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

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Address for reprint requests and other correspondence: M. J. Davis, 1 Hospital Dr., M451, School of Medicine, Univ. of Missouri-Columbia, Columbia, MO 65212 (e-mail: davismj@health.missouri.edu).

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—The activity of persistent Ca2+ sparklets, 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+ sparklets by PKC-α and c-Src, both of which increase whole cell Ca,1.2 current: 1) Does c-Src activation enhance persistent Ca2+ sparklet activity? 2) Does PKC-α activate c-Src to produce persistent Ca2+ sparklets? With the use of total internal reflection fluorescence microscopy, Ca2+ sparklets were recorded from voltage-clamped tsA-201 cells coexpressing wild-type (WT) or mutant Ca,1.2 (the neuronal isoform of Ca,1.2) constructs tagged with GFP, using jetPEI (Polyplus transfection). In all experiments, cells were transfected with either green fluorescent protein (GFP) or protein kinase constructs targeted with A-kinase anchoring protein 150 (AKAP150) is considered essential in this process (27).

However, the mechanism of PKC-α action on Ca,1.2 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 Ca,1.2α (corresponding to S1900 on Ca,1.2c), as a PKC phosphorylation site (36) but whether the phosphorylation of S1928 by PKC-α translates into an increase in channel function remains unresolved. Electrophysiological and imaging studies demonstrating the regulation of persistent Ca2+ sparklet activity by PKC-α have not addressed the question of whether PKC-α phosphorlylates the Ca,1.2α-subunit directly or acts via another kinase. While c-Src, another highly expressed kinase in SMCs, is known to directly phosphorylate the Ca,1.2 channel and potentiate whole cell Ca,1.2 current (2, 12, 14–17, 35, 36), its role in producing persistent Ca2+ sparklet activity through Ca,1.2 channels has not been explored. Regulation of persistent Ca2+ sparklet activity by c-Src may or may not be related to PKC-α as several isoforms of PKC have been shown to activate c-Src (3–5, 11). On the basis of this evidence, we hypothesized that c-Src regulates persistent Ca2+ sparklet activity and that at least part of the effect of PKC-α on persistent sparklet activity is mediated through c-Src.

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 Ca,1.2 channel subunits [wild-type (WT)/mutant α1C, β1a, and α2δ] along with either green fluorescent protein (GFP) or protein kinase constructs targeted with GFP, using jetPEI (Polyplus transfection). In all protocols, 840 ng of α1C cDNA and 200 ng each of α2δ 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 Ca$_{\text{v}}$1.2c cDNA does not alter the expression of Ca$_{\text{v}}$1.2c, we compared current densities in cells expressing Ca$_{\text{v}}$1.2c alone with those of cells coexpressing Ca$_{\text{v}}$1.2c and c-Src. Both groups were found to have similar current densities [WT Ca$_{\text{v}}$1.2c peak calcium current ($I_{\text{Ca}}$) = 8.99 ± 2.18 pA/pF (n = 18); and WT Ca$_{\text{v}}$1.2c + c-Src peak $I_{\text{Ca}}$ = 10.29 ± 2.66 pA/pF (n = 16)] indicating that cotransfection with a kinase cDNA does not significantly impact expression levels of Ca$_{\text{v}}$1.2c.

Rat neuronal WT α$_{\text{v}}$c (accession no. P22002), B$_{\text{v}}$, and α$_{\text{v}}$δ-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 α$_{\text{v}}$c-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 N-methyl-D-glucamine, 5 CsCl, 1 MgCl$_2$, 10 glucose, 10 HEPES, and 0.2 Rhod-2. After gigaseal formation, the bath solution was switched to a solution containing 20 mM CaCl$_2$ 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, 5 MgATP, 10 HEPES, and 0.2 Rhod-2. The pH of bath and patch solution were adjusted to 7.4 using HCl and 7.2 using CsOH, respectively. Patch pipettes filled with pipette solution exhibited resistances between 3 and 6 MΩ. 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 an Olympus PlanApo (×60, 1.45 NA) oil immersion objective and appropriate filter sets to separate excitation (491 nm for GFP and 563 nm for Rhod-2) and emission wavelengths. Images were acquired at a frequency >100 Hz using TILL imaging software (TILL Photonics, Munich, Germany), 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 Ca$^{2+}$ sparklet detection using the amplitude threshold criteria (23, 24). Briefly, a Ca$^{2+}$ 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 Ca$^{2+}$ sparklet activity and Ca$^{2+}$ sparklet density analysis.** To test our hypothesis, we compared two parameters across different test groups: mean Ca$^{2+}$ sparklet activity (nPs) and mean persistent Ca$^{2+}$ sparklet density.

In previous Ca$^{2+}$ sparklet studies (23–27), Ca$^{2+}$ sparklet activity was determined using nP$_{\text{S}}$ analysis (where $n$ = no. of quantal levels and P$_{\text{S}}$ = probability of occurrence of Ca$^{2+}$ sparklet), which is analogous to open probability (nP$_{\text{S}}$) analysis for single-channel data. We followed a similar approach in this study with one difference. Instead of converting fluorescence units to Ca$^{2+}$ concentration, we computed $\Delta F_{\text{total}}$ at each time point in Ca$^{2+}$ sparklet traces. First, the total fluorescence intensity, $F_{\text{total}}$, 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 Ca$^{2+}$ sparklet. Subsequently, $F_{\text{total}}$ of the basal fluorescence value was subtracted from $F_{\text{total}}$ observed during channel opening to determine $\Delta F_{\text{total}}$ value (1). The quantal $\Delta F_{\text{total}}$ value (2,500 arbitrary units), $q$, was calculated by plotting an all-points histogram of several Ca$^{2+}$ sparklet traces and then fitting the histogram with a multicomponent Gaussian function. Following that, nP$_{\text{S}}$ analysis was performed on Ca$^{2+}$ 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 Ca$^{2+}$ sparklet studies, we converted the fluorescence from some of our Ca$^{2+}$ sparklet traces to Ca$^{2+}$ concentration and obtained a Ca$^{2+}$ sparklet quantal value of 34.4 nM (data not shown); this quantal value was similar to that obtained in other Ca$^{2+}$ sparklet studies (23–25).

Persistent Ca$^{2+}$ sparklet density in each cell was determined by dividing the number of persistent Ca$^{2+}$ sparklet sites by the area of the cell visible in its TIRF image. Data from cells that were transfected with Ca$_{\text{v}}$1.2c but did not exhibit measurable $I_{\text{Ca}}$ 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 $I_{\text{Ca}}$, because it was not possible to calculate an accurate nPs value in such cases. However, those cells were accounted for in persistent Ca$^{2+}$ sparklet density analysis since any cell that did not exhibit persistent Ca$^{2+}$ 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 Ca$^{2+}$ sparklet density and Ca$^{2+}$ 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 Ca$^{2+}$ sparklet activities and persistent Ca$^{2+}$ sparklet densities of the control and test groups. A one-tail paired t-test was used to evaluate the effect of PP2 on Ca$^{2+}$ 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**

Ca$^{2+}$ fluorescence events in tsA-201 cells. TIRF imaging of cells expressing WT Ca$_{\text{v}}$1.2c channels confirmed the presence of Ca$^{2+}$ sparklets (Fig. 1). Based on their nPs values, Ca$^{2+}$ sparklets were characterized as either persistent (nP$_{\text{S}}$ ≥ 0.2) or low (nP$_{\text{S}}$ < 0.2) activity events. We also recorded Ca$^{2+}$ fluorescence events similar to Ca$^{2+}$ sparklets from cells expressing GFP alone in the absence of measurable $I_{\text{Ca}}$ upon depolarization (Fig. 2A). Since we had no objective reasons for excluding such anomalous events, they were analyzed in the same manner as Ca$^{2+}$ sparklets. Importantly, the mean number of anomalous event sites per cell in cells expressing GFP alone
Cav1.2c channels were responsible for all of the persistent Ca\textsuperscript{2+} events in nontransfected cells, we also observed anomalous Ca\textsuperscript{2+} events in cells expressing c-Src alone (Fig. 3, B). AU, arbitrary units.

To further test the involvement of c-Src, we coexpressed WT Cav1.1.2c with kinase dead c-Src (kd c-Src) in tsA-201 cells and c-Src were similar to those observed in cells expressing WT CA\textsubscript{V}1.1.2c 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\textsuperscript{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\textsuperscript{2+} sparklet activity through Cav1.1.2c by recording Ca\textsuperscript{2+} sparklets from cells coexpressing WT CA\textsubscript{V}1.1.2c and c-Src before and after treatment with 10 \mu M 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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} sparklets, we tested the effect of PP2 on Ca\textsuperscript{2+} sparklet activity in cells expressing WT CA\textsubscript{V}1.1.2c alone. Indeed, PP2 application decreased persistent Ca\textsuperscript{2+} sparklet density (Fig. 5A) while reducing Ca\textsuperscript{2+} sparklet activity significantly in cells expressing WT CA\textsubscript{V}1.1.2c 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\textsuperscript{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 CA\textsubscript{V}1.1.2c with kinase dead c-Src (kd c-Src) in tsA-201 cells and c-Src were similar to those observed in cells expressing WT CA\textsubscript{V}1.1.2c 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\textsuperscript{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).
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 Cav1.2c and kd c-Src, persistent Ca$$^{2+}$$ sparklet activity was detected in only one cell. Evaluation of persistent Ca$$^{2+}$$ sparklet density revealed a 3.4-fold reduction in cells transfected with WT Cav1.2c and kd c-Src (n = 6 cells) compared with cells coexpressing WT Cav1.2c and c-Src (n = 9 cells; P < 0.05; Fig. 6A). The means of persistent Ca$$^{2+}$$ 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 Cav1.2c + kd c-Src transfection group (Fig. 6B). As expected, the means for nPs of low activity Ca$$^{2+}$$ sparklets in cells coexpressing c-Src or kd c-Src with WT Cav1.2c were similar (Fig. 6C). Collectively, these data suggest that c-Src expression promotes persistent Ca$$^{2+}$$ sparklet activity.

c-Src phosphorylates Ca$$^{2+}$$ at residue Y2122 to produce persistent Ca$$^{2+}$$ sparklets. To elucidate the mechanism by which c-Src enhances Cav1.2c activity, we tested the role of two potential phosphorylation sites (Y2122 and Y2139) on the COOH terminus of rat Ca$$^{2+}$$ (2, 12). Coexpression of the Y2122F Cav1.2c construct with c-Src (n = 19 cells) resulted in a 3.4-fold decrease in the density of persistent Ca$$^{2+}$$ sparklets with respect to cells transfected with WT Cav1.2c + c-Src (n = 19 cells; P < 0.05; Fig. 7A). No such differences were observed between mean persistent Ca$$^{2+}$$ sparklet densities of cells expressing Y2139F Cav1.2c + c-Src and WT Cav1.2c + c-Src (P > 0.05; Fig. 7A). Neither of the single point mutations on Cav1.2c led to significant changes in the mean activities of persistent or low activity Ca$$^{2+}$$ sparklets compared with cells expressing WT Cav1.2c + c-Src (Fig. 7, B and C). These findings suggest that Y2122 on the COOH terminus of Cav1.2c is the major phosphorylation site involved in production of persistent Ca$$^{2+}$$ sparklets by c-Src.

PKC-$$\alpha$$ does not induce persistent Ca$$^{2+}$$ sparklets via c-Src. To test if PKC-$$\alpha$$ induces persistent Ca$$^{2+}$$ sparklet activity by activating endogenous c-Src, we performed Ca$$^{2+}$$ sparklet experiments on cells coexpressing PKC-$$\alpha$$ and Y2122F Cav1.2c, Y2139F Cav1.2c, or WT Cav1.2c. The mean persistent Ca$$^{2+}$$ sparklet densities of the Y2122F Cav1.2c + PKC-$$\alpha$$ (n = 12 cells) and Y2139F Cav1.2c + PKC-$$\alpha$$ (n = 8 cells) transfection groups did not show any significant reduction compared with the WT Cav1.2c + PKC-$$\alpha$$ (n = 9 cells) transfection group (Fig. 8A). Similarly, the mean nPs values of both low and persistent Ca$$^{2+}$$ sparklets in cells expressing Y2122F Cav1.2c + PKC-$$\alpha$$ (n = 7 cells; P > 0.05) or Y2139F Cav1.2c + PKC-$$\alpha$$ (n = 7 cells; P > 0.05) were similar to those in cells expressing WT Cav1.2c + PKC-$$\alpha$$ (n = 9 cells) (Fig. 8, B and C). Furthermore, inhibition of endogenous c-Src by PP2 (10 mM) 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$$^{2+}$$ sparklets in cells coexpressing PKC-$$\alpha$$ and WT Cav1.2c (P > 0.05; Fig. 9, A and B). In contrast, PP2 application led to an increase in the activity of low activity Ca$$^{2+}$$ sparklet sites (N = 9 sparklet sites; n = 7 cells; P < 0.05;
Fig. 3. Ca^{2+} sparklet analysis in tsA-201 cells expressing c-Src alone or with WT Ca_{1.2c}. 

**A:** bar plot of means ± SE persistent Ca^{2+} sparklet density in cells expressing WT Ca_{1.2c} + c-Src (n = 9) and c-Src alone (n = 9). Bar plots of mean nP, ± SE of persistent (B) and low (C) activity Ca^{2+} sparklets in cells expressing WT Ca_{1.2c} + c-Src (n = 8) or c-Src alone (n = 6). Error bars represent SE values; n, number of 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 Y2122. 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 αβ1 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 Y2122 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 Y2122 by c-Src, resulting in an increase in Ca_{1.2} channel current (2, 12, 36). Interestingly, in the present study, mutation of Y2122 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 
Ca\(^{2+}\) sparklets here (Figs. 4 and 6).

An interesting observation was that PP2 application significantly reduced both persistent Ca\(^{2+}\) sparklet activity and density in cells expressing WT Ca\(_{1.2}\)c and c-Src whereas the Y\(^{2122}\)F mutation on Ca\(_{1.2}\)c or expression of kd c-Src had an effect only on persistent Ca\(^{2+}\) sparklet density. Exclusion of cells that did not show any sparklet activity (see MATERIALS AND METHODS) based on nPs analysis may have masked the attenuating effect of the Y\(^{2122}\)F mutation or kd c-Src expression on nPs values. Another plausible explanation could be the presence of a population of persistent Ca\(^{2+}\) sparklets regulated by another kinase like PKC in addition to that promoted by c-Src. Such a population of persistent Ca\(^{2+}\) sparklets might not be affected by the Y\(^{2122}\)F mutation or 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-\(\alpha\), PP2A, and PP2B has previously been suggested to produce different patterns of Cav1.2 activity across the plasma membrane, and these sites with differential activities have been classified as silent, low, and persistent Ca\(^{2+}\) sparklet activity sites (23). Preferential phosphorylation of some Ca\(_{1.2}\) channels over the others by PKC-\(\alpha\), based on the colocalization of PKC-\(\alpha\) with Ca\(_{1.2}\), has been suggested to be responsible for the production of localized persistent Ca\(^{2+}\) sparklet activity (24). Immunofluorescence and confocal imaging experiments indicate that Ca\(_{1.2}\) channels have a diffuse distribution while PKC-\(\alpha\) 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 Ca\(^{2+}\) sparklets occur in cells transfected with WT Ca\(_{1.2}\)c and c-Src, we can speculate that c-Src, like PKC-\(\alpha\), regulates a small population of Ca\(_{1.2}\) 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 Ca\(_{1.2}\)c, it is highly likely that c-Src also induces persistent 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 Ca\(_{1.2}\) current in rat arteriolar, rabbit portal vein, and human colonic smooth muscle cells (5, 12, 14–17, 36). Because Ca\(_{1.2}\)c and Ca\(_{1.2}\)b share identical sequences (in rat) around residue Y\(^{2122}\), these results are likely to be applicable to rat Ca\(_{1.2}\)b. Both rabbit and human Ca\(_{1.2}\)b lack the tyrosine residue corresponding to Y\(^{2122}\), suggesting that in

![Diagram](http://apjcell.physiology.org/)

**Fig. 4.** Effect of PP2 (10 \(\mu\)M) on Ca\(^{2+}\) sparklets in tsA-201 cells coexpressing WT Ca\(_{1.2}\)c + c-Src (\(n = 5\)). A: representative traces of persistent and low activity Ca\(^{2+}\) sparklets before and after application of PP2 (10 \(\mu\)M). B: bar plot of means \(\pm\) SE persistent Ca\(^{2+}\) sparklet density before and after PP2 application (\(N = 5\)). Bar plots of mean nPs, \(\pm\) SE of persistent (C, \(N = 5\)) and low activity (D, \(N = 6\)) Ca\(^{2+}\) sparklet sites before and after PP2 application. Error bars represent SE values; \(n\), number of cells; \(N\), number of sparklet sites; *\(P < 0.05\).
those species Ca,1.2b is phosphorylated by c-Src at other tyrosine residues (17).

Mechanism of PKC-α action on persistent Ca²⁺ sparklet activity. PKC-α activation is linked to an increase in persistent Ca²⁺ sparklet activity in freshly isolated cerebral arterial myocytes and in tsA-201 cells expressing WT Ca,1.2c + 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 Ca²⁺ sparklet activity.

![Graph A](image1.png)

**Fig. 5.** Effect of PP2 (10 μM) on Ca²⁺ sparklets in tsA-201 cells expressing WT Ca,1.2c alone (n = 5). A: bar plot of means ± SE persistent Ca²⁺ sparklet density before and after PP2 application (N = 5). Bar plots of means nPs ± SE of persistent (B; N = 8) and low activity (C; N = 13) Ca²⁺ 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.png)

**Fig. 6.** Ca²⁺ sparklet analysis in tsA-201 cells expressing WT Ca,1.2c with c-Src or kd c-Src. A: bar plot of means ± SE persistent Ca²⁺ sparklet density in cells expressing WT Ca,1.2c + c-Src (n = 9) and WT Ca,1.2c + kd c-Src (n = 6). Bar plots of mean nPs ± SE of persistent (B) and low (C) activity Ca²⁺ sparklets in cells expressing WT Ca,1.2c + c-Src (n = 8) and WT Ca,1.2c + 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 Y\(^{2122}\) should have re-

Fig. 7. Ca\(^{2+}\) sparklet analysis in tsA-201 cells coexpressing c-Src with WT, Y\(^{2122}\)F or Y\(^{2139}\)F Cav1.2c constructs. A: bar plot of means ± SE persistent Ca\(^{2+}\) sparklet density in cells expressing WT Ca1.2c + c-Src (n = 19), Y\(^{2122}\)F Ca1.2c + c-Src (n = 19), and Y\(^{2139}\)F Ca1.2c + c-Src (n = 17). Bar plots of mean nPs ± SE of persistent (B) and low (C) activity Ca\(^{2+}\) sparklets in cells expressing WT Ca1.2c + c-Src (n = 17), Y\(^{2122}\)F Ca1.2c + c-Src (n = 17), and Y\(^{2139}\)F Ca1.2c + c-Src (n = 12). Error bars represent SE values; n, number of cells; *P < 0.05.

Fig. 8. Ca\(^{2+}\) sparklet analysis in tsA-201 cells coexpressing PKC-α with WT, Y\(^{2122}\)F or Y\(^{2139}\)F Cav1.2c constructs. A: bar plot of means ± SE persistent Ca\(^{2+}\) sparklet density in cells expressing WT Ca1.2c + PKC-α (n = 9), Y\(^{2122}\)F Ca1.2c + PKC-α (n = 12), and Y\(^{2139}\)F Ca1.2c + PKC-α (n = 8). Bar plots of mean nPs ± SE of persistent (B) and low (C) activity Ca\(^{2+}\) sparklets in cells expressing WT Ca1.2c + PKC-α (n = 9), Y\(^{2122}\)F Ca1.2c + PKC-α (n = 7), and Y\(^{2139}\)F Ca1.2c + PKC-α (n = 7). Error bars represent SE values; n, number of cells.
PKC-α directly phosphorylates Ca1.2c to produce persistent Ca2+ sparklet density before and after PP2 application (N = 3; n = 3). Bar plots of mean nPs ± SE of persistent Ca2+ sparklet density before and after PP2 application (B; N = 4; n = 3) and low activity (C; N = 9; n = 7) Ca2+ sparklet sites before and after PP2 application. Error bars represent SE values; n, number of cells; N, number of sparklet sites; *P < 0.05.

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 Ca1.2 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 Ca1.2 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.

By what alternative mechanisms could PKC-α induce persistent Ca2+ sparklet activity? It is possible that PKC-α directly phosphorylates Ca1.2c to produce persistent Ca2+ sparklets. The NH2 terminus of the cardiac Ca1.2 isoform has been implicated in both PKC-mediated stimulation and inhibition of Ca1.2 (21, 31, 32), but the NH2 terminus of the (neuronal) Ca1.2c isoform lacks the potential serine and threonine PKC phosphorylation sites of the Ca1.2a (cardiac) and Ca1.2b (smooth muscle) isoforms. The occurrence of persistent Ca2+ sparklets in cells expressing WT Ca1.2c + PKC-α suggests that phosphorylation of the NH2 terminus of Ca1.2c by PKC-α is not required to produce persistent Ca2+ sparklet activity. Site S1904 on Ca1.2c (S1928 on Ca1.2a and b) is conserved among different Ca1.2 channel isoforms in different species and has long been considered the primary 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 Ca1.2 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 Ca1.2 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 Ca1.2c at residue Y2122. However, cells coexpressing Y2122F Ca1.2c 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
No conflicts of interest, financial or otherwise, are declared by the author(s).
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REFERENCES


