Ser\textsuperscript{1928} phosphorylation by PKA stimulates the L-type Ca\textsuperscript{2+} channel Ca\textsubscript{V}1.2 and vasoconstriction during acute hyperglycemia and diabetes

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Hypercontractility of arterial myocytes and enhanced vascular tone during diabetes are, in part, attributed to the effects of increased glucose (hyperglycemia) on L-type Ca\textsubscript{V}1.2 channels. In murine arterial myocytes, kinase-dependent mechanisms mediate the increase in Ca\textsubscript{V}1.2 activity in response to increased extracellular glucose. We identified a subpopulation of the Ca\textsubscript{V}1.2 channel pore-forming subunit (α\textsubscript{1C}) within nanometer proximity of protein kinase A (PKA) at the sarcolemma of murine and human arterial myocytes. This arrangement depended upon scaffolding of PKA by an A-kinase anchoring protein 150 (AKAP150) in mice. Glucose-mediated increases in Ca\textsubscript{V}1.2 channel activity were associated with PKA activity, leading to α\textsubscript{1C} phosphorylation at Ser\textsuperscript{1928}. Compared to arteries from low-fat diet (LFD)–fed mice and nondiabetic patients, arteries from high-fat diet (HFD)–fed mice and from diabetic patients had increased Ser\textsuperscript{1928} phosphorylation and Ca\textsubscript{V}1.2 activity. Arterial myocytes and arteries from mice lacking AKAP150 or expressing mutant AKAP150 unable to bind PKA did not exhibit increased Ser\textsuperscript{1928} phosphorylation and Ca\textsubscript{V}1.2 current density in response to increased glucose or to HFD. Consistent with a functional role for Ser\textsuperscript{1928} phosphorylation, arterial myocytes and arteries from knockin mice expressing a Ca\textsubscript{V}1.2 with Ser\textsuperscript{1928} mutated to alanine (S1928A) lacked glucose-mediated increases in Ca\textsubscript{V}1.2 activity and vasoconstriction. Furthermore, the HFD-induced increases in Ca\textsubscript{V}1.2 current density and myogenic tone were prevented in S1928A knockin mice. These findings reveal an essential role for α\textsubscript{1C} phosphorylation at Ser\textsuperscript{1928} in stimulating Ca\textsubscript{V}1.2 channel activity and vasoconstriction by AKAP-targeted PKA upon exposure to increased glucose and in diabetes.

INTRODUCTION

Diabetes mellitus is a major risk factor underlying multiple pathological complications such as stroke, hypertension, heart disease, and retinal degeneration (1). These pathologies have been linked, in part, to enhanced contractility of arterial myocytes in the resistance vasculature during hyperglycemia, that is, high blood glucose that is a signature metabolic feature of diabetes (2–7). The contractile state of arterial myocytes, and thereby arterial diameter, is predominantly controlled by the amount of Ca\textsuperscript{2+} influx through L-type Ca\textsubscript{V}1.2 channels. We reported that Ca\textsubscript{V}1.2 channel activity in murine cerebral arterial myocytes is augmented in response to increased glucose and in a mouse model of diabetes through a process that is independent of changes in membrane potential (8). However, the molecular mechanisms underlying the enhanced Ca\textsubscript{V}1.2 channel function associated with increased glucose and whether similar phenomena are engaged in the human vasculature during diabetes are unclear.

The physiological importance of Ca\textsubscript{V}1.2 channels is underscored by their essential role in cardiac contractility, neuronal excitability, and control of vascular tone in health and disease (9,10). Ca\textsubscript{V}1.2 channels are composed of pore-forming α\textsubscript{1C} subunits in association with several regulatory accessory subunits (11). Regulation of Ca\textsubscript{V}1.2 function occurs through reversible phosphorylation of the channel complex by serine/threonine kinases and phosphatases, including protein kinase A (PKA) (10–12). Modulation of Ca\textsubscript{V}1.2 channel function by PKA has been extensively studied in the context of β-adrenergic stimulation in the heart and brain (9,12). Although PKA-dependent stimulation of Ca\textsubscript{V}1.2 channels occurs in myocytes from arteries and portal veins (13–15), the molecular mechanisms and functional implications are unknown.

Regulation of Ca\textsubscript{V}1.2 channel activity by PKA in other cell types involves direct phosphorylation of one or more subunits that compose the channel (16). A highly conserved PKA consensus phosphorylation site is Ser\textsuperscript{1928}, located within the intracellular C terminus of α\textsubscript{1C}. Stimulation of Ca\textsubscript{V}1.2 by PKA activity has been associated with increased phosphorylation of this residue in neurons (17–20) and cardiac cells (19), although its physiological significance in cardiac Ca\textsubscript{V}1.2 channel function is controversial (21,22). However, evidence for this modification and elucidation of its functional role in the vasculature are lacking.

PKA-dependent stimulation of heterologously expressed (23), as well as native cardiac (24,25) and neuronal (20,26,27), Ca\textsubscript{V}1.2 channels requires an A-kinase anchoring protein (AKAP) scaffold. Consistent with these findings, we demonstrated that potentiation of Ca\textsubscript{V}1.2 channels in arterial myocytes during acute increases in extracellular d-glucose requires an AKAP-bound PKA (8). Yet, the functional implications of PKA-dependent stimulation of Ca\textsubscript{V}1.2 channels in response to increased glucose, as well as the identity of the AKAP, are unknown. Here, super-resolution nanoscopy and analysis by proximity ligation assay (PLA) revealed the existence of a subpopulation of Ca\textsubscript{V}1.2 and PKA catalytic subunit (PKA\textsubscript{cat}) clusters that are within nanometer range of each other in mouse and human arterial myocytes. We identified the
scaffold protein AKAP150 (the murine ortholog of human AKAP79) and the binding of PKA to AKAP150 as key determinants of PKA-mediated α1C phosphorylation at Ser1928. PKA-mediated phosphorylation of this residue resulted in potentiation of vascular CaV1.2 channel activity and vasoconstriction in response to increased extracellular d-glucose. Enhanced CaV1.2 channel activity and intracellular Ca2+ concentrations ([Ca2+]i), which augmented vasoconstriction in response to increased d-glucose and in mice fed a high-fat diet (HFD), were abolished upon prevention of Ser1928 phosphorylation by selective mutation of this residue to alanine in S1928A knockin mice. We also observed PKA-dependent phosphorylation of α1C at Ser1928 and enhanced CaV1.2 channel activity in human arterial myocytes in response to increased extracellular d-glucose and in human cells from diabetic subjects, thus highlighting the translational relevance of our findings. Together, these results revealed a fundamental role for AKAP-dependent, PKA-mediated phosphorylation of α1C as a key molecular signaling event underlying potentiation of CaV1.2 channel activity and vasoconstriction upon acute increases in extracellular d-glucose and in diabetes.

RESULTS

Super-resolution spatial maps and PLA reveal sarcolemmal CaV1,2-PKA interactions

Enhanced CaV1.2 channel activity in rodent cerebral arterial myocytes after an increase in extracellular d-glucose depends upon PKA activity (8). We therefore hypothesized that a sub-population of PKA is located near CaV1.2 channels in these cells, enabling functional regulation of channel activity. To test this, we examined the spatial relationship between CaV1.2 and PKA in freshly dissociated cerebral arterial myocytes from wild-type mice using stochastic optical reconstruction microscopy (STORM) in the total internal reflection fluorescence (TIRF) configuration with antibodies against CaV1.2 (28) and PKAcat. This method achieves a lateral resolution of ~30 nm with selective axial illumination of sarcolemma and subsarcolemmal regions. Although conventional TIRF images showed diffuse and regionally varied fluorescence associated with CaV1.2 and PKAcat (Fig. 1A, top), STORM-rendered spatial maps revealed that both proteins were broadly distributed in clusters of various sizes (CaV1.2, 2485 ± 47 nm2; PKAcat, 3272 ± 72 nm2) at the sarcolemma of arterial myocytes [Fig. 1, A (bottom) and B]. We observed sites of close interaction between CaV1.2 and PKAcat (Fig. 1C). Histograms of the CaV1.2-to-nearest PKAcat distances were fit with a two-component Gaussian function with centers at ~93 and ~256 nm (Fig. 1D), suggesting that a pool of PKA is localized closer to a sub-population (~5 to 10%) of CaV1.2 channels.

We used PLA as an additional test of CaV1.2-PKA association in arterial myocytes. PLA generates fluorescent puncta when proteins of interest are at or less than 40 nm apart (29). Mouse arterial myocytes labeled for CaV1.2 and PKAcat (Fig. 1A, top) showed diffuse and regionally varied fluorescence associated with both proteins. When proteins of interest are at or less than 40 nm apart (29), PLA signals were nearly absent when either CaV1.2 or PKAcat primary antibodies were omitted (Fig. 1, E and F). PLA was also performed on cells

![Fig. 1. Spatial organization of CaV1,2 and PKA in murine arterial myocytes.](http://stke.sciencemag.org/)
colabeled for CaV1.2 and transferrin receptor (TfR) or CaV1.2 and the Ca2+-activated Cl− channel anoctamin 1 (ANO1), which are cell surface proteins that have limited to no interaction with CaV1.2 (30–33). Arterial myocytes were robustly stained for TR and ANO1 when primary antibodies were included in the preparation (fig. S1A), yet no PLA signal was detected in cells colabeled for CaV1.2 and TfR or CaV1.2 and ANO1 (fig. S1, B and C). As a positive control, cells colabeled with two distinct CaV1.2 antibodies [rabbit FP1 (28) and monoclonal NeuroMab clone N263/31] showed surface membrane staining (fig. S1A) and produced robust PLA signals (fig. S1, B and C). Together, these data indicated close (≤40 nm) spatial proximity between a subpopulation of CaV1.2 channels and PKAcat in mouse arterial myocytes.

**AKAP150 facilitates sarcolemmal interactions between CaV1.2 and PKA in murine arterial myocytes**

The scaffolding protein AKAP150, which is the murine ortholog of human AKAP79, binds and coordinates a multienzyme complex that includes PKA, protein kinase C (PKC), and the protein phosphatase calcineurin to facilitate signaling to specific substrates, including CaV1.2 (20, 26, 27, 34–37). We found that the PKA RIIα regulatory subunit and PKCa coimmunoprecipitated with AKAP150 in arteries from wild-type mice (Fig. 2A). We tested whether the AKAP150-PKA complex facilitates the proximity between PKAcat and CaV1.2 in arterial myocytes by examining their distribution in cells isolated from two genetically engineered mouse strains. One expresses a truncated form of AKAP150 in which the binding site for PKA has been deleted (ΔA; fig. S2) (26, 38), and the other lacks the entire AKAP150 protein (AKAP150−/−) (37). The association between AKAP150 and PKA RIIα was either absent (AKAP150−/−) or markedly reduced (ΔA) in arteries from these mice (Fig. 2A). Coimmunoprecipitation of PKCa within the AKAP150 complex was maintained in ΔA arteries but was absent in the arteries from the AKAP150−/− mice (Fig. 2A).

We used structured illumination microscopy (SIM; lateral resolution, ~114 nm) (39) in combination with an object-based analysis to compare CaV1.2 and PKAcat proximity in arterial myocytes from wild-type, ΔA, and AKAP150−/− animals. Consistent with findings we obtained by STORM, object-based analysis identified sites of spatial overlap between CaV1.2 and PKAcat at the sarcolemma of wild-type arterial myocytes (Fig. 2, B to D). In contrast, cells from ΔA and AKAP150−/− mice exhibited significantly fewer sites of overlap between CaV1.2 and PKAcat (Fig. 2, B and D). Furthermore, ΔA and AKAP150−/− myocytes had a reduction in the density of PKAcat but not CaV1.2 particles at the sarcolemma identified by STORM/TIRF (Fig. 2E). These findings suggest that AKAP150 facilitates targeting of a pool of PKAcat to a subpopulation of CaV1.2 in arterial myocytes.

**Enhanced vascular CaV1.2 current in 20 mM d-glucose requires AKAP150-anchored PKA**

We tested the hypothesis that increasing extracellular d-glucose from 10 to 20 mM augments CaV1.2 channel activity in an AKAP150- and PKA-dependent manner in mouse arterial myocytes. These extracellular d-glucose concentrations are within the range of observed...
enhanced CaV1.2 and metabolic utilization. Note that there was no change in CaV1.2 channel activity in response to 20 mM D-glucose requires its internalization and metabolic utilization.

Contrary to findings in wild-type arterial myocytes, 20 mM D-glucose did not stimulate I_{Ba} that were elicited with a voltage pulse from −70 to +10 mV in AKAP150−/− and ΔA myocytes (Fig. 3, C and D). Note that there was no change in I-V relationship by 20 mM D-glucose in AKAP150−/− cells (Fig. S5) with V_{max} similar to that in wild-type cells (V_{max} = 8.75 ± 1.26 mV in wild-type and V_{max} = 7.98 ± 2.06 mV in AKAP150−/−; P = 0.7836, extra sum-of-squares F test). Consistent with the electrophysiological data, we observed an increase in [Ca^{2+}]_i in cells from wild-type mice loaded with the Ca^{2+}-sensitive indicator Fluo-4 in response to 20 mM D-glucose (Fig. 3, E and F). This response was not observed in the presence of nifedipine or when D-glucose was substituted with L-glucose (fig. S6), and it was significantly attenuated in AKAP150−/− and ΔA cells (Fig. 3, E and F). Together, these findings indicated that AKAP150-anchored PKA signaling is required for enhanced CaV1.2 channel activity and an increase in global [Ca^{2+}] in response to increasing concentration of extracellular D-glucose.

We also investigated whether AKAP150-anchored PKC participates in enhancing I_{Ba} in response to increasing extracellular D-glucose to 20 mM with a transgenic mouse expressing a form of AKAP150 that cannot bind PKC, yet retains binding to PKA (ΔC; Fig. 4A and fig. S2). Consistent with results in arteries from AKAP150−/− mice (37), basal tone in pressurized (60 mmHg) middle cerebral arteries was slightly reduced in the ΔC mice, and receptor-mediated activation of PKC with angiotensin II evoked less constriction in arteries from ΔC mice than in arteries from littermate controls (Fig. 4, B to D). Moreover, angiotensin II did not increase I_{Ba} in arterial myocytes from ΔC mice, yet I_{Ba} in these cells was significantly augmented by 20 mM D-glucose (Fig. 4, E and F). These data indicated that AKAP150-anchored PKA, but not AKAP150-anchored PKC, is responsible for enhanced CaV1.2 channel activity in response to increased D-glucose in arterial myocytes.

Phosphorylation of α1C at Ser^{1928} underlies enhanced vascular CaV1.2 activity in response to 20 mM D-glucose

We tested whether increased CaV1.2 channel activity was associated with an alteration in the phosphorylation state of the α1C subunit at nonfasting blood glucose concentrations reported for non-diabetic and experimental diabetic mice, respectively (3, 8, 40). To assess the effects of increased extracellular glucose on CaV1.2 channel activity, we used patch-clamp electrophysiology in the whole-cell configuration with barium (Ba^{2+}) as the charge carrier before and after application of the L-type calcium channel blocker nifedipine to determine the nifedipine-sensitive Ba^{2+} current (I_{Ba}) associated with CaV1.2 channel activity. We found that 20 mM D-glucose produced a robust increase in I_{Ba} across a range of membrane potentials with no change in the current-voltage (I-V) relationship (V_{max} = 8.75 ± 1.26 mV for 10 mM D-glucose and V_{max} = 9.45 ± 0.56 mV for 20 mM D-glucose; P = 0.6174, extra sum-of-squares F test) in arterial myocytes (Fig. 3, A and B). We also observed a similar increase in I_{Ba} in response to 20 mM D-glucose in mesenteric and femoral arterial myocytes (fig. S3, A and B), suggesting that increased extracellular D-glucose potentiates CaV1.2 current density in myocytes from different vascular beds. Incubation of arterial myocytes with the selective inhibitor of the glucose transporter GLUT4 indinavir (41) prevented the increase in CaV1.2 current density in response to 20 mM D-glucose (fig. S4A). Furthermore, I_{Ba} was unchanged when D-glucose was substituted with equimolar concentrations of the metabolically inactive enantiomer L-glucose (10 mM D-glucose + 10 mM L-glucose; fig. S4B), suggesting that enhanced CaV1.2 channel activity in response to increased D-glucose requires its internalization and metabolic utilization.

Fig. 3. Enhanced CaV1.2 channel activity and elevation of global [Ca^{2+}], in arterial myocytes in response to 20 mM D-glucose require anchoring of PKA by AKAP150. (A) Representative I_{Ba} in WT arterial myocytes evoked by 200-ms depolarization steps from holding potential of −70 mV to voltages ranging from −20 to +40 mV in 10 mM D-glucose (D-glu) and 5 min after application of 20 mM D-glucose. (B) I_{Ba}-voltage relationship (−60 to +60 mV) in WT arterial myocytes in the presence of 10 and 20 mM D-glucose (n = 8 cells; **P < 0.05, paired t test). (C) Representative I_{Ba} from ΔA and AKAP150−/− arterial myocytes in response to step depolarizations from −70 to +10 mV before and after application of 20 mM D-glucose. (D) IAMamagated I_{Ba} (to +10 mV) before and after application of 20 mM D-glucose in WT, ΔA, and AKAP150−/− arterial myocytes (n = 8 to 11 cells; *P < 0.05, paired t test). (E) Exemplary [Ca^{2+}]^\text{in} traces in isolated WT, AKAP150−/−, and ΔA arterial myocytes in the presence of 10 mM D-glucose and in response to 20 mM D-glucose in shaded time frame. (F) Plot of change in Ca^{2+} (Δ[Ca^{2+}]/[Ca^{2+}]/F_{0}) to 20 mM D-glucose in isolated WT (n = 7 cells), AKAP150−/− (n = 8 cells), and ΔA (n = 7 cells) arterial myocytes (*P < 0.05; one-way analysis of variance (ANOVA) with Tukey post hoc test).
Ser<sup>1928</sup>, which is a conserved PKA target site in the distal C terminus and which has been implicated in Cav<sub>1.2</sub> regulation (17–19). Using Western blot analysis with a specific antibody that recognizes α<sub>C</sub> phosphorylation at Ser<sup>1928</sup> (42), we found increased phosphorylation of this residue in wild-type mouse arterial lysates incubated for 5 min with 20 mM D-glucose as compared with those with 10 mM D-glucose (Fig. 5, A and B). In contrast to wild-type arteries, no change in Ser<sup>1928</sup> phosphorylation in response to 20 mM D-glucose was observed in arterial lysates from ΔA or AKAP150<sup>−/−</sup> mice (Fig. 5, A and B). These results suggested that AKAP150-anchored PKA is required for phosphorylation of α<sub>C</sub> at Ser<sup>1928</sup> in response to increased D-glucose.

Considering an apparent relationship in molecular and functional data described above, we postulated that Ser<sup>1928</sup> serves as a functional PKA regulatory site in vascular Cav<sub>1.2</sub> channels. This hypothesis was tested using patch-clamp electrophysiology to record I<sub>Ba</sub> in arterial myocytes isolated from a genetically engineered mouse expressing Cav<sub>1.2</sub> in which Ser<sup>1928</sup> has been mutated to an alanine residue (S1928A) (21). Our data revealed that I<sub>Ba</sub> was not altered by an increase in extracellular D-glucose in S1928A myocytes (Fig. 5, C and D). Pretreatment of wild-type myocytes with the PKA inhibitor Rp-cyclic 3′,5′-adenosine monophosphate (cAMPS) also blocked the glucose-induced stimulation of I<sub>Ba</sub> (Fig. 5, C and D). In contrast to the response of wild-type cells, we found no significant change in global [Ca<sup>2+</sup>] in response to 20 mM D-glucose in cells from S1928A mice (Fig. 5E). We confirmed that protein abundance of Kv1.2, BK<sub>α</sub>, and BK<sub>β</sub> subunits, which regulate arterial myocyte excitability and are glucose-sensitive (43–46), is similar between wild-type and S1928A arteries (fig. S7), thus suggesting that loss of glucose-induced stimulation of global Ca<sup>2+</sup> in S1928A myocytes is not due to changes in basal expression of these K<sup>+</sup> channel subunits.

Increases in extracellular D-glucose could modulate protein function by promoting O-linked N-acetylglucosamine (O-GlcNAc) (47, 48). Therefore, we examined whether posttranslational O-GlcNAc modification modulates Cav<sub>1.2</sub> channel activity in response to increased D-glucose by treating wild-type cells with the inhibitor of glutamine-fructose amidotransferase, 6-diazo-5-oxo-L-norleucine (DON), and measuring the effect of glucose on I<sub>Ba</sub>. Inhibition of this enzyme prevented production of the substrate for O-GlcNAc transferase. Perfusion of 20 mM D-glucose enhanced I<sub>Ba</sub> in wild-type mouse arterial myocytes in the presence of DON, indicating that the effect of glucose on Cav<sub>1.2</sub> channel activity was not mediated by O-GlcNAcylation (fig. S8).

To explore the functional significance of the glucose-induced Ser<sup>1928</sup> phosphorylation, we performed measurements of arterial diameter in pressurized (60 mmHg) middle cerebral arteries in the presence of 10 and 20 mM D-glucose. Arteries isolated from wild-type mice developed stable myogenic tone and constricted in response to 20 mM D-glucose (Fig. 5F). This constriction response was independent of endothelial function, because we observed a similar response in wild-type endothelium-denuded arteries (fig. S9). Although basal myogenic tone was not significantly different in arteries from S1928A and wild-type mice, the response to 20 mM D-glucose was abolished in arteries from S1928A mice (Fig. 5F).

Together, data indicated that phosphorylation of α<sub>C</sub> at Ser<sup>1928</sup> by AKAP150-anchored PKA is necessary for increased Cav<sub>1.2</sub> channel activity, global [Ca<sup>2+</sup>], and vasoconstriction in response to 20 mM D-glucose.

**The single point mutation S1928A restores normal Cav<sub>1.2</sub> activity and vascular tone in HFD animals.**

We tested whether phosphorylation of α<sub>C</sub> at Ser<sup>1928</sup> is enhanced in mice on an HFD. Wild-type, AKAP150<sup>−/−</sup>, ΔA, and S1928A animals...
were maintained on either a low-fat diet (LFD) (10% kcal fat) or HFD (60% kcal fat) for a period of 12 to 16 weeks. This HFD model recapitulates clinical features observed in patients with type 2 diabetes (49, 50), including enhanced arterial tone and increased blood pressure (3, 40). HFD animals exhibited significantly higher body weight and increased blood glucose concentration (table S1). Arteries and arterial myocytes from LFD and HFD mice were isolated, maintained, and immediately used in experiments with extracellular solutions containing 10 mM D-glucose. We found that α1C at Ser1928 was significantly more phosphorylated in arteries from wild-type HFD mice compared with those from wild-type LFD mice (Fig. 6A and B). Additionally, arteries from the LFD and HFD ΔΔa mice had similar amounts of Ser1928 phosphorylation, which were not different compared with arteries from wild-type LFD mice (Fig. 6A and B). Arterial myocytes from wild-type HFD mice exhibited significant enhancement of Iba over a range of membrane potentials with no change in I-V relationship (Vmax = 7.66 ± 0.87 mV for LFD and Vmax = 6.94 ± 1.21 mV for HFD; P = 0.7075, extra sum-of-squares F test) (Fig. 6C and fig. S10). Yet, enhanced Iba was absent in arterial myocytes from AKAP150−/−, ΔΔa, or S1928A HFD mice (Fig. 6C and fig. S10).

We also examined the relevance of Ser1928 phosphorylation on the development of myogenic tone in diabetic mice. Vasconstriction in response to 60 mM K+, in which membrane potential closely follows the equilibrium potential for K+ (−20 mV) (51), was significantly higher in arteries from wild-type HFD mice, but not in arteries from S1928A HFD mice, compared with those from corresponding LFD mice (table S2). In line with other reports (3, 40), arteries from wild-type HFD mice consistently developed greater myogenic tone compared to arteries from wild-type LFD mice over a range of intravascular pressures (Fig. 6D and E). Arterial tone development was not different between arteries from HFD or LFD wild-type mice in the presence of the L-type calcium channel blocker nifedipine (fig. S11), suggesting that the greater myogenic response in HFD mice requires CaV1.2 activity. In contrast to the findings in arteries from wild-type mice, arterial tone was similar between arteries from S1928A mice on LFD and HFD at all intravascular pressures tested (Fig. 6D and E). Together, these findings indicated a key role for AKAP150-anchored PKA, leading to phosphorylation of α1C at Ser1928 in stimulation of CaV1.2 channel activity in HFD mice. Our results suggested that substitution of a single amino acid (Ser to Ala at residue 1928) to block Ser1928 phosphorylation in the α1C is sufficient to prevent the increase in CaV1.2 channel activity and vascular tone in HFD mice.

**PKA mediates phosphorylation of α1C at Ser1928 and increased CaV1.2 activity in native human arterial myocytes from diabetic subjects**

To test the translational significance of our observations, we analyzed native human arterial myocytes and arteries from non diabetic and diabetic patients (table S3). In native contractile human arterial myocytes...
DISCUSSION

Many studies have revealed impaired myogenic autoregulation that may contribute to increased vascular reactivity in the diabetic state (7). This has been associated, at least in part, with enhanced contractility of arterial myocytes in response to increased blood d-glucose (hyperglycemia) during diabetes (2–6). The mechanisms underlying this hypercontractility remain the subject of intense investigation, but links have been made to changes in PKC and Rho kinase signaling (52, 53), transcriptional remodeling due to activation of the prohypertensive transcription factor NFATc3 (3, 54), and reduced K⁺ channel abundance and channel activity (3, 40, 55, 56). The results from nondiabetic patients, STORM/TIRF and PLA imaging revealed a subpopulation of CaV1.2 within close proximity to PKAcat (Fig. 7, A and B, and fig. S12), similar to our results in murine arterial myocytes (Fig. 1). We tested a role for PKA in Ser1928 phosphorylation and CaV1.2 channel activity in response to increasing extracellular d-glucose from 5 to 15 mM in native human arterial myocytes from nondiabetic patients. We selected these concentrations because they are comparable to the blood glucose concentrations observed in nondiabetic and diabetic subjects. We found that 15 mM d-glucose increased I[Na] in cells from nondiabetic human subjects (Fig. 7, C and D). Global [Ca²⁺] also significantly increased in response to 15 mM d-glucose in these cells, and this effect was attenuated by inhibition of PKA with rpcAMPS (Fig. 7, E and F). Moreover, incubation with 15 mM d-glucose for 5 min stimulated a significant increase in phosphorylation of α1C at Ser1928 in intact arteries from the nondiabetic subjects (Fig. 7, G and H). Preincubating the arteries in rpcAMPS abolished this increase, indicating that Ser1928 phosphorylation during increased d-glucose depends upon PKA activity.

Consistent with our findings in HFD animals, we found that phosphorylation of α1C at Ser1928 was significantly increased in arterial lysates prepared from diabetic compared to those from nondiabetic human subjects, without any change in total CaV1.2 protein abundance (Fig. 8A). We recorded single L-type Ca²⁺ channel activity in the cell-attached configuration from freshly isolated arterial myocytes from nondiabetic and diabetic human subjects. Cells from diabetic subjects had significantly enhanced L-type Ca²⁺ channel open probability (nPo) (Fig. 8B and C). Preincubation of the cells from diabetic subjects with rpcAMPS significantly reduced L-type Ca²⁺ channel nPo to values similar to those observed in cells from nondiabetic patients (Fig. 8B and C), suggesting that PKA plays a key role in stimulation of L-type Ca²⁺ channel activity during diabetes in human subjects. Together, our results are consistent with a mechanistic model whereby PKA activity determines phosphorylation of α1C at Ser1928, leading to functional enhancement of L-type Ca²⁺ channel activity in human arterial myocytes in response to increased d-glucose and in diabetes.
of the present study provide strong support for an additional model in which direct phosphorylation of $\alpha_{1C}$ at Ser$^{1928}$ by an AKAP150-anchored PKA plays a key role in stimulating $\text{Ca}^{2+}$ influx through $\text{Ca}_{v1.2}$ channels, which leads to vasocnstriction during increases in extracellular glucose and in diabetes (Fig. 8D).

A central observation in this study was that increasing the concentration of glucose triggered an increase in global $[\text{Ca}^{2+}]_{i}$ in arterial myocytes, leading to vasocnstriction through a PKA-dependent pathway. These findings are paradoxical, because PKA activation plays a well-established role in arterial myocyte relaxation in response to endogenous and exogenous vasodilatory agents (57–61). Data reported here provide insight into this paradox by supporting a role for precise subsarcolemmal compartmentalization of a subpopulation of PKA in $\text{Ca}_{v1.2}$ channel regulation and vasocnstriction. Accordingly, targeting PKA to $\text{Ca}_{v1.2}$ channels by scaffolding proteins, such as AKAPs, may provide a means for selective phosphorylation of the channel to promote increased global $\text{Ca}^{2+}$ and vasocnstriction rather than vasodilation. When PKA is tethered by an AKAP, dissociation of the catalytic subunit may not be required for substrate phosphorylation (62). Hence, association with the AKAP complex may be indispensable for spatial limitation of kinase activity within discrete cellular compartments. To extend the range of activity of an AKAP-anchored PKA, intrinsically disordered regions within RIIs subunits afford a high degree of flexibility within the anchored PKA holoenzyme, giving the catalytic subunits a ~20-nm radius of motion. Considering the length of antibodies used to label proteins (~10 to 15 nm), the intermolecular distances between $\text{Ca}_{v1.2}$ and PKAcat reported in the current study are conceivably within the limits required for selective modulation of $\text{Ca}_{v1.2}$ channel activity by PKA-mediated phosphorylation to promote contraction. In contrast, stimuli that induce cell-wide activation of PKA, such as forskolin, or activate a different PKA pool to stimulate other substrates, such as the phosphorylation of K$^{+}$ channels in response to $\beta$-adrenergic agonists (61, 63), may cause relaxation. Therefore, we predict that the spatial and temporal characteristics of the activated PKA signal determine the functional effects of PKA activity on vascular reactivity.

Another fundamental finding was that enhanced $\text{Ca}_{v1.2}$ channel activity in arterial myocytes in response to increases in extracellular D-glucose and in diabetes relies on phosphorylation of $\alpha_{1C}$ at Ser$^{1928}$. This is a highly conserved residue among $\alpha_{1C}$ subunits and is phosphorylated by PKA in response to various stimuli, such as $\beta$-adrenergic signals (17–21). Here, we showed that, in murine arterial myocytes and arteries, PKA-dependent phosphorylation of $\alpha_{1C}$ at Ser$^{1928}$ enhances vascular $\text{Ca}_{v1.2}$ channel activity and vasocnstriction in response to increased extracellular D-glucose and in diabetic mice. Similar increases
in PKA-mediated Ser\textsuperscript{1928} phosphorylation, Ca\textsubscript{V}1.2 channel activity, and global Ca\textsuperscript{2+} signals were found in nondiabetic human arteries and myocytes in response to increasing the concentration of glucose and in arteries and myocytes from diabetic human subjects, thus highlighting the consistency of the results from the murine model with human pathology. In agreement with an important role for Ser\textsuperscript{1928}, we found that this single Ser-to-Ala substitution, which precludes phosphorylation of this site in S1928A knockin mice (21), prevented the enhancement in Ca\textsubscript{V}1.2 channel activity and vasoconstriction in response to increased D-glucose. Moreover, enhanced Ca\textsubscript{V}1.2 channel activity and myogenic tone in an HFD mouse model of diabetes were blocked in S1928A knockin mice on HFD, with no effect on basal Ca\textsubscript{V}1.2 channel function. A reduction in K\textsuperscript+ channel function has also been proposed to promote membrane depolarization and vasoconstriction in response to acute increases in extracellular D-glucose (55, 56). Our data showing no functional impact of acute high glucose on arterial diameter in arteries from S1928A mice argue that direct phosphorylation of α\textsubscript{1}C at Ser\textsuperscript{1928}, leading to membrane potential–dependent increase in [Ca\textsuperscript{2+}], is required for enhanced vasoconstriction. However, this Ca\textsubscript{V}1.2-dependent response could also act in concert with mechanisms of membrane potential dysregulation to exacerbate vascular tone in diabetes.

The essential influence of Ser\textsuperscript{1928} on Ca\textsubscript{V}1.2 channel activity during PKA signaling in arterial myocytes is conserved in neurons, as revealed by Qian et al. (64). However, this phosphorylation event does not seem to be important for PKA-dependent regulation of Ca\textsubscript{V}1.2 channel activity in cardiac cells (21). β-Adrenergic stimulation of Ca\textsubscript{V}1.2 channels in ventricular myocytes from S1928A mice was normal, suggesting that Ser\textsuperscript{1928} phosphorylation by PKA is not necessary for sympathetic stimulation of channel activity and contraction in murine hearts (21). In contrast, Qian et al. report that S1928A mice have impaired long-term potentiation and compromised β-adrenergic–mediated stimulation of Ca\textsubscript{V}1.2 activity by PKA in neurons, demonstrating a key role for Ser\textsuperscript{1928} in β-adrenergic regulation of Ca\textsubscript{V}1.2 channel activity and neuronal function (64). The molecular mechanisms underlying these marked tissue-specific differences are unclear but may involve functional heterogeneity among distinct tissue-specific channel splice variants, a unique phosphorylation profile of α\textsubscript{1}C, posttranslational regulation of other interacting proteins at distinct sites, or differential interaction with or regulation by different scaffold proteins and accessory subunits.

Ser\textsuperscript{1928} can also be phosphorylated by PKC (65), and activation of PKC increases Ca\textsubscript{V}1.2 channel activity in arterial myocytes (66, 67). Enhanced activity of this kinase has also been associated with increased Ca\textsubscript{V}1.2 channel activity and vascular reactivity during hypertension (68). We speculate that increased Ca\textsubscript{V}1.2 channel activity during hypertension could be attributed to PKC-dependent phosphorylation of the same target residue. Thus, Ser\textsuperscript{1928} could represent a point of convergence to regulate Ca\textsubscript{V}1.2 channel function by diverse signaling pathways. Furthermore, this phosphorylation site may be a distinct critical site of action for signal transduction in arterial myocytes, as well as a potential therapeutic target to treat vascular complications in diabetes and perhaps other pathological conditions.

Our previous studies have demonstrated that AKAP150 is necessary for regulation of vascular Ca\textsubscript{V}1.2 channel activity by receptor-stimulated signals, such as angiotensin II (37, 68). This potent vasoconstrictor acts on G\textsubscript{q}-coupled AT\textsubscript{1} receptors to stimulate phospholipase C, generate diacylglycerol, and activate PKC. AKAP150 associates with AT\textsubscript{1} receptors (69), and binding of PKC to AKAP150 is necessary for both acute and chronic angiotensin II stimulation of vascular Ca\textsubscript{V}1.2 channels (37). Here, we confirmed the importance of AKAP150-bound PKC in modulating Ca\textsubscript{V}1.2 channel activity and vascular reactivity during angiotensin II signaling using the ΔC knockin mouse. We also discovered that anchored PKC was not necessary for enhanced Ca\textsubscript{V}1.2 channel activity in response to increased D-glucose. In contrast, the AKAP150 mutant lacking the PKA-binding site (ΔA) failed to enhance D-glucose–mediated phosphorylation of α\textsubscript{1}C at Ser\textsuperscript{1928} to promote Ca\textsubscript{V}1.2 channel activity. Although the upstream mechanisms by which an increase in extracellular
D-glucose leads to activation of AKAP150-anchored PKA in arterial myocytes are unclear, research from several groups suggested that glucose might stimulate a G\textsubscript{\textalpha} signal (70, 71). This would activate adenylyl cyclase to produce cyclic adenosine 3',5'-monophosphate (cAMP), which leads to cAMP-dependent activation of PKA. The incorporation of AKAP150 in this pathway may coordinate the clustering of specialized G protein (heterotrimeric guanine nucleotide–binding protein)–coupled receptors, G proteins, and the downstream effectors to enable the decoding of different stimuli.

The augmented Ca\textsuperscript{2+} influx observed in cells exposed to prolonged increases in extracellular D-glucose or cells from animals or humans with diabetes may also activate a prohypertensive transcriptional cascade involving calcineurin and NFATc3 (Fig. 8D) (54, 68, 72, 73). Consistent with this, NFATc3 signaling is activated in an HFD mouse model of diabetes through a process that requires AKAP150-anchored calcineurin and Ca\textsuperscript{2+} influx through Ca\textsubscript{V1.2} channels (3). However, the abundance of Ca\textsubscript{V1.2} in arteries from wild-type HFD mice (3) and humans with diabetes (Fig. 8A) is similar to that in arteries from the corresponding LFD mice and nondiabetic subjects. Yet, NFATc3 activation leads to selective reduction in BK\textsubscript{Ca} \( \beta \) and \( \kappa \text{v}2.1 \) subunit abundance and function, which may further contribute to enhanced vascular tone during diabetes (3, 40). Thus, we propose that AKAP150-anchored PKA and phosphorylation of \( \alpha_1 \text{C} \) at Ser\textsuperscript{1928}, leading to increased Ca\textsubscript{V1.2} channel activity during chronic increases in extracellular D-glucose and diabetes, may activate AKAP150-anchored calcineurin to promote NFATc3 nuclear accumulation, transcriptional changes, and vascular dysfunction. Findings of restored myogenic tone in S1928A HFD animals may reflect the absence of an effect of diabetes on PKA-mediated Ca\textsubscript{V1.2} channel stimulation and a normalization of the steady-state global [Ca\textsuperscript{2+}], as well as minimization of K\textsuperscript{+} channel remodeling and normalization of membrane potential regulation through prevention of downstream NFATc3-dependent signaling. Therefore, regulatory events that are independent of membrane potential (PKA-mediated potentiation of Ca\textsubscript{V1.2} channel activity) and those that are dependent on membrane potential (down-regulation of K\textsuperscript{+} channel activity, leading to depolarization and further Ca\textsubscript{V1.2} channel activation) may both modulate arterial myocyte [Ca\textsuperscript{2+}], and contractility during diabetes.

We found that Ca\textsubscript{V1.2} channel activity is higher in cells and arteries from HFD mice and human subjects with diabetes even when measurements were performed in the presence of normal low concentrations of D-glucose. These results are similar to those observed in arterial myocytes from a db/db mouse model of diabetes (8). Both Navedo et al. (8) and we here found that these differences were eliminated by PKA inhibition, suggesting that increased Ca\textsubscript{V1.2} channel function is the result of higher PKA activity in cells from diabetic animals and human subjects, thus providing evidence for “molecular memory.” At present, it is unclear whether this increase in PKA activity results from altered upstream signaling (for example, activity of adenylyl cyclase), impaired activity of a phosphodiesterase, or changes in PKA subunit abundance or regulation.

In summary, our data indicated that, upon an increase in extracellular glucose, phosphorylation of \( \alpha_1 \text{C} \) at Ser\textsuperscript{1928} by AKAP-anchored PKA is essential for enhanced Ca\textsubscript{V1.2} channel activity and increased global [Ca\textsuperscript{2+}]. Because contractility of arterial myocytes depends upon changes in global [Ca\textsuperscript{2+}], higher Ca\textsubscript{V1.2} channel open probability in response to increased glucose and during diabetes may stimulate vasoconstriction in the resistance vasculature. Thus, activation of this pathway may have profound implications for tissue perfusion and blood pressure regulation during chronic hyperglycemia and diabetes.

**Materials and Methods**

**Animals**

Male mice (5 to 8 weeks of age) were used for this study in strict accordance with protocols approved by the Animal Care and Use Committee of the University of California, Davis. In some experiments, mice were maintained on either an LFD (10% kcal) or HFD (60% kcal) (Research Diets) starting at 5 weeks of age for 12 to 16 weeks. The composition of these diets and the propensity of mice maintained on this HFD to develop type 2 diabetes and induce vascular dysfunction of small resistance arteries have been well documented in other studies (3, 50, 74). Wild-type (C57BL/6J), AKAP150\textsuperscript{1C} (C57BL/6J), and knockin mice expressing AKAP150 lacking its PKA RI binding site (\( \Delta \alpha; \) C57BL/6J) or PKC (\( \Delta \alpha; \) C57BL/6J) or in which Ser\textsuperscript{1928} of \( \alpha_1 \text{C} \) was mutated to Ala (S1928A) were euthanized by intraperitoneal injection of sodium pentobarbital (250 mg/kg). Pial cerebral arteries were acutely dissected, and arterial myocytes were isolated using enzymatic digestion techniques as described (3, 75). For some experiments, third-order mesenteric and femoral arteries were isolated, and arterial myocytes were dissociated following the procedure above. Arteries and arterial myocytes were maintained in ice-cold nominally Ca\textsuperscript{2+}-free Ringer’s solution (5 mM KCl, 140 mM NaCl, 2 mM MgCl\textsubscript{2}, 10 mM Hepes, and 10 mM D-glucose adjusted to pH 7.4 with NaOH) containing normal 10 mM D-glucose until use.

**Human Arteries and Cells**

Excised human arteries from surgical patients undergoing gastric bypass procedure were obtained after institutional review board (IRB) approval and written consent and in accordance with the guidelines of the Declaration of Helsinki. Because our IRB exemption requires the use of noncoded tissue, a detailed therapeutic profile of the patient is unknown. Available patient information is included in table S3. Tissue was collected and placed in cold phosphate-buffered saline (PBS) solution containing normal D-glucose [138 mM NaCl, 3 mM KCl, 10 mM Na\textsubscript{2}HPO\textsubscript{4}, 2 mM NaH\textsubscript{2}PO\textsubscript{4}, 5 mM D-glucose, 0.1 mM CaCl\textsubscript{2}, and 0.1 mM MgSO\textsubscript{4} (pH adjusted to 7.4)] and transferred to the laboratory. Small-diameter (~150- to 250-\( \mu \)m-diameter) arteries were carefully dissected out of surrounding tissue and used for experimentation. Arterial myocytes were enzymatically isolated using a two-step digestion process as described (3, 75). Cells were maintained in ice-cold nominally Ca\textsuperscript{2+}-free Ringer’s solution with normal D-glucose concentration (5 mM) until use.

**Immunolabeling and STORM**

Isolated arterial myocytes were allowed to adhere to a coverslip before fixing with PBS containing 3% paraformaldehyde + 0.1% glutaraldehyde followed by 0.1% sodium borohydride. After washing with PBS, cells were permeabilized and blocked with 0.5% Triton X-100 and 3% bovine serum albumin and exposed overnight to custom antibodies against Ca\textsubscript{V1.2} (28, 76) and antibody against PKA\textsubscript{cat} \( \alpha, \beta, \gamma \) (Santa Cruz Biotechnology). For secondary antibodies, we used a goat FLIP 565–conjugated antibody recognizing rabbit immunoglobulin G (IgG) and goat Alexa Fluor 647–conjugated antibody recognizing mouse IgG. The specificity of secondary antibodies to detect corresponding primary antibodies was tested in control experiments in which cells were only treated with secondary antibodies before imaging (–1° antibody control). We used a Nikon N-STORM super-resolution microscopy system to capture images after TIRF illumination with 561- and 640-nm laser light. During imaging, cells were bathed in PBS (pH 7.4) containing glucose oxidase (0.56 mg/ml), catalase (0.3 mg/ml), and cysteamine (10 mM). A series of 20,000 images were acquired using a Plan Apochromat.
TIRF 100× oil immersion lens [numerical aperture (NA), 1.49] and an iXon3 DU-897E electron-multiplying charge-coupled device (EMCCD) camera (Andor Technology). STORM images were rendered by plotting molecule localization as a Gaussian that was proportional to the number of photons released per photoswitching event. The full width at half maximum measured for single-molecule signals was ~31 nm. Reconstructions were filtered to reject localizations generated from less than 600 photons or from events that were detected on eight or greater consecutive frames. Half-maximal intensity for single molecules in reconstructed images was used to set a threshold for binary analysis, and the shortest intermolecular distance was determined for CaV1.2 and PKAcat in freshly dissociated arterial myocytes. Cells were then permeabilized with 0.1% Triton X-100 (20 min) and blocked (1 hour, 37°C) in 50% Odyssey blocking solution (LI-COR Biosciences). Cells were incubated overnight with a specific combination of primary antibodies [mouse antibody against PKAcat (Santa Cruz Biotechnology, sc-365615, 1:1000), custom rabbit antibody specific for CaV1.2 (1:1000) (28, 76), monoclonal antibody specific for CaV1.2 (NeuroMab, clone N263/31 AB_11000167, 1:1000) and TIR (Thermo Scientific, clone H684.4, #13-6800, 1:1000), and goat antibody against ANO1 (Santa Cruz Biotechnology, clone S-20, sc-69343, 1:1000)] in Duolink antibody diluent solution. As a control, cells were incubated with only one primary antibody. Secondary antibodies conjugated with oligonucleotides (PLA probes, antibody against rabbit PLUS and antibody against mouse MINUS) were used to detect CaV1.2 and PKA, CaV1.2 and TIR, and CaV1.2 and ANO1 (1 hour, 37°C). After incubation with probes, a ligation solution consisting of two distinct oligonucleotides and ligase was added and incubated for 30 min at 37°C to allow hybridization and formation of a circular DNA template at sites of dual labeling. The ligation step was followed by a rolling circle amplification reaction (100 min, 37°C) using the ligated circle as a template. The ligation reaction was followed by washing in Duolink buffer B (2 × 10 min) and in 1% buffer B (1 × 10 min). Coverslips were allowed to dry and subsequently mounted on a microscope slide with Duolink mounting medium. The fluorescence signal was visualized using an Olympus FV1000 confocal microscopy system on an Olympus IX81 microscope with a 60× oil immersion lens (NA, 1.54). Images were acquired at different optical planes (z-axis step size, 0.5 μm). The stack of images for each sample was then combined into a single-intensity projection image that was subsequently used for analysis of number of puncta per square micrometer per cell. For presentation, representative PLA images at the center of the z axis are shown. Before labeled specimens were imaged, photomultiplier gain and laser power parameters were determined using control cells in which primary antibodies were omitted. Images for all conditions were obtained using the same acquisition parameters.

**Immunofluorescence**

Immunofluorescence labeling of freshly isolated arterial myocytes was performed as described (37) using a monoclonal antibody against CaV1.2 (NeuroMab, clone N263/31; 1:1000) and TIR (Thermo Scientific, clone H684.4; 1:1000) and goat antibody against ANO1 (Santa Cruz Biotechnology, clone S-20; 1:1000). The secondary antibody was either a donkey Alexa Fluor 568–conjugated antibody against mouse (5 mg/ml) or donkey Alexa Fluor 568–conjugated antibody against goat (5 mg/ml) from Molecular Probes. Cells were imaged (512 × 512–pixel images) using an Olympus FV1000 confocal microscope coupled with an Olympus 60× water immersion lens (NA, 1.2) and a zoom of 3.0 (pixel size, 0.138 μm). Images were collected at multiple optical planes (z-axis step size, 0.35 μm). The specificity of the primary antibody was tested in negative control experiments in which the primary antibody was substituted with PBS. CaV1.2-, TIR-, and ANO1-associated fluorescence was not detected under this experimental condition. The same laser power, gain settings, and pinhole were used to image arterial myocytes under different experimental conditions.

**Proximity ligation assay**

A Duolink In Situ PLA kit was used to detect complexes consisting of CaV1.2 and PKAcat in freshly dissociated arterial myocytes. Cells were plated on glass coverslips and allowed to adhere (30 min, room temperature) before fixing with 4% paraformaldehyde (20 min), quenching in 100 mM glycine (15 min), and washing in PBS (2 × 3 min). Cells were then permeabilized with 0.1% Triton X-100 (20 min) and blocked (1 hour, 37°C) in 50% Odyssey blocking solution (LI-COR Biosciences). Cells were incubated overnight with a specific combination of primary antibodies [mouse antibody against PKAcat (Santa Cruz Biotechnology, sc-365615, 1:1000), custom rabbit antibody specific for CaV1.2 (1:1000) (28, 76), monoclonal antibody specific for CaV1.2 (NeuroMab, clone N263/31 AB_11000167, 1:1000) and TIR (Thermo Scientific, clone H684.4, #13-6800, 1:1000), and goat antibody against ANO1 (Santa Cruz Biotechnology, clone S-20, sc-69343, 1:1000)] in Duolink antibody diluent solution. As a control, cells were incubated with only one primary antibody. Secondary antibodies conjugated with oligonucleotides (PLA probes, antibody against rabbit PLUS and antibody against mouse MINUS) were used to detect CaV1.2 and PKA, CaV1.2 and TIR, and CaV1.2 and ANO1 (1 hour, 37°C). After incubation with probes, a ligation solution consisting of two distinct oligonucleotides and ligase was added and incubated for 30 min at 37°C to allow hybridization and formation of a circular DNA template at sites of dual labeling. The ligation step is followed by a rolling circle amplification reaction (100 min, 37°C) using the ligated circle as a template. The ligation reaction was followed by washing in Duolink buffer B (2 × 10 min) and in 1% buffer B (1 × 10 min). Coverslips were allowed to dry and subsequently mounted on a microscope slide with Duolink mounting medium. The fluorescence signal was visualized using an Olympus FV1000 confocal microscopy system on an Olympus IX81 microscope with a 60× oil immersion lens (NA, 1.54). Images were acquired at different optical planes (z-axis step size, 0.5 μm). The stack of images for each sample was then combined into a single-intensity projection image that was subsequently used for analysis of number of puncta per square micrometer per cell. For presentation, representative PLA images at the center of the z axis are shown. Before labeled specimens were imaged, photomultiplier gain and laser power parameters were determined using control cells in which primary antibodies were omitted. Images for all conditions were obtained using the same acquisition parameters.

**Electrophysiology**

Freshly isolated arterial myocytes were allowed to adhere to a glass coverslip in a recording chamber for 10 min. CaV1.2 channel activity was examined using the perforated whole-cell patch-clamp technique to record macroscopic currents with Ba2+ as a charge carrier. The pipette solution contained 120 mM CsCl, 20 mM tetraethylammonium chloride (TEA-Cl), 1 mM EGTA, and 20 mM Hepes with amphotericin B (250 μg/ml; pH adjusted to 7.2 with CsOH). Cells were bathed in a solution containing 115 mM NaCl, 10 mM TEA-Cl, 0.5 mM MgCl₂, 10 mM d-glucose, 5 mM CsCl, 20 mM BaCl₂, and 20 mM Hepes (pH adjusted to 7.4 with CsOH). All electrophysiological experiments were performed at 7 to 23°C. Experiments were terminated by application of the experimental solution containing 1 μM nifedipine to determine the nifedipine-sensitive component, which is produced by CaV1.2 channels containing α₁C subunits. To obtain the I-V relationship of nifedipine-sensitive Ba2+ currents, we depolarized cells for 200 ms from the holding potential of −70 mV to voltages ranging from −60 to +60 mV. The I-V relationship for averaged data sets was fit with a peak Gaussian function: (V) = I_Imax × exp(−0.5((V − Vmax)/b)²), where Imax is peak I, Vmax is V at Imax and b is the slope of the distribution, as described previously (79).

Single-channel Ca2+ currents were recorded from arterial myocytes in the cell-attached configuration of the patch-clamp technique as described...
Briefly, data were acquired at a sampling rate of 50 μs and low pass-filtered at 2 kHz with an Axopatch 200B amplifier and Digidata 1440 digitizer (Molecular Devices). Data were subsequently filtered with a Gaussian filter (400 Hz) during analysis. Pipettes were pulled from borosilicate capillary glass using a micropipette puller (model P-97, Sutter Instruments). Pipettes were polished to achieve resistances that range from 3.5 to 6.5 megohms. The pipette solution contained 120 mM 

![Image](https://example.com/image.png)
REFERENCES AND NOTES


Ser^{1928} phosphorylation by PKA stimulates the L-type Ca^{2+} channel Ca_{V1.2} and vasoconstriction during acute hyperglycemia and diabetes

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