-201 cells between passages 6 and 25 were plated on autoclaved glass coverslips in 35-mm Petri dishes, 24–48 h before transfection. These cells are derived from a human embryonic kidney cell line and immortalized by expression of the T antigen; they do not express any endogenous voltage-gated calcium channels. Cells were transfected with Cav1.2 channel subunits [wild-type (WT)/mutant αC, β3, and α2.5] along with either green fluorescent protein (GFP) or protein kinase constructs tagged with GFP, using jetPEI (Polyplus transfection). In all protocols, 840 ng of αC and 200 ng each of α2-δ and β1b were used for transfection in a 35-mm dish with ~70% cell confluence. When appropriate, 200 ng c-Src-GFP, PKC-α, and/or GFP cDNA were added to the transfection mixture. Successfully transfected cells were identified on the basis of GFP fluorescence. To
confirm that cotransfection of a kinase cDNA with Cav1.2c cDNA does not alter the expression of Cav1.2c, we compared current densities in cells expressing Cav1.2c alone with those of cells coexpressing Cav1.2c and c-Src. Both groups were found to have similar current densities [WT Cav1.2c peak calcium current (Ica) = 8.99 ± 2.18 pA/pF (n = 18); and WT Cav1.2c + c-Src peak Ica = 10.29 ± 2.66 pA/pF (n = 16)] indicating that cotransfection with a kinase cDNA does not significantly impact expression levels of Cav1.2c.

Rat neuronal WT α1c (accession no. P22002), B10, and α2δ6 DNA, subcloned into pcDNA3.1 vectors, were gifts from Dr. G. W. Zamponi. Site-directed mutagenesis was carried out in the laboratory of Zamponi (12) to obtain Y2122F and Y2139F mutations of the rat neuronal α1c-subunit. PKC-α tagged with GFP was a gift from Dr. J. Exton. Kinase-dead (kd) c-Src and c-Src tagged with GFP were gifts from Dr. A. P. Braun and Dr. G. E. Davis, respectively.

Electrophysiology. tsA-201 cells were voltage clamped at −70 mV in the whole cell configuration using an EPC10 (HEKA, Bellmore, NY) or Axopatch 200B (Axon Instruments, Union City, CA) patch-clamp amplifier controlled by Patchmaster (HEKA) or pClamp (Axon Instruments) software, respectively. The cells were perfused with a solution composed of the following (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, 25 HEPES, and 2CaCl2. After gigaseal formation, the bath solution was switched to a solution containing 20 mM CaCl2 and 120 N-methyl-d-glucamine without any change in the concentrations of the other constituents. The patch pipette solution consisted of the following (in mM): 87 Cs-Asp, 20 CsCl, 1 MgATP, 10 MgCl2, 10 EGTA, 0.2 Rhod-2. The pH of bath and patch pipette solutions were adjusted to 7.4 using NaOH. A perfusion system was used to perfuse the patch pipette with solutions at a flow rate of 1 ml/min. All experiments were performed at room temperature (23, 24).

TIRF microscopy. Voltage-clamped tsA-201 cells were imaged using a through-the-lens TIRF system coupled to an IX-71 Olympus microscope that was integrated with a high-speed Andor iXON EMCCD camera (Belfast, Ireland). The microscope was equipped with appropriate filter sets to separate excitation (491 nm for GFP and 647 nm for Rhod-2) and emission wavelengths. Images were acquired at a frequency >100 Hz using TILL imaging software (TILL Photonics, Rochester, NY), which also controlled the laser intensity and angle of laser incidence on the objective.

The acquired images were imported into a custom analysis program written in Interactive Data Language (IDL; ITTVIS, Boulder, CO) to perform Ca2+ sparklet detection using the amplitude threshold criteria (23, 24). Briefly, a Ca2+ fluorescence transient qualified as a sparklet if the average fluorescence amplitude of the 3 × 3 grid of the adjoining pixels (centered at the pixel with the highest fluorescence amplitude) was equal to or greater than the mean basal fluorescence plus 2.5 times its standard deviation.

Mean Ca2+ sparklet activity and Ca2+ sparklet density analysis. To test our hypothesis, we compared two parameters across different test groups: mean Ca2+ sparklet activity (nPs) and mean persistent Ca2+ sparklet density.

In previous Ca2+ sparklet studies (23–27), Ca2+ sparklet activity was determined using nPs analysis (where n = no. of quanta levels and P = probability of occurrence of Ca2+ sparklet), which is analogous to open probability (nPs) analysis for single-channel data. We followed a similar approach in this study with one difference. Instead of converting fluorescence units to Ca2+ concentration, we computed ΔFtotal at each time point in Ca2+ sparklet traces. First, the total fluorescence intensity, Ftotal, for each time point was calculated by summing the Rhod-2 fluorescence over a predefined region from the raw image stacks acquired over a fixed length of time. The area of the predefined region was greater than the entire fluorescence signal produced by the Ca2+ sparklet. Subsequently, Ftotal of the basal fluorescence value was subtracted from Ftotal observed during channel opening to determine ΔFtotal Value (1). The quantal ΔFtotal Value (2,500 arbitrary units), q, was calculated by plotting an all-points histogram of several Ca2+ sparklet traces and then fitting the histogram with a multicomponent Gaussian function. Following that, nPs analysis was performed on Ca2+ sparklet traces using “threshold detection analysis” with no duration constraints and a q value of 2,500 arbitrary units in pClamp 9.0. For the purpose of quantal value comparison with previous Ca2+ sparklet studies, we converted the fluorescence from some of our Ca2+ sparklet traces to Ca2+ concentration and obtained a Ca2+ sparklet quantal value of 34.4 nM (data not shown); this quantal value was similar to that obtained in other Ca2+ sparklet studies (23–25).

Persistent Ca2+ sparklet density in each cell was determined by dividing the number of persistent Ca2+ sparklet sites by the area of the cell visible in its TIRF image.

Data from cells that were transfected with Cav1.2c but did not exhibit measurable Ica upon depolarization were not included in the analyses. For mean nPs calculation, we did not consider cells in which sparklet activity could not be detected even if they expressed measurable Ica because it was not possible to calculate an accurate nPs value in such cases. However, those cells were accounted for in persistent Ca2+ sparklet density analysis since any cell that did not exhibit persistent Ca2+ sparklets could be assigned a sparklet density value of 0. In addition, we found that 9–10% of our traces fell into the nonstationary category (e.g., Fig. 4A). This was due to the limitation of our image acquisition software where some of the images were acquired during the occurrence of a sparklet event. Inclusion of such traces would lead to an inaccurate estimate (on the lower side) of nPs. However, every nonstationary trace in this study could be very clearly classified as a persistent or low sparklet activity group except for two traces. Putting those data in either sparklet activity group did not alter our conclusions.

Application of drugs. Xestospongin C (90 μM) was included in the patch-pipette solution to block inositol 1,4,5-trisphosphate receptors. Tetracaine (50 μM) was added to the bath solution to inhibit ryanodine receptors. For some experiments, PP2 (10 μM) was included in the bath solution to inhibit c-Src activity.

Statistical analysis. Previous analyses of Ca2+ sparklet density and Ca2+ sparklet activity by two of our laboratories (1, 23–28) have shown that while sparklet density follows a normal distribution, sparklet activity does not if it is not divided into low and persistent sparklet sites. Parametric statistical analyses are appropriate for normally distributed data and nonparametric analyses for nonnormally distributed data. Here, the data as presented followed a normal distribution and thus parametric analyses were used. A one-tail unpaired t-test was used to compare the Ca2+ sparklet activities and persistent Ca2+ sparklet densities of the control and test groups. A one-tail paired t-test was used to evaluate the effect of PP2 on Ca2+ sparklets. P < 0.05 was considered to be statistically significant.

Chemical reagents. Xestospongin C and PP2 were purchased from Calbiochem (San Diego, CA). Rhod-2 was procured from Invitrogen (San Diego, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

RESULTS

Ca2+ fluorescence events in tsA-201 cells. TIRF imaging of cells expressing WT Ca1.2c channels confirmed the presence of Ca2+ sparklets (Fig. 1). Based on their nPs values, Ca2+ sparklets were characterized as either persistent (nPs ≥ 0.2) or low (nPs < 0.2) activity events. We also recorded Ca2+ fluorescence events similar to Ca2+ sparklets from cells expressing GFP alone in the absence of measurable Ica upon depolarization (Fig. 2A). Since we had no objective reasons for excluding such anomalous events, they were analyzed in the same manner as Ca2+ sparklets. Importantly, the mean number of anomalous event sites per cell in cells expressing GFP alone

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Cav1.2c channels were responsible for all of the persistent Ca\(^{2+}\) sparklets (A) and persistent Ca\(^{2+}\) sparklet density analysis in further protocols.

To further test the involvement of c-Src, we coexpressed WT Cav1.2c with kinase dead c-Src (kd c-Src) in tsA-201 cells and c-Src were similar to those observed in cells expressing WT Cav1.2c alone (compare Figs. 2 and 3). This observation suggests at least two possible explanations. First, c-Src may not be involved in the production of persistent Ca\(^{2+}\) sparklets. Second, endogenous c-Src expression may be sufficient to promote persistent sparklet activity. Indeed, tsA-201 cells have been shown previously to express endogenous c-Src (12).

We substantiated the role of c-Src in producing persistent Ca\(^{2+}\) sparklet activity through Cav1.2c by recording Ca\(^{2+}\) sparklets from cells coexpressing WT Cav1.2c and c-Src before and after treatment with 10 μM PP2, a membrane-permeable c-Src antagonist (Fig. 4). Application of PP2 resulted in a 2.3- and 6.5-fold decrease in the mean density and activity respectively, of persistent Ca\(^{2+}\) sparklets (N = 5 sparklet sites; n = 5 cells; P < 0.05; Fig. 4, A–C) without significantly changing the mean activity of low activity Ca\(^{2+}\) sparklet sites (N = 6 sparklet sites; n = 5 cells; P > 0.05; Fig. 4D). To address whether endogenous c-Src was responsible for producing persistent Ca\(^{2+}\) sparklets, we tested the effect of PP2 on Ca\(^{2+}\) sparklet activity in cells expressing WT Cav1.2c alone. Indeed, PP2 application decreased persistent Ca\(^{2+}\) sparklet density (Fig. 5A) while reducing Ca\(^{2+}\) sparklet activity significantly in cells expressing WT Cav1.2c alone (N = 8 sparklet sites; n = 5 cells; P < 0.05; Fig. 5B). Interestingly, PP2 treatment also resulted in a significant reduction in the activity of low activity Ca\(^{2+}\) sparklets (N = 13 sparklet sites; n = 5 cells; P < 0.05; Fig. 5C).

To further test the involvement of c-Src, we coexpressed WT Cav1.2c with kinase dead c-Src (kd c-Src) in tsA-201 cells...
to competitively inhibit endogenous c-Src (Fig. 6). kd c-Src harbors a single point mutation in the kinase domain of the enzyme resulting in ablation of c-Src kinase activity. Out of six cells cotransfected with WT Ca\textsubscript{1.2c} and kd c-Src, persistent Ca\textsuperscript{2+} sparklet activity was detected in only one cell. Evaluation of persistent Ca\textsuperscript{2+} sparklet density revealed a 3.4-fold reduction in cells transfected with WT Ca\textsubscript{1.2c} and kd c-Src (n = 6 cells) compared with cells coexpressing WT Ca\textsubscript{1.2c} and c-Src (n = 9 cells; P < 0.05; Fig. 6A). The means of persistent Ca\textsuperscript{2+} sparklet activities of the two transfection groups were comparable but could not be statistically compared due to an insufficient number of persistent sparklets in the WT Ca\textsubscript{1.2c} + kd c-Src transfection group (Fig. 6B). As expected, the means for nPs of low activity Ca\textsuperscript{2+} sparklets in cells coexpressing c-Src or kd c-Src with WT Ca\textsubscript{1.2c} were similar (Fig. 6C). Collectively, these data suggest that c-Src expression promotes persistent Ca\textsuperscript{2+} sparklet activity.

c-Src phosphorylates Ca\textsubscript{1.2c} at residue Y\textsuperscript{2122} to produce persistent Ca\textsuperscript{2+} sparklets. To elucidate the mechanism by which c-Src enhances Ca\textsubscript{1.2c} activity, we tested the role of two potential phosphorylation sites (Y\textsuperscript{2122} and Y\textsuperscript{2139}) on the COOH terminus of rat Ca\textsubscript{1.2c} (2, 12). Coexpression of the Y\textsuperscript{2122}F Ca\textsubscript{1.2c} construct with c-Src (n = 19 cells) resulted in a 3.4-fold decrease in the density of persistent Ca\textsuperscript{2+} sparklets with respect to cells transfected with WT Ca\textsubscript{1.2c} + c-Src (n = 19 cells; P < 0.05; Fig. 7A). No such differences were observed between mean persistent Ca\textsuperscript{2+} sparklet densities of cells expressing Y\textsuperscript{2139}F Ca\textsubscript{1.2c} + c-Src and WT Ca\textsubscript{1.2c} + c-Src (P > 0.05; Fig. 7A). Neither of the single point mutations on Ca\textsubscript{1.2c} led to significant changes in the mean activities of persistent or low activity Ca\textsuperscript{2+} sparklets compared with cells expressing WT Ca\textsubscript{1.2c} + c-Src (Fig. 7, B and C). These findings suggest that Y\textsuperscript{2122} on the COOH terminus of Ca\textsubscript{1.2c} is the major phosphorylation site involved in production of persistent Ca\textsuperscript{2+} sparklets by c-Src.

PKC-\(\alpha\) does not induce persistent Ca\textsuperscript{2+} sparklets via c-Src. To test if PKC-\(\alpha\) induces persistent Ca\textsuperscript{2+} sparklet activity by activating endogenous c-Src, we performed Ca\textsuperscript{2+} sparklet experiments on cells coexpressing PKC-\(\alpha\) and Y\textsuperscript{2122}F Ca\textsubscript{1.2c}, Y\textsuperscript{2139}F Ca\textsubscript{1.2c}, or WT Ca\textsubscript{1.2c}. The mean persistent Ca\textsuperscript{2+} sparklet densities of the Y\textsuperscript{2122}F Ca\textsubscript{1.2c} + PKC-\(\alpha\) (n = 12 cells) and Y\textsuperscript{2139}F Ca\textsubscript{1.2c} + PKC-\(\alpha\) (n = 8 cells) transfection groups did not show any significant reduction compared with the WT Ca\textsubscript{1.2c} + PKC-\(\alpha\) (n = 9 cells) transfection group (Fig. 8A). Similarly, the mean nPs values of both low and persistent Ca\textsuperscript{2+} sparklets in cells expressing Y\textsuperscript{2122}F Ca\textsubscript{1.2c} + PKC-\(\alpha\) (n = 7 cells; P > 0.05) or Y\textsuperscript{2139}F Ca\textsubscript{1.2c} + PKC-\(\alpha\) (n = 7 cells; P > 0.05) were similar to those in cells expressing WT Ca\textsubscript{1.2c} + PKC-\(\alpha\) (n = 9 cells) (Fig. 8, B and C). Furthermore, inhibition of endogenous c-Src by PP2 (10 \(\mu\)M) did not change the activity (N = 4 sparklet sites; n = 3 cells) or the density (N = 3 sparklet sites; n = 3 cells) of PKC-\(\alpha\)-induced persistent Ca\textsuperscript{2+} sparklets in cells coexpressing PKC-\(\alpha\) and WT Ca\textsubscript{1.2c} (P > 0.05; Fig. 9, A and B). In contrast, PP2 application led to an increase in the activity of low activity Ca\textsuperscript{2+} sparklet sites (N = 9 sparklet sites; n = 7 cells; P < 0.05;
Fig. 9C). Overall, these data suggest that PKC-α does not induce persistent Ca\(^{2+}\) sparklet activity through c-Src.

**DISCUSSION**

The present study is the first to investigate the role of c-Src in the production of persistent Ca\(^{2+}\) sparklets. Our findings suggest that c-Src promotes persistent Ca\(^{2+}\) sparklets by phosphorylation of Ca\(_{1.2c}\) on residue Y\(^{2122}\). We could not find evidence that PKC-α acts via c-Src to induce persistent Ca\(^{2+}\) sparklet activity of Ca\(_{1.2c}\). The possibility of parallel actions of c-Src and PKC-α conforms to existing ideas about the complex regulation of Ca\(_{1.2}\) channels to fully explain their central roles in the regulation of many physiological functions.

One challenge that arose in the present study was dealing with the presence of anomalous Ca\(^{2+}\) events in untransfected tsa-201 cells. The origin of these anomalous events could not be resolved, but they might be related to the endogenous expression of Ca\(^{2+}\)-permeable transient receptor potential channels in this cell line. Similar events have been observed in Xenopus oocytes expressing N-type Ca\(^{2+}\) channels at negative (−60 to −120 mV) holding potentials and have been reported to have similar amplitudes, durations and spatial spread as N-type Ca\(^{2+}\) channel fluorescence events (7). Reportedly, the amplitudes of those anomalous events decreased with the application of depolarizing pulses that were used to record events associated with N-type Ca\(^{2+}\) channels. We could not use a similar strategy to eliminate anomalous Ca\(^{2+}\) events for at least two reasons: 1) Ca\(_{1.2}\) channels exhibit a higher open probability with membrane depolarization, which results in increased basal Ca\(^{2+}\) fluorescence (24); and 2) the driving force for Ca\(^{2+}\) decreases at higher potentials, leading to smaller changes in Ca\(^{2+}\) fluorescence upon Ca\(_{1.2}\) channel opening that are more difficult to resolve. Due to these limitations, we focused primarily on the analysis of persistent Ca\(^{2+}\) sparklet activity. The frequency of anomalous Ca\(^{2+}\) events resembling persistent Ca\(^{2+}\) sparklets was very low, thus making our results less prone to errors caused by the presence of such events among persistent Ca\(^{2+}\) sparklets in cells that expressed Ca\(_{1.2c}\) alone or with kinases.

**Effect of c-Src on persistent Ca\(^{2+}\) sparklet activity.** c-Src activation has previously been shown to increase whole cell Ca\(_{1.2c}\) current in response to insulin-like growth factor-I and αβ\(_1\) integrin activation (2, 12, 36), and c-Src has been implicated in the basal regulation of Ca\(_{1.2c}\) current as well (36). In the present study, we tested the involvement of c-Src in the production of persistent Ca\(^{2+}\) sparklet activity. Our results suggest that c-Src promotes persistent Ca\(^{2+}\) sparklet activity by acting on residue Y\(^{2122}\) of the COOH terminus of Ca\(_{1.2c}\) (Fig. 7). Of the various intracellular regions on rat Ca\(_{1.2c}\), only the region from amino acid residues 1,932 to 2,143, containing tyrosine residues at positions 2,122 and 2,139, is known to be phosphorylated by c-Src (2). Furthermore, previous studies have provided evidence for phosphorylation of Y\(^{2122}\) by c-Src, resulting in an increase in Ca\(_{1.2}\) channel current (2, 12, 36). Interestingly, in the present study, mutation of Y\(^{2122}\) on Ca\(_{1.2c}\) did not completely eliminate persistent Ca\(^{2+}\) sparklets (Fig. 7, A and B). There are at least two possible explanations for the residual persistent Ca\(^{2+}\) sparklet activity: 1) c-Src phosphorylates another tyrosine residue on Ca\(_{1.2c}\), or 2) the presence of another Ca\(_{1.2c}\) activating mechanism independent of c-Src. Here, we used PP2 treatment and kd c-Src overexpression to selectively inhibit c-Src-induced persistent Ca\(^{2+}\) sparklets. These c-Src inhibition strategies have been used successfully in previous studies (2, 12, 36), and both
approaches attenuated the occurrence of persistent $\text{Ca}^{2+}$ sparklets here (Figs. 4 and 6).

An interesting observation was that PP2 application significantly reduced both persistent $\text{Ca}^{2+}$ sparklet activity and density in cells expressing WT $\text{Ca}_{1.2c}$ and c-Src whereas the $Y^{2122}$F mutation on $\text{Ca}_{1.2c}$ or expression of $\text{kd}$ c-Src had an effect only on persistent $\text{Ca}^{2+}$ sparklet density. Exclusion of cells that did not show any sparklet activity (see MATERIALS AND METHODS) based on $nP_s$ analysis may have masked the attenuating effect of the $Y^{2122}$F mutation or $\text{kd}$ c-Src expression on $nP_s$ values. Another plausible explanation could be the presence of a population of persistent $\text{Ca}^{2+}$ sparklets regulated by another kinase like PKC in addition to that promoted by c-Src. Such a population of persistent $\text{Ca}^{2+}$ sparklets might not be affected by the $Y^{2122}$F mutation or $\text{kd}$ c-Src expression.

The activity of a channel at any given time is determined by the balance of the activity of specific kinases and phosphatases in its immediate vicinity. The balance between the local activities of PKC-α, PP2A, and PP2B has previously been suggested to produce different patterns of Cav1.2 activity across its plasma membrane, and these sites with differential activities have been classified as silent, low, and persistent $\text{Ca}^{2+}$ sparklet activity sites (23). Preferential phosphorylation of some Cav1.2 channels over the others by PKC-α, based on the colocalization of PKC-α with Cav1.2, has been suggested to be responsible for the production of localized persistent $\text{Ca}^{2+}$ sparklet activity (24). Immunofluorescence and confocal imaging experiments indicate that $\text{Ca}_{1.2c}$ has a diffuse distribution while PKC-α is distributed in clusters near the plasma membrane (24). Whether c-Src has a clustered distribution on the cell membrane under the conditions of our experiments is not known; however, c-Src has been shown in multiple studies to localize preferentially to focal adhesions (36, 20). Based on the observation that both low activity and persistent $\text{Ca}^{2+}$ sparklets occur in cells transfected with WT $\text{Ca}_{1.2c}$ and c-Src, we can speculate that c-Src, like PKC-α, regulates a small population of $\text{Ca}_{1.2c}$ channels on the plasma membrane. In that case, the activity of a sparklet site may be determined by the activities of nearby c-Src and possibly unidentified phosphatases.

Although the experiments in this study were performed on tsA-201 cells expressing $\text{Ca}_{1.2c}$, it is highly likely that c-Src also induces persistent $\text{Ca}^{2+}$ sparklet activity in native vascular smooth muscle cells. Most vascular smooth muscle cells have high expression levels of c-Src (30). c-Src activation has been shown to increase $\text{Ca}_{1.2c}$ current in rat arteriolar, rabbit portal vein, and human colonic smooth muscle cells (5, 12, 14 –17, 36). Because $\text{Ca}_{1.2c}$ and $\text{Ca}_{1.2b}$ share identical sequences (in rat) around residue $Y^{2122}$, these results are likely to be applicable to rat $\text{Ca}_{1.2b}$. Both rabbit and human $\text{Ca}_{1.2b}$ lack the tyrosine residue corresponding to $Y^{2122}$, suggesting that in
those species Cav1.2b is phosphorylated by c-Src at other tyrosine residues (17).

Mechanism of PKC-α action on persistent Ca^{2+} sparklet activity. PKC-α activation is linked to an increase in persistent Ca^{2+} sparklet activity in freshly isolated cerebral arterial myocytes and in tsA-201 cells expressing WT Ca_{1.2}c + PKC-α (23, 24); however, the mechanism by which PKC-α acts on Ca_{1.2} channels is not well understood. In this study, we tested whether c-Src acts downstream of PKC-α in the series of events that lead to increased persistent Ca^{2+} sparklet activity.

![Graph A](image1)

**Fig. 5.** Effect of PP2 (10 μM) on Ca^{2+} sparklets in tsA-201 cells expressing WT Ca_{1.2}c alone (n = 5). A: bar plot of means ± SE persistent Ca^{2+} sparklet density before and after PP2 application (N = 5). Bar plots of means nP_S ± SE of persistent (B; N = 8) and low activity (C; N = 13) Ca^{2+} sparklet sites before and after PP2 application. Error bars represent SE values; n, number of cells; N, number of sparklet sites; *P < 0.05.

![Graph B](image2)

![Graph C](image3)

**Fig. 6.** Ca^{2+} sparklet analysis in tsA-201 cells expressing WT Ca_{1.2}c with c-Src or kd c-Src. A: bar plot of means ± SE persistent Ca^{2+} sparklet density in cells expressing WT Ca_{1.2}c + c-Src (n = 9) and WT Ca_{1.2}c + kd c-Src (n = 6). Bar plots of mean nP_S ± SE of persistent (B) and low (C) activity Ca^{2+} sparklets in cells expressing WT Ca_{1.2}c + c-Src (n = 8) and WT Ca_{1.2}c + kd c-Src (n = 3). Error bars represent SE values; n, number of cells; *P < 0.05.
PKC has been shown to lie upstream of c-Src in signaling pathways controlling actin reorganization (3, 4), podosome formation (11), as well as in the regulation of smooth muscle Cav1.2 channels in smooth muscle (5). PKC may activate c-Src directly or indirectly, and c-Src activation mechanisms may also vary among the various PKC isoforms expressed by any particular cell type. If PKC-α acts solely via c-Src to produce persistent Ca\(^{2+}\) sparklets, mutation of Y2122 should have re-
After PP2

*rectly phosphorylates Cav1.2c to produce persistent Ca2

Cav1.2c by PKC-

By what alternative mechanisms could PKC-

PKA phosphorylation site on Ca1.2 channels (6, 10, 22, 33). However, some recent studies have challenged the role of S1928 phosphorylation in PKA-mediated potentiation of Cav1.2 current (8, 9, 19) by suggesting that PKA acts primarily by phosphorylation of a more proximal residue, S1700 to relieve the inhibition by the noncovalently associated distal COOH-terminal inhibitory domain (8, 37). The S1928 residue of Cav1.2 is also implicated as a PKG phosphorylation site using in vitro kinase assays, but PKG phosphorylation of S1928 does not seem to be involved in PKG-mediated inhibition of Cav1.2 current. In addition, biochemical experiments provide evidence for S1928 phosphorylation by PKC-α (38), but whether this phosphorylation event translates into increased channel function or sparklet activity remains unknown.

In conclusion, our data show that c-Src can enhance the activity of persistent Ca2+ sparklets in transfected tsA-201 cells through phosphorylation of Cav1.2c at residue Y2122. However, cells coexpressing Y2122F Cav1.2c and PKC-α continue to show an increase in persistent Ca2+ sparklet activity. These findings indicate that PKC-α does not act upstream of c-Src to produce persistent Ca2+ sparklets under these conditions.

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DISCLOSURES

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