Protein Kinase Cθ Negatively Regulates Store-independent Ca²⁺ Entry and Phosphatidylserine Exposure Downstream of Glycoprotein VI in Platelets*

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Platelet activation must be tightly controlled to provide an effective, but not excessive, response to vascular injury. Cytosolic calcium is a critical regulator of platelet function, including granule secretion, integrin activation, and phosphatidylserine (PS) exposure. Here we report that the novel protein kinase C isoform, PKCθ, plays an important role in negatively regulating Ca²⁺ signaling downstream of the major collagen receptor, glycoprotein VI (GPVI). This limits PS exposure and so may prevent excessive platelet procoagulant activity. Stimulation of GPVI resulted in significantly higher and more sustained Ca²⁺ signals in PKCθ⁻/⁻ platelets. PKCθ acts at multiple distinct sites. PKCθ limits secretion, reducing autocrine ADP signaling that enhances Ca²⁺ release from intracellular Ca²⁺ stores. PKCθ thereby indirectly regulates activation of store-operated Ca²⁺ entry. However, PKCθ also directly and negatively regulates store-independent Ca²⁺ entry. This pathway, activated by the diacylglycerol analogue, 1-oleoyl-2-acetyl-*sn*-glycerol, was enhanced in PKCθ⁻/⁻ platelets, independently of ADP secretion. Moreover, LOE-908, which blocks 1-oleoyl-2-acetyl-*sn*-glycerol-induced Ca²⁺ entry but not store-operated Ca²⁺ entry, blocked the enhanced GPVI-dependent Ca²⁺ signaling and PS exposure seen in PKCθ⁻/⁻ platelets. We propose that PKCθ normally acts to restrict store-independent Ca²⁺ entry during GPVI signaling, which results in reduced PS exposure, limiting platelet procoagulant activity during thrombus formation.

The central role of platelets in both physiological hemostasis and pathological thrombosis means that platelet activation must be tightly controlled. Blood vessel damage or atherosclerotic plaque rupture exposes subendothelial matrix proteins, in particular collagens, to flowing blood, leading to platelet adhesion and activation (1, 2). Activated platelets expose phosphatidylserine (PS),[2] which is an efficient surface for assembly of the tenase (FIXa/VIIIa) and prothrombinase (FXa/Va) complexes

(3), generating a burst of thrombin that is responsible for producing a stable hemostatic clot or an occlusive thrombus.

PS-exposing platelets are essential to thrombus growth in arterioles and, to a lesser extent, in venules (4). Restricting platelet PS exposure may therefore restrict thrombus growth.

A rise in intracellular calcium concentration ($[Ca^{2+}]_i$) is required for many platelet responses. Sustained Ca²⁺ signaling is required for platelets to expose PS and accelerate thrombin generation (5–8). Loss of plasma membrane asymmetry takes several minutes, so a sustained increase in intracellular Ca²⁺ is required. If $[Ca^{2+}]_i$ is restored to basal levels, membrane asymmetry can be restored (9). Tight control of Ca²⁺ signaling therefore closely regulates PS exposure and platelet-dependent thrombin generation, and any factor that negatively regulates sustained Ca²⁺ signaling may reduce PS exposure and limit occlusive thrombus development.

Most platelet activators induce Ca²⁺ release from intracellular Ca²⁺ stores via the second messenger, inositol 1,4,5-trisphosphate (10). The initial transient increase in $[Ca^{2+}]_i$ due to Ca²⁺ release is amplified Ca²⁺ entry from the extracellular medium. Ca²⁺ entry is also likely to be required to provide a sufficiently sustained signal to induce prolonged PS exposure, because agonist-induced Ca²⁺ release is usually transient (11). Store-operated Ca²⁺ entry (SOCE), a major pathway for Ca²⁺ entry into platelets and other nonexcitable cells, is activated by a decrease in the Ca²⁺ content of the intracellular Ca²⁺ stores (12–14). Platelets also express at least one store-independent Ca²⁺ entry pathway, activated by diacylglycerol (DAG) (15). The relative contributions of these different Ca²⁺ entry pathways to agonist-induced Ca²⁺ signaling and Ca²⁺-dependent platelet responses, in particular PS exposure, are less well understood. Moreover, how Ca²⁺ signaling is regulated during platelet activation, to provide sufficient signal without excessive PS exposure, is not known.

Protein kinase C (PKC) isoforms have long been known to play important roles in platelet activation. Although often considered a positive regulator of platelet activation, PKC may also negatively regulate some signaling events (16). In particular, it has been suggested that PKC negatively regulates platelet Ca²⁺ signaling (17). We have previously reported that the novel PKC isoform, PKCθ, negatively regulates platelet activation downstream of the major collagen receptor, glycoprotein VI (GPVI), with PKCθ⁻/⁻ platelets displaying enhanced collagen-related peptide (CRP)-induced integrin activation (18) and granule secretion (19) at low agonist concentrations. Here, we have

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[2] The abbreviations used are: PS, phosphatidylserine; ARC, AR-C69931M-X; BIM, bisindolylmaleimide I; $[Ca^{2+}]_i$, intracellular Ca²⁺ concentration; CRP, collagen-related peptide; DAG, diacylglycerol; GPVI, glycoprotein VI; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PLC, phospholipase C; PKC, protein kinase C; SOCE, store-operated Ca²⁺ entry; WT, wild type; AUC, area under curve.

investigated whether PKCθ regulates GPVI-dependent Ca^{2+} signaling in mouse platelets. In particular, we report that PKCθ negatively regulates store-independent Ca^{2+} entry, which leads to higher sustained [Ca^{2+}]$_i$ levels in PKCθ$^{-/-}$ platelets and enhanced PS exposure, revealing a novel mechanism by which platelet procoagulant activity may be tightly controlled.

EXPERIMENTAL PROCEDURES

Materials—Unless stated, all materials were from Sigma and were of analytical grade. Cross-linked CRP was from Professor Richard Farndale (Biochemistry, University of Cambridge, UK). Fura-PE3 was from TefLabs (Austin, TX). Annexin V-fluorescein isothiocyanate was obtained from Abcam (Cambridge, UK). Bisindolylmaleimide I (BIM), LOE-908, and MRS-2279 were from Tocris Bioscience (Bristol, UK). AR-C69931M-X was a kind gift from AstraZeneca.

Mice—PKCθ$^{-/-}$ C57B6/J mice have been previously described (20). No compensatory change in expression of other PKC isoforms has been observed in PKCθ$^{-/-}$ platelets (18), and we have found no differences in GPVI surface expression between PKCθ$^{-/-}$ and WT platelets (data not shown). Wild-type C57B6/J mice were used as control. Use of mouse platelets was approved by local research ethics committee at the University of Bristol, UK, and mice were bred for this purpose under UK Home Office license (PPL 30/2386) held by A. W. P.

Preparation of Fura-PE3-loaded Mouse Platelets—Washed mouse platelets were prepared essentially as described previously (21) and suspended in a modified Tyrode's/HEPES buffer (134 mM NaCl, 0.34 mM Na$_2$HPO$_4$, 2.9 mM KCl, 12 mM NaHCO$_3$, 20 mM HEPES, 5 mM glucose, and 1 mM MgCl$_2$, pH 7.3) supplemented with 0.35% bovine serum albumin, 1 μM prostaglandin E$_1$, 10 μM indomethacin, 0.02 units/ml apyrase (grade VII). Platelets were incubated with Fura-PE3 (3 μM) for 30 min at room temperature. Acid citrate dextrose (20 mM citric acid, 110 mM sodium citrate, 5 mM glucose) was added (1:9), and platelets were collected by centrifugation and resuspended in modified HEPES/Tyrode's, supplemented with indomethacin and apyrase, to a concentration of 1 × 10^8 platelets/ml.

[Ca^{2+}]$_i$ Measurements—Platelets were stimulated at 37 °C with continuous stirring. Fura-PE3 was excited alternately at 340 and 380 nm, and fluorescence emission was detected at 510 nm. Fluorescence signals were corrected for autofluorescence. Fluorescence ratios, R (340/380), were calibrated in terms of [Ca^{2+}]$_i$ using the following equation: [Ca^{2+}]$_i$ = K_d·(S_0/S_s)·($R - R_{min}$)/($R_{max} - R$), where S_O and S_s are the fluorescence at 380 nm in the absence of Ca^{2+} and in saturating Ca^{2+}, respectively (22), and K_d = 290 nM (23). Where indicated, the area under the Ca^{2+} trace (AUC) above basal was calculated for 3 min after stimulation. For store-operated Ca^{2+} entry, AUC was calculated for 3 min after re-addition of Ca^{2+} (see Fig. 3A), corrected by subtraction of the increase in fluorescence due to leaked Fura-PE3.

Mn^{2+} Quench as a Marker of Divalent Cation Entry—Platelets were stimulated in the presence of MnCl$_2$ (100 μM) and CaCl$_2$ (1 mM), and fluorescence was monitored at an excitation wavelength of 360 nm. As with Fura-2 (24), at this wavelength Fura-PE3 fluorescence is insensitive to changes in Ca^{2+} concentration but is quenched by Mn^{2+}. Autofluorescence was

subtracted from the fluorescence intensity, which was then normalized to initial fluorescence intensity (F/F_0). The extent of decrease 3 min after stimulation was measured and corrected by subtraction for the decrease in fluorescence due to Mn^{2+} or dye leakage (monitored as the quench in the absence of agonist over the same time period).

Flow Cytometry—Annexin V-fluorescein isothiocyanate was used to detect surface PS exposure. Platelets (5 × 10^7/ml) were stimulated in the presence of CaCl$_2$ (2 mM) for 10 min. Annexin V binding was detected by flow cytometry, with platelets gated by their forward and side scatter profile. Analysis of 20,000 platelets was performed using a FACSCalibur (BD Biosciences). Data were analyzed using WinMDI version 2.8.

Statistical Analyses and Data Presentation—Where presented, mean data are given ± S.E. Statistical significance was determined by two-way analysis of variance with Bonferroni post-test, performed using Prism 4.0 (GraphPad Software). $p < 0.05$ was considered significant.

RESULTS

PKCθ Negatively Regulates CRP-induced Ca^{2+} Signaling and PS Exposure—The role of PKCθ in GPVI-dependent Ca^{2+} signaling was investigated in Fura-PE3-loaded and washed mouse platelets. The platelets were treated with indomethacin (10 μM) to prevent any thromboxane generation, because this may also be regulated by PKCθ (25).

A low concentration of the GPVI-selective agonist, CRP (1 μg/ml), induced an increase in [Ca^{2+}]$_i$ in wild-type (WT) platelets, which reached a peak within 2 min and then declined toward a sustained level (Fig. 1A). In PKCθ$^{-/-}$ platelets, the peak increase was significantly greater than seen in WT (363 ± 42 nM above basal in PKCθ$^{-/-}$ compared with 236 ± 24 nM in WT; $n = 4$; $p < 0.01$). Furthermore, the sustained [Ca^{2+}]$_i$ level was significantly greater in PKCθ$^{-/-}$ platelets (in PKCθ$^{-/-}$ platelets, [Ca^{2+}]$_i$ was 158 ± 19 nM above basal, 5 min after stimulation, compared with 46 ± 8 nM in WT; $n = 4$; $p < 0.001$). These data indicate that PKCθ negatively regulates GPVI-dependent Ca^{2+} signaling.

We have previously shown that CRP-induced granule secretion is greater in PKCθ$^{-/-}$ platelets, under some conditions (18, 19). To test whether enhanced ADP secretion could explain the enhanced Ca^{2+} signal, platelets were treated with MRS-2279 (10 μM) and AR-C69931M-X (1 μM), antagonists of P2Y$_1$ and P2Y$_{12}$, respectively. Although P2Y antagonism abolished the enhancement in peak Ca^{2+} increase (Fig. 1, B and C), [Ca^{2+}]$_i$ was still sustained at a greater level in PKCθ$^{-/-}$ platelets (Fig. 1, B and D; the increase in [Ca^{2+}]$_i$ 5 min after stimulation was 74 ± 20 nM in PKCθ$^{-/-}$ platelets, compared with 24 ± 10 nM in WT; $n = 4$; $p < 0.05$). These data indicate that ADP signaling is not required for the enhanced sustained [Ca^{2+}]$_i$ level.

PS exposure on the platelet surface forms an assembly site for coagulation complexes and accelerates thrombin generation. PS exposure requires a sustained increase in [Ca^{2+}]$_i$. CRP induced a proportion of WT platelets to expose PS (9.8 ± 2.9% were stained positively with annexin V; $n = 9$). This proportion was doubled in PKCθ$^{-/-}$ platelets (21.5 ± 2.5%; $n = 9$; Fig. 1E). Consistent with these data, pretreatment of WT platelets with the broad spectrum PKC inhibitor BIM (10 μM) increased the
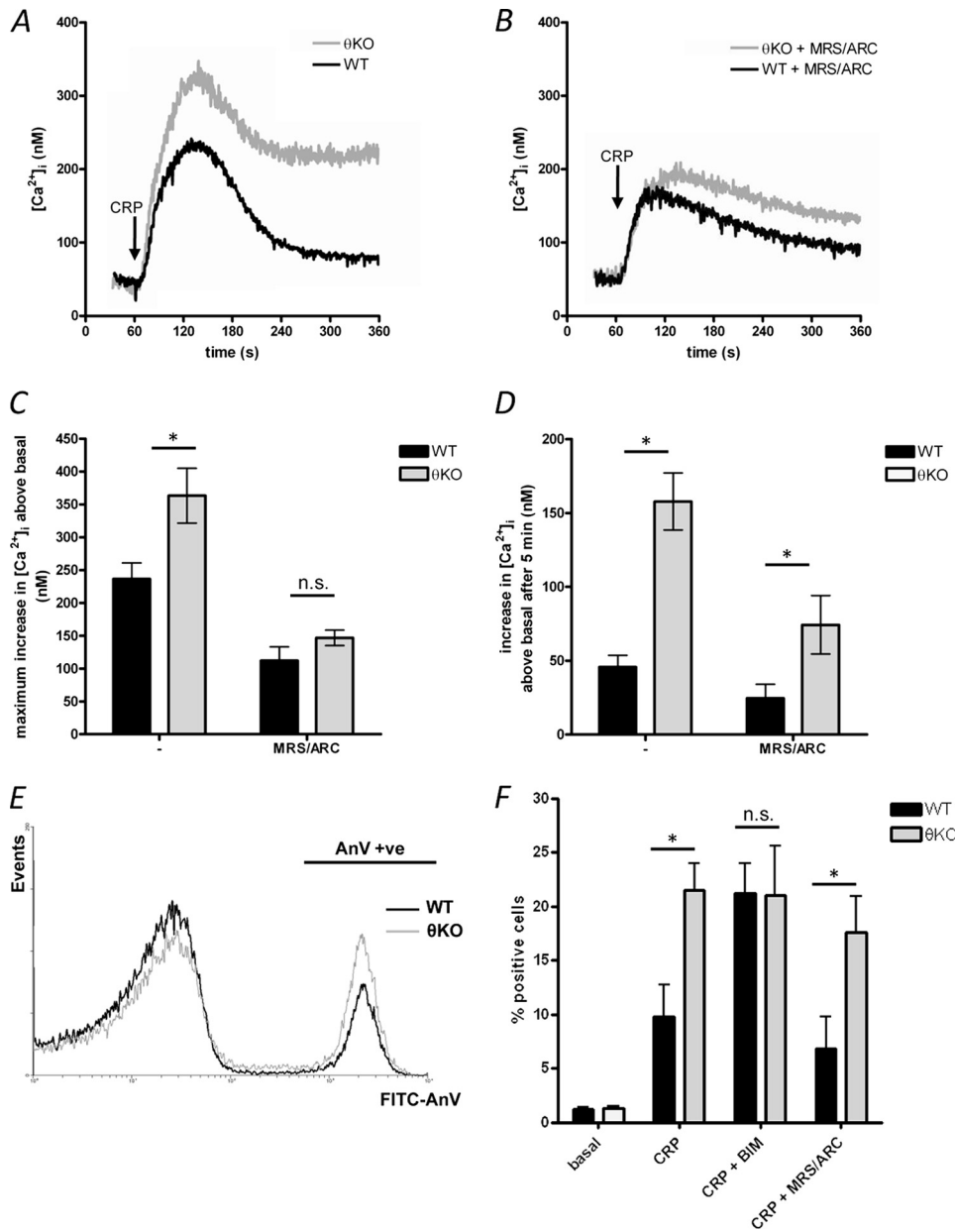
FIGURE 1. **PKCθ negatively regulates GPVI-dependent Ca²⁺ signaling and PS exposure.** *A,* washed platelets from wild-type (*WT*) and PKCθ⁻/⁻ (*θKO*) mice were stimulated by CRP (1 μg/ml) in the presence of extracellular Ca²⁺ (1 mM CaCl₂ added). Platelets had been treated with indomethacin to prevent thromboxane synthesis, which may also be affected by PKCθ. [Ca²⁺]ᵢ was monitored by Fura-PE3 fluorescence. *B,* platelets were treated with MRS-2279 (10 μM; MRS-2279) and AR-C69931M-X (1 μM; ARC), antagonists of P2Y₁ and P2Y₁₂, respectively, prior to stimulation. *C* and *D,* maximum increase in [Ca²⁺]ᵢ above basal (*C*) and the increase in [Ca²⁺]ᵢ above basal 5 min after stimulation (*i.e.* at 360 s in *A* and *B*) were determined. Data are means ± S.E. of four independent experiments. * indicates $p < 0.05$ for WT *versus* PKCθ⁻/⁻ platelets. *E,* platelets were stimulated with CRP (5 μg/ml) in the presence of annexin V-fluorescein isothiocyanate for 10 min. The annexin V-positive population is indicated (*AnV +ve*). The *histogram* is representative of nine independent experiments. *F,* mean data (± S.E.) showing the percentage of annexin V-positive platelets ($n = 9$). In some experiments, platelets were treated with BIM (10 μM, 10 min) or MRS-2279 + ARC prior to stimulation ($n = 4$ for CRP + BIM and CRP + MRS-2279/ARC). *n.s.,* not significant.

proportion of annexin V-positive platelets (21.3 ± 2.7%; $n = 4$). In contrast, BIM had no additional effect on PKCθ⁻/⁻ platelets (21.0 ± 4.6%; $n = 4$; Fig. 1*F*).

Pretreatment with MRS-2279 and AR-C69931M-X partially reduced annexin V binding in WT platelets (6.8 ± 2.9% were annexin V-positive; $n = 4$). Even with P2Y1/P2Y12 blockade, however, annexin V binding was significantly enhanced in

PKCθ⁻/⁻ platelets (17.6 ± 3.4%; $n = 4$; $p < 0.05$). These data suggest that PKCθ negatively regulates CRP-induced PS exposure in an ADP-independent manner.

PKCθ Negatively Regulates CRP-induced Ca²⁺ Release Indirectly by Reducing ADP Secretion—CRP induced an increase in [Ca²⁺]ᵢ in the absence of extracellular Ca²⁺ (200 μM EGTA added), indicating that CRP induces Ca²⁺ release from intracellular Ca²⁺ stores (Fig. 2*A*). In PKCθ⁻/⁻ platelets, CRP-induced Ca²⁺ release was enhanced (Fig. 2, *A* and *B*). However, the enhanced Ca²⁺ release in PKCθ⁻/⁻ platelets was blocked by MRS-2279, indicating that it required P2Y₁ (Fig. 2*B*). Because PKCθ does not directly regulate ADP-induced Ca²⁺ release (Fig. 2*C*), these data suggest that enhanced ADP secretion in PKCθ⁻/⁻ platelets is responsible for the observed increase in Ca²⁺ release. However, this also suggests that altered Ca²⁺ release does not underlie the enhanced sustained Ca²⁺ signal seen in the presence of extracellular Ca²⁺.

PKCθ Negatively Regulates CRP-induced Ca²⁺ Entry Independently of ADP Secretion—To confirm that PKCθ regulates a Ca²⁺ entry pathway, Mn²⁺-induced quench of Fura-PE3 fluorescence was used as a marker for Ca²⁺ entry. The extent of Mn²⁺ quench was greater in PKCθ⁻/⁻ platelets compared with WT (Fig. 2*E*). This enhancement was also unaffected by P2Y antagonism (Fig. 2*F*). Together, these data indicate that PKCθ negatively regulates a Ca²⁺ entry pathway following stimulation of GPVI.

PKCθ Does Not Directly Regulate SOCE—SOCE, a major Ca²⁺ entry pathway in nonexcitable cells, was activated by treating platelets with the sarco/endoplasmic reticulum Ca²⁺-ATPase inhibitor, thapsigargin (1 μM; 5 min), in the absence to extracellular Ca²⁺ (200 μM EGTA added). SOCE was assessed following addition of CaCl₂ (1.2 mM). The Ca²⁺ signal was much greater in PKCθ⁻/⁻ platelets (Fig. 3*A*). However, P2Y antagonists blocked the apparent enhancement, suggesting that enhanced ADP secretion was responsible for this, rather than a direct effect on SOCE itself (Fig. 3, *B* and *C*). Furthermore, these data
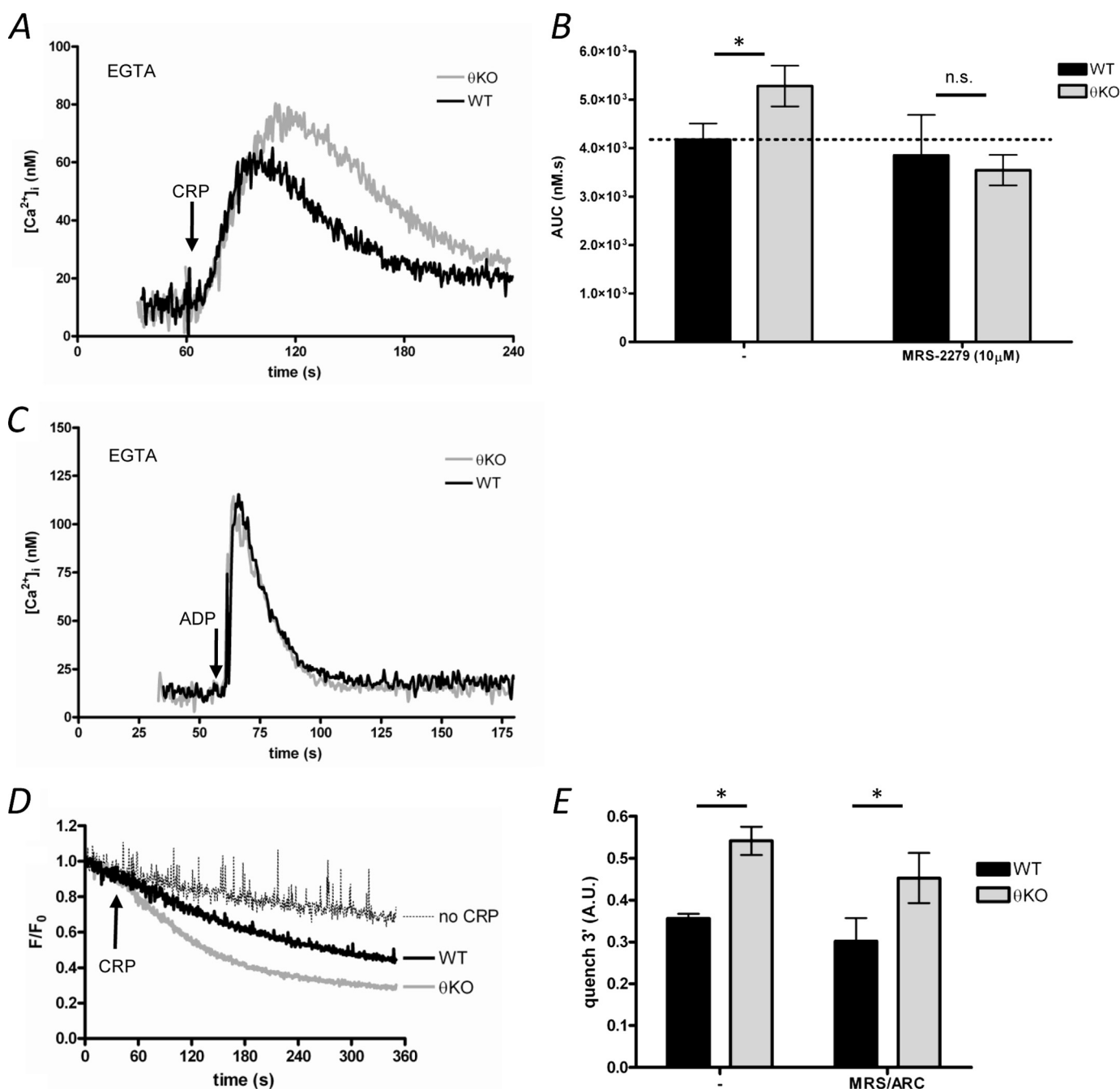
FIGURE 2. **PKCθ negatively regulates Ca²⁺ entry but does not directly regulate Ca²⁺ release.** *A,* washed platelets were stimulated with CRP in the absence of extracellular Ca²⁺ (200 μM EGTA added). *B,* platelets were treated with the P2Y$_1$ antagonist, MRS-2279, then stimulated as in *A*. Ca²⁺ release was analyzed as the area under the Ca²⁺ trace (*AUC*) above basal (see "Experimental Procedures"). Treatment with MRS-2279 blocked the enhanced Ca²⁺ release. *C,* platelets were stimulated by ADP (10 μM) in the absence of extracellular Ca²⁺ (200 μM EGTA added). *D,* divalent cation entry was monitored using Mn²⁺-dependent quench of Fura-PE3 fluorescence. The quench in the absence of CRP is indicated. *E,* analysis of the quench experiments (see "Experimental Procedures") indicates that Mn²⁺ entry is increased in PKCθ$^{-/-}$ platelets, even in the presence of P2Y antagonists (MRS-2279/ARC). * indicates $p < 0.05$. *KO,* knock-out; *n.s.,* not significant.

suggest PKCθ may instead regulate a store-independent Ca²⁺ entry pathway.

PKCθ Negatively Regulates Store-independent Ca²⁺ Entry—Store-independent Ca²⁺ entry pathways (also known as noncapacitative Ca²⁺ entry) have been described in many cells, including platelets (15). In many cells, Ca²⁺ entry can be directly activated by DAG analogues, such as OAG. In WT platelets, OAG (20 μM) induced a sustained increase in [Ca²⁺]$_i$ (Fig. 4A) that was unaffected by P2Y blockade (Fig. 4B). OAG-induced Ca²⁺ entry was significantly greater in PKCθ$^{-/-}$ platelets (Fig. 4A) but also unaffected by P2Y antagonists (Fig. 4B). Furthermore, OAG-induced Mn²⁺ entry was enhanced in PKCθ$^{-/-}$ platelets independently of P2Y signaling (Fig. 4, C and D). These data indicate that PKCθ negatively regulates OAG-induced store-independent Ca²⁺ entry.

Role of Store-independent Ca²⁺ Entry in CRP-induced Ca²⁺ Signaling—LOE-908 is a blocker of some nonselective cation channels (26, 27). LOE-908 (30 μM) blocked OAG-induced
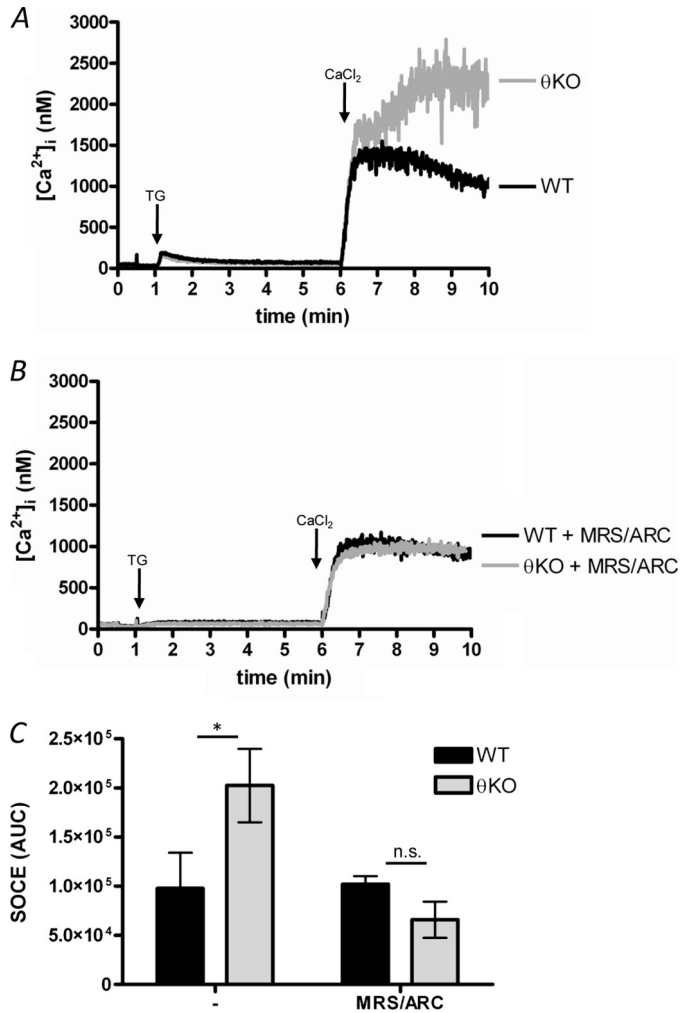
FIGURE 3. **Store-operated Ca²⁺ entry is not directly regulated by PKCθ independently of ADP secretion.** *A* and *B,* SOCE was activated by treating platelets with thapsigargin (*TG*; 1 μM) for 5 min in the absence of extracellular Ca²⁺ (200 μM EGTA added). SOCE was then assessed by addition of 1.2 mM CaCl₂. *B,* platelets were first treated with P2Y antagonists (MRS-2279/ARC). *C,* quantification of SOCE, as AUC. *n.s.,* not significant; *KO,* knock-out. * indicates $p < 0.05$; $n = 3$.

store-independent Ca²⁺ entry (Fig. 5*A*),but at this concentration has no effect on SOCE (Fig. 5*B*). LOE-908 significantly reduced the CRP-induced Ca²⁺ signal in WT platelets, although the sustained [Ca²⁺]ᵢ level reached after 5 min was not affected (Fig. 5, *C* and *D*). Importantly, the Ca²⁺ signal was no different between WT and PKCθ⁻/⁻ platelets in the presence of LOE-908 (Fig. 5*C*). The increase in [Ca²⁺]ᵢ above basal, 5 min after stimulation, was 42 ± 7 nM in WT compared with 39 ± 9 nM in PKCθ⁻/⁻ platelets ($p > 0.05$; Fig. 5, *C* and *D*). These data suggest that store-independent Ca²⁺ entry is required for the higher, sustained [Ca²⁺]ᵢ in PKCθ⁻/⁻ platelets.

PKCθ Negatively Regulates PS Exposure—LOE-908 had little effect on CRP-induced PS exposure in WT platelets, consistent with its lack of effect on the sustained [Ca²⁺]ᵢ in these cells. Importantly, however, there was no difference in PS exposure between WT and PKCθ⁻/⁻ platelets in the presence of LOE-908 (10.5 ± 2.1% in WT, 12.1 ± 3.1% in PKCθ⁻/⁻, $p > 0.05$; Fig. 5*E*). These data are summarized in Fig. 5*F*. These data strongly suggest that PKCθ negatively regulates CRP-induced Ca²⁺ sig-
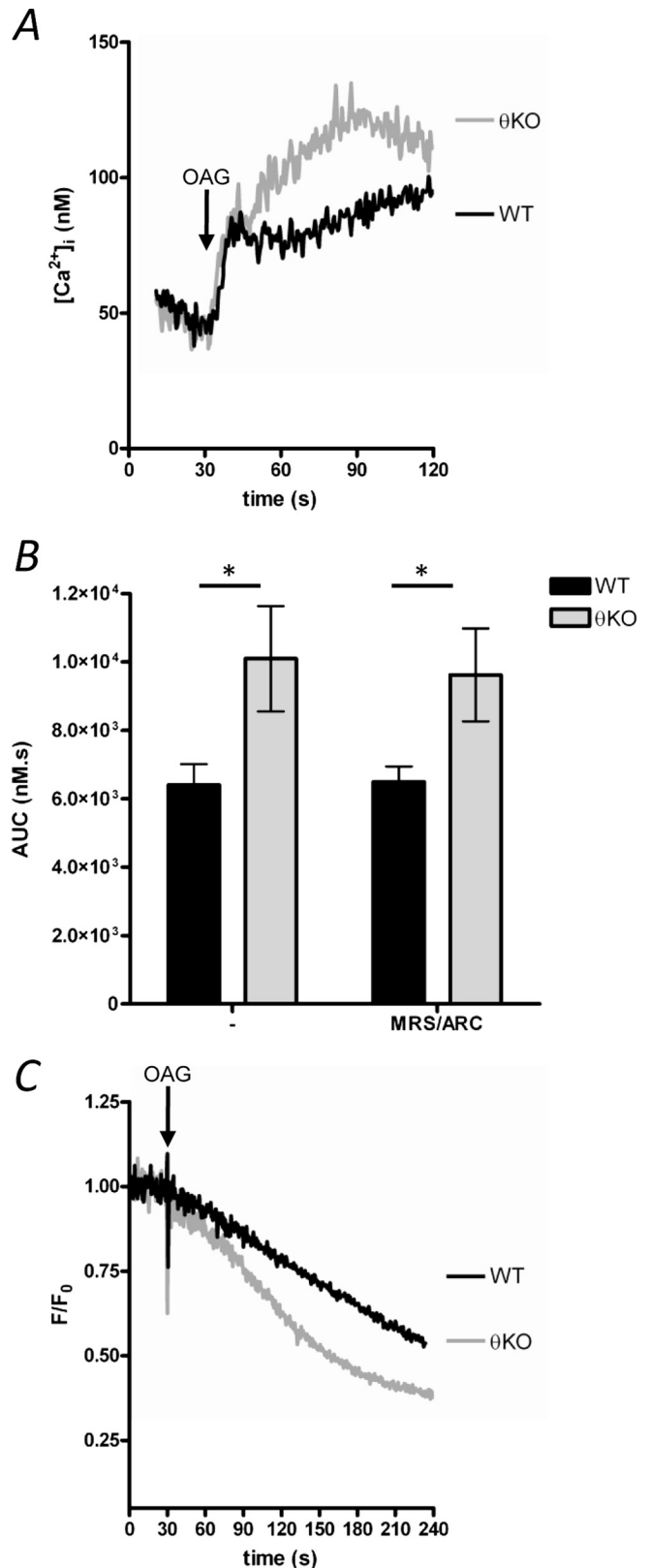

FIGURE 4. **Store-independent Ca²⁺ entry is negatively regulated by PKCθ.** *A,* platelets were stimulated with the DAG analogue, OAG (20 μM), in the presence of extracellular CaCl₂ (1 mM). At this concentration, OAG does not induce Ca²⁺ release (data not shown). *B,* quantification of OAG-induced Ca²⁺ signaling, as AUC. Treatment with P2Y antagonists (MRS-2279/ARC) did not affect OAG-induced Ca²⁺ signaling. * indicates $p < 0.05$; $n = 4$. *C,* OAG-induced Mn²⁺ entry was monitored by quench of Fura-PE3 fluorescence. Trace is representative of three independent experiments. *KO,* knock-out.
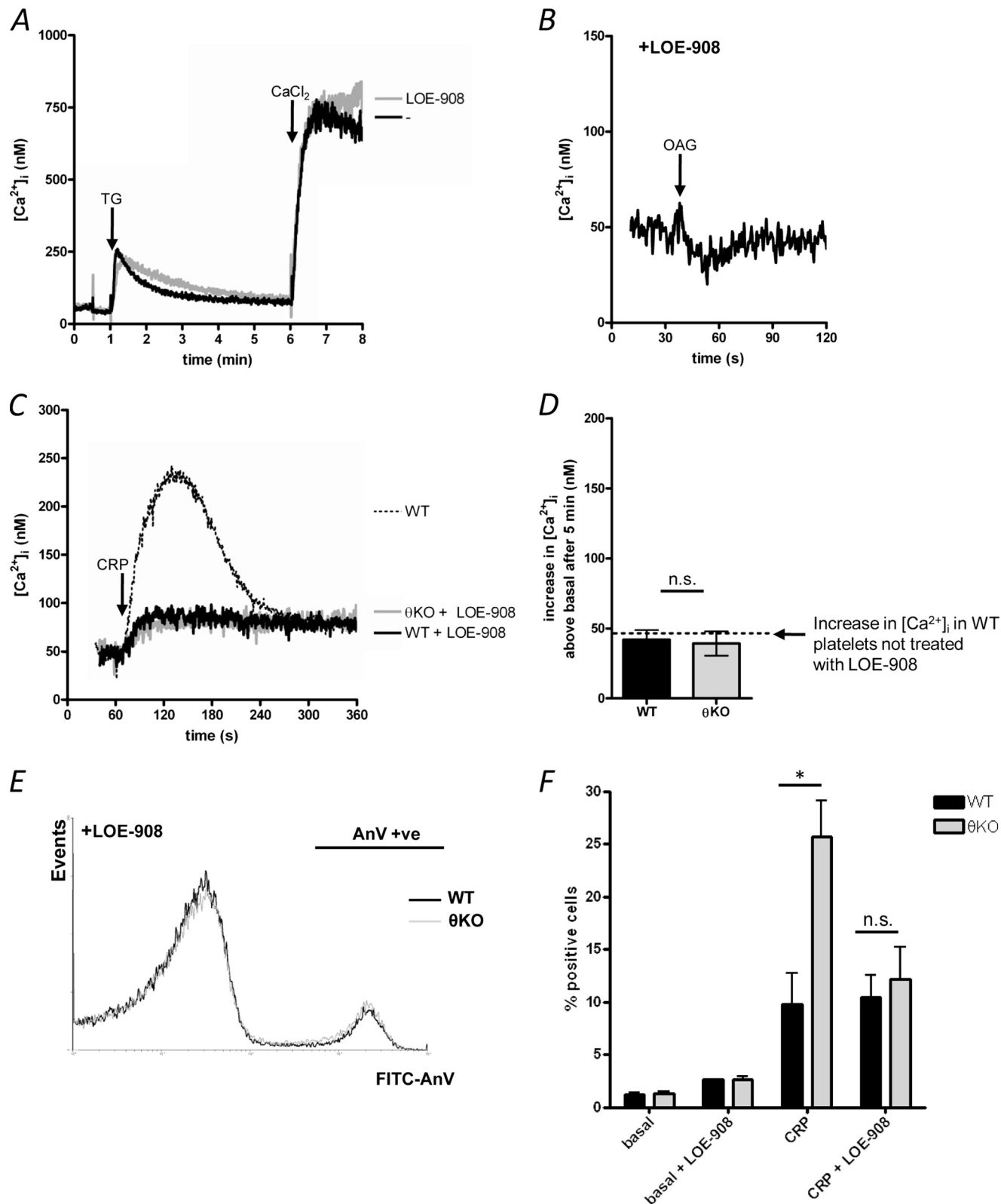
FIGURE 5. **Role of store-independent Ca²⁺ entry in GPVI-dependent Ca²⁺ signaling and PS exposure.** *A,* WT platelets were treated with LOE-908 (30 μM) or the vehicle (Tyrode's) prior to activation of SOCE. No significant difference in SOCE was seen between LOE-908-treated and untreated cells ($n = 3$). *TG,* thapsigargin. *B,* WT platelets treated with LOE-908 were stimulated with OAG. In contrast to untreated cells (see Fig. 4*A*), no Ca²⁺ signal was seen in LOE-908-treated cells ($n = 3$). *C,* WT and PKC$\theta^{-/-}$ (θKO) platelets were treated with LOE-908 and then stimulated with CRP. The *dotted line* indicates the Ca²⁺ signal seen in untreated WT cells, for comparison. The increase in [Ca²⁺]$_i$, 5 min after stimulation, is shown in *D.* The *dashed line* indicates the [Ca²⁺]$_i$ after 5 min in untreated, CRP-stimulated WT platelets (see Fig. 1*D*). *E,* PS exposure was monitored in LOE-908-treated WT and PKC$\theta^{-/-}$ platelets by annexin V binding, as in Fig. 1*E*. The annexin V-positive population is indicated (*AnV +ve*). *F,* mean data ($\pm$ S.E.) showing the percentage of annexin V positive platelets. Control data (basal, CRP) are the same as Fig. 1*F*, for comparison. LOE-908 blocks the enhanced PS exposure in CRP-stimulated PKC$\theta^{-/-}$ platelets. * indicates $p < 0.05$ for WT *versus* PKC$\theta^{-/-}$. *n.s.,* not significant; *FITC,* fluorescein isothiocyanate.
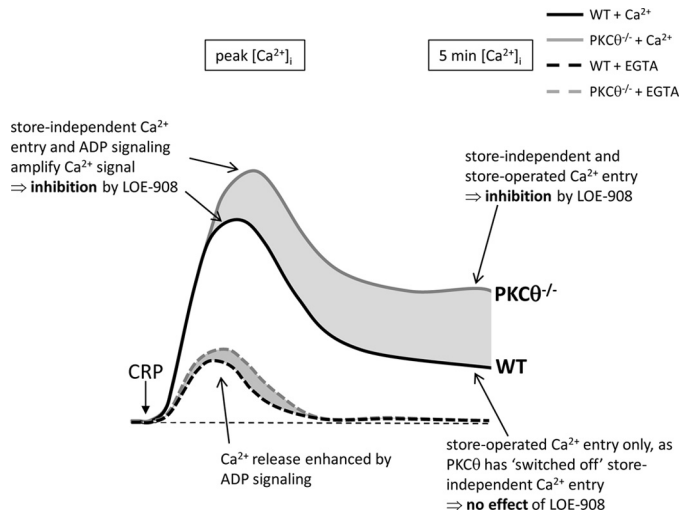
FIGURE 6. **Store-independent Ca²⁺ entry in GPVI-dependent Ca²⁺ signaling.** Stimulation of GPVI by CRP induces Ca²⁺ release from intracellular Ca²⁺ stores and Ca²⁺ entry. The initial peak includes Ca²⁺ release (amplified by ADP) and Ca²⁺ entry (mainly via store-independent Ca²⁺ entry and so blocked by LOE-908). Following the peak Ca²⁺ signal, PKCθ down-regulates store-independent Ca²⁺ entry in WT platelets, and so the sustained Ca²⁺ signal is not affected by LOE-908. In PKCθ⁻/⁻ platelets, however, store-independent Ca²⁺ entry remains active leading to a higher sustained Ca²⁺ signal. This can be normalized to WT levels by LOE-908.

naling by reducing store-independent Ca²⁺ entry, which in turn reduces platelet PS exposure.

DISCUSSION

In this study we have shown that PKCθ negatively regulates GPVI-dependent Ca²⁺ signaling, leading to reduced PS exposure. The negative regulation occurs at two distinct points as follows: 1) negative regulation of dense granule secretion and subsequent ADP-dependent Ca²⁺ release; 2) negative regulation of store-independent Ca²⁺ entry. We suggest that under normal conditions, store-independent Ca²⁺ entry contributes to the initial, transient increase in $[Ca^{2+}]_i$ but is then down-regulated by PKCθ. This limits the sustained increase in $[Ca^{2+}]_i$ and so reduces PS exposure. This is represented in Fig. 6. The work therefore reveals a novel pathway for regulation of platelet Ca²⁺ signaling and procoagulant activity, which plays a key role in thrombus growth.

We have previously shown that PKCθ negatively regulates GPVI-dependent platelet activation (18). Here, we were first able to show that GPVI-dependent Ca²⁺ signaling is greater in amplitude and sustained at a higher level in PKCθ⁻/⁻ platelets. The more sustained Ca²⁺ signal is associated with a PS exposure in a greater proportion of cells. The normal function of PKCθ therefore may be to limit GPVI-dependent Ca²⁺ signaling and so restrict PS exposure and platelet procoagulant activity during thrombus formation. The sustained Ca²⁺ signal was not entirely due to enhanced ADP secretion. We have previously demonstrated that, under some conditions, CRP-induced dense granule secretion is enhanced in PKCθ⁻/⁻ platelets (19) (although this is partly dependent on assay conditions and agonist concentration). However, Ca²⁺ signaling was sustained at a higher level in PKCθ⁻/⁻ platelets even in the presence of P2Y receptor antagonists, indicating that ADP signaling was not required.

Similarly, although Ca²⁺ release from intracellular Ca²⁺ stores was enhanced in PKCθ⁻/⁻ platelets, this also does not underlie the more sustained Ca²⁺ signaling that we have observed. Instead, our data indicate that the enhanced Ca²⁺ release was secondary to ADP secretion and P2Y₁ activation. P2Y₁ is coupled via Gα_q to phospholipase Cβ, resulting in inositol 1,4,5-trisphosphate formation and subsequent Ca²⁺ release. PKCθ does not appear to directly regulate Ca²⁺ release downstream of P2Y₁ because ADP-induced Ca²⁺ was unaffected. In the absence of P2Y₁ signaling, CRP-induced Ca²⁺ release was also unaffected, indicating that PKCθ does directly regulate this process. Therefore, under normal conditions PKCθ acts to reduce dense granule secretion (19), limiting P2Y₁-dependent amplification of the initial Ca²⁺ release. However, this is not responsible for the greater sustained Ca²⁺ signal.

In contrast, we found that PKCθ negatively regulates Ca²⁺ entry, independently of ADP secretion. Mn²⁺ entry, a marker of divalent cation entry, was enhanced, even when P2Y receptors were blocked. Multiple Ca²⁺ entry pathways have been described in platelets. SOCE is activated by a decrease in the Ca²⁺ content within the intracellular Ca²⁺ stores (12) and can regulate PS exposure (28). Although we found that thapsigargin-activated SOCE was enhanced in PKCθ⁻/⁻ platelets, it is known that part of the apparent SOCE signal may in fact be due to dense granule release (29). When the contribution of autocrine P2Y signaling was blocked, SOCE was not significantly different between PKCθ⁻/⁻ and WT platelets. This indicates that SOCE is not directly regulated by PKCθ. Rather, increased dense granule secretion may further enhance Ca²⁺ release and store depletion, leading to greater SOCE. These data suggest that the negative regulation of CRP-induced Ca²⁺ entry is not through regulation of SOCE.

Ca²⁺ entry can occur through store-independent pathways, although much less is known about these pathways in platelets. In common with many cells, DAG analogues induce Ca²⁺ entry in platelets (15). Interestingly, this Ca²⁺ entry was enhanced in PKCθ⁻/⁻ platelets. Furthermore, unlike SOCE, this enhancement was seen in the presence of P2Y antagonists. This means that OAG-activated, store-independent Ca²⁺ entry pathway is directly regulated by PKCθ, whereas SOCE is not.

OAG-induced Ca²⁺ entry pathway was blocked by LOE-908, whereas SOCE was unaffected by this drug. This makes LOE-908 a useful tool to discriminate between different Ca²⁺ entry pathways during platelet activation. LOE-908 significantly reduced CRP-induced Ca²⁺ signaling. In WT platelets, LOE-908 reduced the initial Ca²⁺ signal but not the sustained signal. This suggests that normally, store-independent Ca²⁺ entry amplifies the initial response but is not involved in the sustained Ca²⁺ signal. Importantly, in the presence of LOE-908, PKCθ⁻/⁻ platelets showed identical Ca²⁺ signaling to WT, indicating that the enhanced response requires store-independent Ca²⁺ entry. We conclude, therefore, that PKCθ negatively regulates store-independent Ca²⁺ entry following stimulation of GPVI and that normally (*i.e.* in WT cells) store-independent Ca²⁺ entry is not involved in the sustained response because it has been switched off by PKCθ.

Negative regulation of store-independent Ca²⁺ entry by PKC has been proposed in some cultured cell lines (30), although, to

the best of our knowledge, this is the first report that describes regulation of store-independent Ca^{2+} entry by a specific PKC isoform in a native cell type. The mechanism of regulation by PKC is still unclear. TRPC3, which may form part of store-independent channels in some cells (although its expression in platelets is uncertain (31, 32)), is phosphorylated at Ser-712 in a PKC-dependent manner in HEK-293 cells (33), which leads to inhibition of TRPC3. The store-independent channel in platelets, yet to be unambiguously identified, may also be directly phosphorylated. Alternatively, PKCθ may act indirectly, for example by modulating channel expression in the plasma membrane (34, 35).

SOCE is commonly considered to provide the majority of Ca^{2+} entry in nonexcitable cells (12). This view is strengthened by the large reduction in Ca^{2+} signaling in Orai1$^{-/-}$ platelets, in which SOCE is almost completely abolished (36). In contrast, LOE-908, which does not affect SOCE under the conditions used in this study, significantly reduced CRP-induced Ca^{2+} signaling. The effect of LOE-908 was restricted to the earlier portion of the Ca^{2+} signal, whereas the sustained $[Ca^{2+}]_i$ was unaffected. These data suggest that the importance of different Ca^{2+} entry pathways may be temporally controlled, with DAG-activated/store-independent Ca^{2+} entry contributing to the early peak and SOCE more important for sustained Ca^{2+} signaling. Our data suggest that one mechanism behind this temporal control is PKCθ, which may "switch off" store-independent Ca^{2+} entry (see Fig. 6).

The major functional consequence of this negative regulation of store-independent Ca^{2+} entry may be to limit platelet PS exposure, which is a key component of thrombin generation during vascular damage. Although tissue factor-exposing cells initiate thrombin generation through the extrinsic coagulation cascade, propagation of thrombin generation occurs on PS-exposing platelets (3). Platelets provide an efficient and regulated procoagulant surface because they are localized to sites of vascular injury and can coordinate assembly of the tenase and prothrombinase complexes. Furthermore, inhibition of these coagulation complexes by plasma protease inhibitors is less effective when the proteases are on cell surfaces than when they are in solution (3). Central in inducing PS exposure is a sustained increase in $[Ca^{2+}]_i$ (9, 37, 38). Our data suggest that PKCθ negatively regulates this process by inhibiting store-independent Ca^{2+} entry and so reducing $[Ca^{2+}]_i$. Importantly, this regulation appears to be the normal situation, because LOE-908 had little effect on PS exposure in WT platelets, despite the large inhibition of the earlier phase of the Ca^{2+} signal. Interestingly, LOE-908 reduced infarct volume following focal cerebral ischemia in a rat middle cerebral artery occlusion model of stroke (39, 40), highlighting the importance of controlling store-independent Ca^{2+} entry.

We have previously reported that PKCθ negatively regulates GPVI-dependent granule secretion, integrin activation, and thrombus formation on collagen (18). In contrast, another group has reported that PKCθ positively regulates GPVI-dependent granule secretion, integrin activation, and platelet aggregation (25). With regards to granule secretion, this may reflect agonist concentration (with negative regulation seen at low CRP concentrations by both groups (19)). The alterations

in integrin activation are likely to be related to changes in dense granule secretion, because P2Y$_{12}$ is a major regulator of integrin activation (41). It is important to stress, however, that these previous studies were performed in the absence of extracellular Ca^{2+}, and so the novel regulation of store-independent Ca^{2+} entry that we describe here cannot account for the regulation of granule secretion. Rather, PKCθ is likely to regulate granule secretion and Ca^{2+} entry through distinct mechanisms. In summary, we have identified a novel role for PKCθ in reducing GPVI-dependent Ca^{2+} signaling and PS exposure. This is mediated by reduced Ca^{2+} entry through a store-independent Ca^{2+} channel. Because platelet PS exposure is a key determinant of thrombin generation and thrombus formation, PKCθ may act to limit collagen-dependent thrombin generation and hence occlusive thrombus formation at sites of vascular injury.

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