PECAM-1 (CD31) regulates a hydrogen peroxide–activated nonselective cation channel in endothelial cells

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Hydrogen peroxide (H_2O_2) released by neutrophils is an important mediator of endothelial cell (EC) injury and vascular inflammation via its effect on EC-free Ca^{2+}, $[Ca^{2+}]_i$. Although the underlying mechanisms are not well understood, platelet endothelial cell adhesion molecule (PECAM)-1/CD-31 is a critical modulator of neutrophil–EC transmigration. PECAM-1 is also known to regulate EC calcium signals and to undergo selective tyrosine phosphorylation. Here, we report that PECAM-1 molecules transduce EC responses to hydrogen peroxide. In human umbilical vein EC and REN cells (a PECAM-1–negative EC-like cell line) stably transfected with PECAM-1 (RHP), noncytolytic H_2O_2 exposure (100–200 μM H_2O_2) activated a calcium-permeant, nonselective cation current, and a transient rise in $[Ca^{2+}]_i$ of similar time course. Neither response was observed in untransfected REN cells, and H_2O_2-evoked cation current was ablated in REN cells transfected with PECAM-1 constructs mutated in the cytoplasmic tyrosine–containing domain. The PECAM-dependent H_2O_2 current was inhibited by dialysis of anti–PECAM-1 cytoplasmic domain antibodies, required Src family tyrosine kinase activity, was independent of inositol trisphosphate receptor activation, and required only an intact PECAM-1 cytoplasmic domain. PECAM-1–dependent H_2O_2 currents and associated $[Ca^{2+}]_i$ transients may play a significant role in regulating neutrophil–endothelial interaction, as well as in oxidant-mediated endothelial response and injury.

Introduction

Reactive oxygen species (H_2O_2, $^{\bullet}O_2^-$,$^{\bullet}OH$) are important mediators of endothelial cell (EC)* injury and likely play a central role in cardiovascular, cerebrovascular, and pulmonary syndromes such as myocardial infarction, ischemic stroke, acute respiratory distress syndrome, and acute organ transplant rejection (Lounsbury et al., 2000). H_2O_2 appears to be particularly important in the context of neutrophil–endothelial interaction in inflammation (Martin, 1984; Lewis et al., 1988; Gasic et al., 1991), during which local H_2O_2 levels may exceed 100–400 μM (Test and Weiss, 1984). H_2O_2 causes cell depolarization (Matoba et al., 2000) and increases cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) in EC through a range of concentration-dependent $[Ca^{2+}]_i$ responses, including Ca^{2+} influx into EC (Bowles et al., 2001) and Ca^{2+} release from intracellular stores (Schilling and Elliott, 1992; Doan et al., 1994; Siflinger-Birnboim et al., 1996; Yolk et al., 1997; Hu et al., 1998). This rise in $[Ca^{2+}]_i$ triggers the release of platelet-activating factor (Lewis et al., 1988) and PGI_2 (Harlan and Callahan, 1984), endothelial retraction, adhesion molecule redistribution (Bradley et al., 1995), EC permeability (Siflinger-Birnboim et al., 1996; Carbajal and Schaeffer, 1998), gene transcription (Barchowsky et al., 1995), and apoptosis (Martin, 1984; Suzucki et al., 1997). H_2O_2 can also activate tyrosine kinases and inhibit protein tyrosine phosphatases in ECs (Barchowsky et al., 1995; Carbajal and Schaeffer, 1998). Despite the recognized importance of these EC responses to oxidant exposure, the key

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*Abbreviations used in this paper: EC, endothelial cell; HSVTK, herpes simplex virus thymidine kinase; HUVEC, human umbilical vein endothelial cell(s); IgG, immunoglobin; IL, interleuken; IP_3, inositol trisphosphate; $LaCl_3$, lanthanum chloride; NSC, nonselective cation; PCD, PECAM cytoplasmic domain; PECAM, platelet endothelial cell adhesion molecule; SOC, store-operated channel.

Key words: hydrogen peroxide; capacitative current; calcium; tyrosine kinase; ion channels

regulatory proteins modulating EC calcium responses remain largely unknown.

One potential participant in this regulation is platelet endothelial adhesion molecule (PECAM)-1/CD-31, a 130-kD type 1 membrane glycoprotein belonging to the Ig superfamily of CAMs that plays an important role in the modulation of EC–neutrophil interaction and transmigration in inflammation (Vaporciyan et al., 1993; Nakada et al., 2000) and oxidant injury (Scalia and Lefer, 1998). PECAM-1, which functions as both an adhesion and signaling protein, is highly expressed on ECs where it localizes to cell–cell borders, as well as on leukocytes and platelets (Newman, 1997). Recently, it has been shown that engagement of PECAM-1 elicits prolonged EC calcium transients through a calcium-permeant, plasmalemmel nonselective cation (NSC) channel. Activation of this channel is dependent on Src family kinase activity, but independent of inositol trisphosphate (IP_3)-mediated store release or phosphoinositide turnover (Gurubhagavatula et al., 1998; O'Brien et al., 2001). In addition to a large extracellular domain containing six Ig-like loops, PECAM-1 has a 118–amino acid cytoplasmic domain containing a dual tyrosine SH2 binding motif (Y663/Y686). Under a range of conditions, including phosphatase inhibition (Jackson et al., 1997; Sagawa et al., 1997), Src and Csk overexpression (Lu et al., 1997; Cao et al., 1998), and anti–PECAM-1 antibody engagement (Varon et al., 1998), this motif is selectively tyrosine phosphorylated. Once phosphorylated, PECAM-1 can associate with SH2 domain proteins such as SHP-2, SHP-1, PLCγ, and PI3-kinase, although the physiologic relevance of these interactions in EC are unknown (Newman, 1999). In light of the evidence that PECAM-1 participates in ionic signaling and may serve as a substrate for phosphotyrosine modification, we hypothesized that PECAM-1 might participate in the regulation of oxidant-mediated calcium signals in ECs.

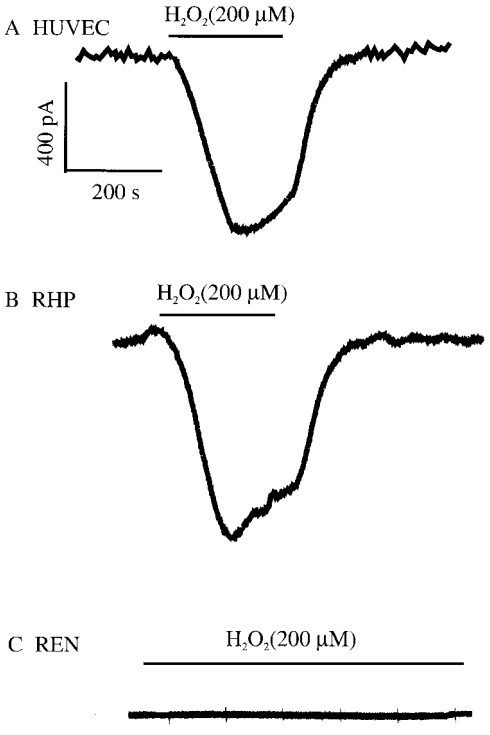

Figure 1. **Hydrogen peroxide activates a PECAM-1–dependent inward current in HUVEC and in endothelia-like PECAM-1–transfected REN cells (RHP).** HUVEC, RHP, and REN cells were voltage clamped at a holding potential of −60 mV in HBSS with CsCl pipette solution. Cells were puffed with 200 μM H_2O_2 for 3–5 min. (A) H_2O_2-treated HUVEC yield a prolonged inward current (B) RHP cells identically treated yield a current with similar kinetics. (C) Untransfected REN cells do not manifest detectable current following 200 or 400 μM H_2O_2 (unpublished data). Instantaneous voltage ramps from −60 mV up to +50 mV yielded Vrev = 0 in all trials. Experiments using the nystatin perforated patch technique yielded similar results.

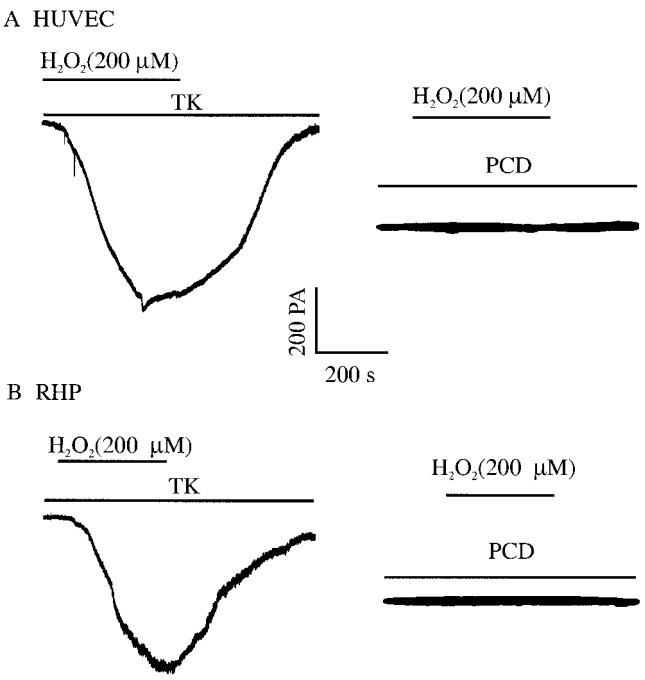

Figure 2. **Intracellular dialysis with anti–PECAM-1 cytoplasmic domain antibodies inhibits the H_2O_2-activated, PECAM-1–dependent current.** Voltage-clamped HUVEC (A) and RHP cells (B) were dialyzed through the patch pipette with CsCl pipette solution containing either (10 μg/ml) polyclonal anti–PECAM-1 cytoplasmic domain antibody (PCD) or a polyclonal anti–HSVTK antibody (TK) negative control. Cells were puffed with 200 μM H_2O_2 for 3–5 min.

Results

H_2O_2 activates a PECAM-1–dependent, inward-directed current in human umbilical vein EC and PECAM-1–transfected REN cells

In order to test our hypothesis that PECAM-1 may play a role in oxidant-mediated signaling, we performed voltage clamp experiments on human umbilical vein EC (HUVEC), REN cells, and REN cells stably transfected with PECAM-1 (RHP). We have previously utilized REN cells, a mesothelioma cell line that does not express endogenous PECAM-1, as an EC model, and have found that many EC signaling processes may be reconstituted after PECAM-1 expression (Gurubhagavatula et al., 1998; O'Brien et al., 2001). Puffer pipette application of 200 μM H_2O_2 (a dose based on preliminary studies described below) to HUVEC and RHP cells

voltage clamped at a holding potential of –60 mV, elicited a large inward current in 93% of HUVEC (n = 15) with a peak amplitude of 648.4 ± 36.6 pA. A similar current (peak amplitude 682.5 ± 41.55 pA) was detected in 89% of RHP (n = 18), whereas no currents were observed in untransfected REN cells exposed to 200 (n = 16) or 400 μM (n = 11) H_2O_2 (Fig. 1). H_2O_2 currents were typically activated 30–90 s after H_2O_2 exposure and were of prolonged time course, features similar to cation currents evoked in HUVEC and RHP cells after PECAM-1 engagement (O'Brien et al., 2001). The response to H_2O_2 was concentration dependent in that RHP cells treated with H_2O_2 at concentrations of 100 μM or lower elicited current less consistently (though those responding manifested similar current kinetics). Cells that responded to 400 or 500 μM H_2O_2 displayed evident cell toxicity over 24 h (unpublished data), consistent with previous reports (Barchowsky et al., 1994). Puffer application of vehicle alone did not elicit a current, further eliminating a potential role for shear stress–gated NSC channels or transient loss of seal in the response (unpublished data).

To further confirm the role of PECAM-1 in both HUVEC and RHP cells, H_2O_2 current responses were recorded in cells dialyzed with polyclonal anti–PECAM-1 cytoplasmic domain (PCD) antibody or control polyclonal antibody directed against herpes simplex virus thymidine kinase ([anti-] HSVTK). 200 μM H_2O_2 elicited currents with identical kinetics to nondialyzed cells in 83% of HUVEC (n = 12) and 80% RHP cells (n = 5) dialyzed with anti-HSVTK antibody. By contrast, in cells dialyzed with PCD, currents were completely blocked in 81% of HUVEC (n = 16) and 75% of RHP (n = 4) cells, and markedly attenuated in those cells in which currents were observed (Fig. 2).

The PECAM-1–regulated current is mediated by a nonselective, calcium-conducting channel

The ion selectivity and voltage dependence of the PECAM-1 current were examined in HUVEC and RHP. The reversal potential of the evoked current was determined by voltage ramp (−60 up to +50 mV for 500 ms) and voltage step (−60 to +40 mV in 20 mV increments) protocols in both RHP cells (Fig. 3) and HUVEC (unpublished data). The current–voltage relationship of the PECAM-1 current was ohmic, with no evidence of voltage-dependent gating or rectification. Similarly, initial trials with instantaneous voltage ramps to +100 mV showed no rectification (unpublished data). The reversal potential in standard physiologic solutions was ∼0 mV, close to the theoretical monovalent cation equilibrium potential of −1.2 mV. Consistent with a NSC current, replacement of extracellular Na^+ ions with impermeant Tris resulted in a left shift of the current reversal potential and a decrease in the slope conductance of the inward current. Conversely, asymmetric impermeant anion and symmetric cation solutions (CsAcetate pipette and CsCl

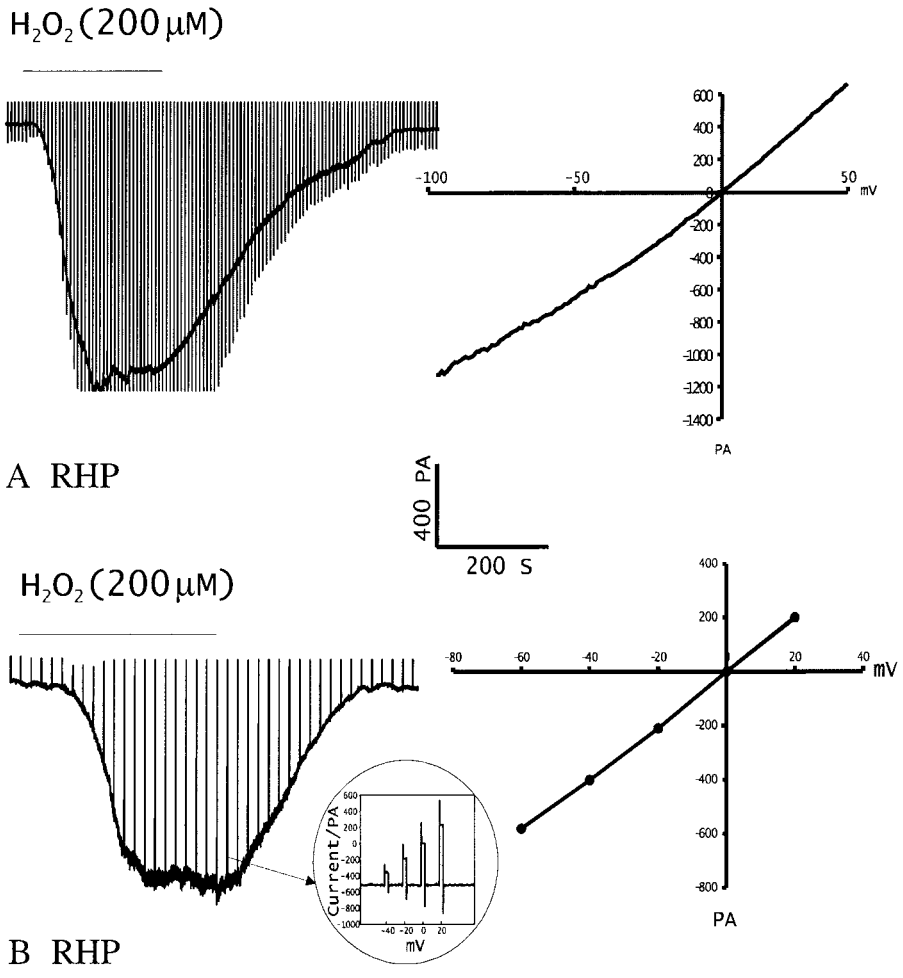

Figure 3. **The H_2O_2-activated PECAM-1–dependent current is mediated by a voltage-independent, nonrectifying, NSC channel.** RHP cells and HUVEC (unpublished data) voltage clamped at a holding potential of −60 mV in HBSS and CsCl pipette solution were puffed with 200 μM H_2O_2 for 3–5 min. Reversal potential, voltage gating, and rectification were assessed for both cell types after an instantaneous voltage ramp protocol from −60 mV to +40 mV (A) and a 20-mV/20mS incremental step-gradient protocol (inset) (B). RHP cells demonstrate a Vrev = 0 with a linear I/V relationship derived from instantaneous voltage ramp and step-gradient trials with step-gradient trials manifesting no voltage dependence, consistent with a voltage-independent, NSC channel. Identical results were obtained in similar analyses on HUVEC.

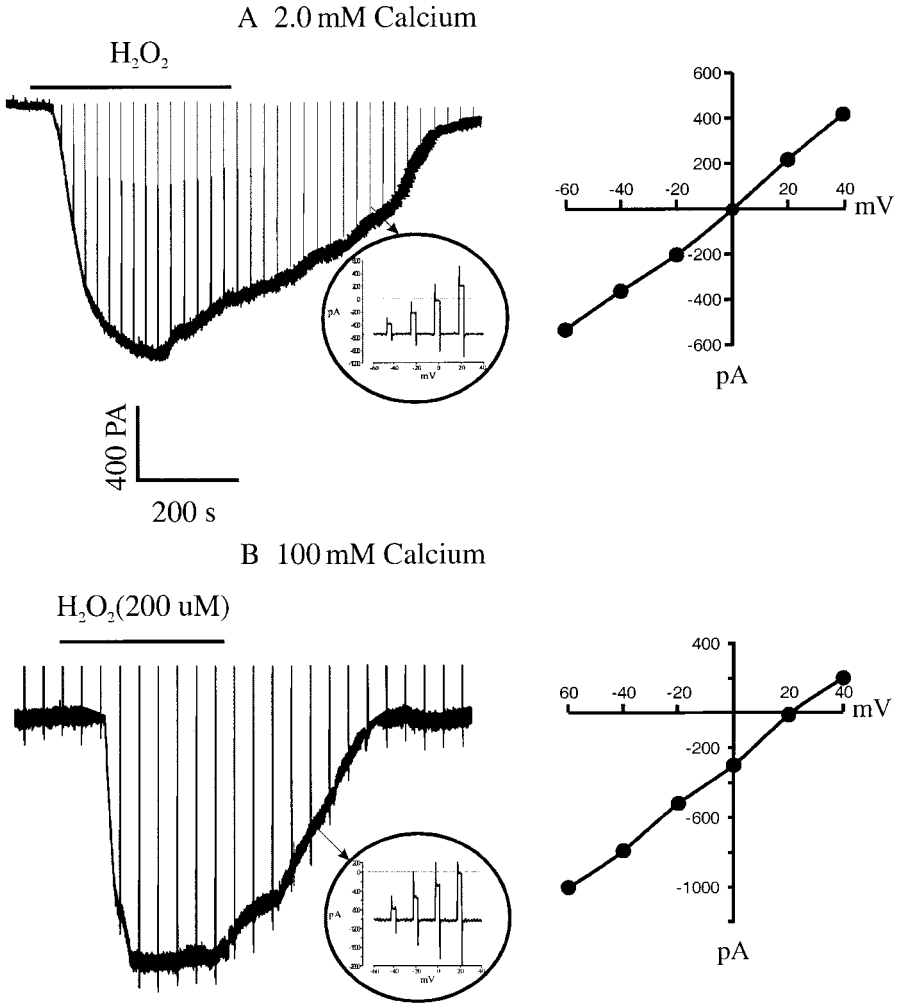

Figure 4. **The PECAM-1–regulated channel is calcium permeant.** RHP cells and HUVEC (unpublished data) were voltage clamped at a holding potential of −60 mV in (A) Hepes-buffered saline solution containing 2 mM $CaCl_2$ or (B) Hepes-buffered solution containing 100 mM Ca^{+2} as the only permeant cation with CsCl pipette solution. Cells were puffed with 200 μM H_2O_2 for 3–5 min. Cells in high-calcium solution manifested characteristic current with a right shift in Vrev toward E_{Ca+2}.

bath) did not shift the reversal potential nor alter the slope conductance (unpublished data).

To assess the permeability of PECAM-1–activated NSC channels to Ca^{2+}, we examined ionic signaling in extracellular solution containing 100 mM Ca^{2+} as the only permeant cation. As shown in Fig. 4, exposure of cells to 200 μM H_2O_2 activated a prolonged Ca^{2+} current in 5/6 (83%) HUVEC and 6/6 (100%) RHP cells. The kinetics of this current were similar to those observed in monovalent cation solutions, and the current reversal potential was shifted to positive potentials, as predicted for Ca^{2+}-permeant channels. These results are identical to those previously obtained after homophilic PECAM-1 engagement and anti–PECAM-1 antibody ligation (O'Brien et al., 2001), suggesting that the same channel mediates both PECAM-1–dependent ionic signaling events.

H_2O_2 elicits prolonged intracellular calcium transients in PECAM-1–transfected cells

To further examine the role of PECAM-1 in mediating H_2O_2-activated calcium signaling, we measured H_2O_2-induced Ca^{2+} fluorescence with laser scanning confocal microscopy in individual Fluo-4–loaded REN and RHP cells exposed to 200 μM H_2O_2. As shown in Fig. 5, H_2O_2 evoked a prolonged rise in intracellular calcium lasting about 5 min (Fig. 5, A and B), a time course quite similar to that of the PECAM-1–dependent cation current (compare Figs. 1 and 5). Conversely, in REN cells, no increase in intracellular calcium was observed, confirming the requirement for PECAM-1 in this calcium response (Fig. 5 B, inset). Addition of thrombin yielded calcium signals in both cell lines that were of rapid onset, but of much shorter duration, whereas buffer did not produce a rise in $[Ca^{2+}]_i$ in either cell type (Fig. 5 B). These findings suggest that EC PECAM-1 regulates the gating of a calcium-permeant, voltage-independent, NSC channel that mediates prolonged calcium signals observed in ECs after physiologic H_2O_2 exposure.

The PECAM-1–regulated channel is independent of IP_3 receptor activation or phosphoinositide turnover, is not calcium or voltage dependent, and is partially lanthanum sensitive

We have previously reported that PECAM-1 engagement activates a calcium-permeant channel that is independent of intracellular Ca^{2+} store release (O'Brien et al., 2001). We next sought to determine the relationship of the PECAM-1–dependent H_2O_2 cation current to phosphoinositide-mediated intracellular calcium store release and to subsequently activated endothelial plasmalemmal nonspecific cation channels termed depletion-activated channel or store-operated

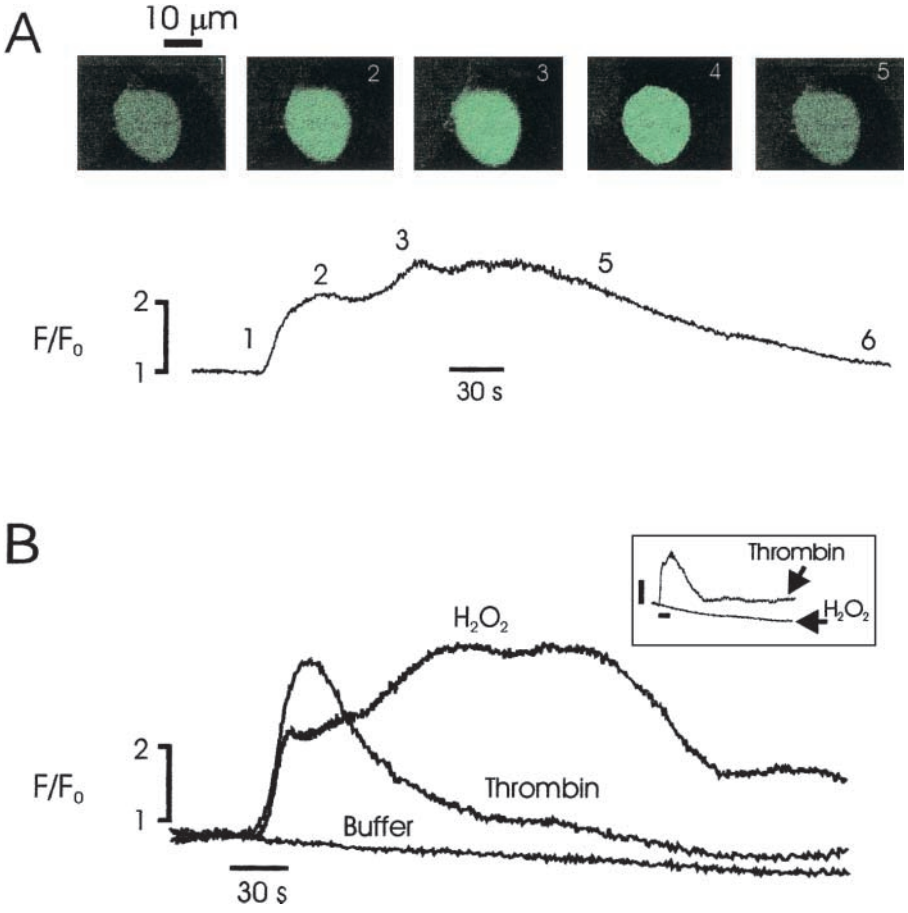

Figure 5. **Hydrogen peroxide elicits prolonged intracellular calcium transients in PECAM-1–transfected cells.** Fluo-4–loaded RHP and REN cells were treated with 200 μmH_2O_2 during acquisition of confocal images every 43 ms. (A) After H_2O_2 exposure, there was a homogenous, prolonged rise in Fluor-4 fluorescence in PECAM-1–expressing cells (images 1–5, above). The fluorescence profile (below, derived from all of the images as described in Materials and methods) indicates a roughly threefold increase in fluorescence, similar in magnitude to that observed when cells were exposed to thrombin (B). (B) Comparison of responses to H_2O_2 (200 μM) and thrombin (1 U/ml) from confocal images as in A shows a similar magnitude increase in $[Ca^{2+}]_i$, although H_2O_2 responses are substantially increased in duration. Puff application of buffer was without effect, and thrombin, but not H_2O_2, increased Ca^{2+} fluorescence in REN cells (inset).

channels (SOCs) that mediate capacitative calcium entry (Vaca and Kunze, 1995; Parekh and Penner, 1997; Kamouchi et al., 1999). After dialysis with 5 mg/ml heparin, a compound known to act as an IP_3 receptor antagonist as well as a potential inhibitor of G protein receptor interaction (Taylor and Broad, 1998), H_2O_2- and thrombin-induced currents were measured in RHP cells (Fig. 6) and HUVEC (unpublished data). Treatment with 200 μM H_2O_2 evoked typical NSC currents in 8/9 (89%) RHP cells and 3/3 (100%) HUVEC, suggesting that the PECAM-1–activated NSC is neither IP_3 gated nor dependent on IP_3-mediated intracellular calcium store release mechanisms, similar to findings after antibody engagement in whole-cell fluorimetry and voltage clamp studies (O'Brien et al., 2001). Conversely, thrombin (1.5 U/ml), a G protein–coupled receptor agonist known to activate intracellular calcium store release and subsequent NSC activation in HUVEC and REN cells (Gurubhagavatula et al., 1998; O'Brien et al., 2001), activated a large inward current in control recordings ($n = 9$), but this current was completely blocked in cells dialyzed with heparin (Fig. 6, inset). Phosphoinositol turnover assays were also performed in RHP cells to confirm these findings as previously described (Krymskaya et al., 1999; O'Brien et al., 2001). Treatment with 200 μM H_2O_2 failed to elicit significant stimulation of phosphoinositide formation, whereas addition of thrombin yielded a robust increase in phosphoinositide turnover (unpublished data). These findings further support a phosphoinositide-independent activation pathway for the PECAM-1 NSC current.

The kinetic characteristics of the PECAM-1–dependent current are similar to DAC/SOC NSC currents activated by intracellular calcium release (capacitative calcium entry), which are sensitive to trivalent cations such as lanthanum chloride ($LaCl_3$) (Vaca and Kunze, 1995; Parekh and Penner, 1997; Kamouchi et al., 1999). Therefore, we evaluated the dependence on intracellular Ca^{2+} release and the La^{3+} sensitivity of H_2O_2-activated PECAM-1–dependent currents. RHP cells and HUVEC were dialyzed with CsCl pipette solution containing 10 mM EGTA and 0 mM Ca^{2+} (with a calculated $[Ca^{2+}]_i$ of <1 nM) and current recorded 10 min after break-in. Dialysis with this intracellular solution did not itself elicit large cation currents similar to those activated by H_2O_2, but subsequent exposure (after 3–5 min) to 200 μM H_2O_2 activated currents similar to those observed in cells dialyzed with normal intracellular solution. H_2O_2 currents were observed in 5/5 (100%) RHP cells and 3/3 (100%) HUVEC tested, whereas no currents were observed prior to H_2O_2. Moreover, the H_2O_2 current was only partially inhibited in the presence of 100 μM $LaCl_3$ ($n = 3$), whereas the current activated by thrombin (1.5 U/ml) was completely inhibited by 50 μM $LaCl_3$ ($n = 6$). Interestingly, thrombin-induced capacitative currents mediated by DAC/SOC NSC channels manifest time course, reversal potential (Vrev = 0), mean peak current amplitude (730 ± 47pA), and current–voltage relationships quite similar to those observed for PECAM-1 currents. However, the differences in trivalent sensitivity, calcium gating, and phosphoinositide dependence suggest that the PECAM-1–dependent

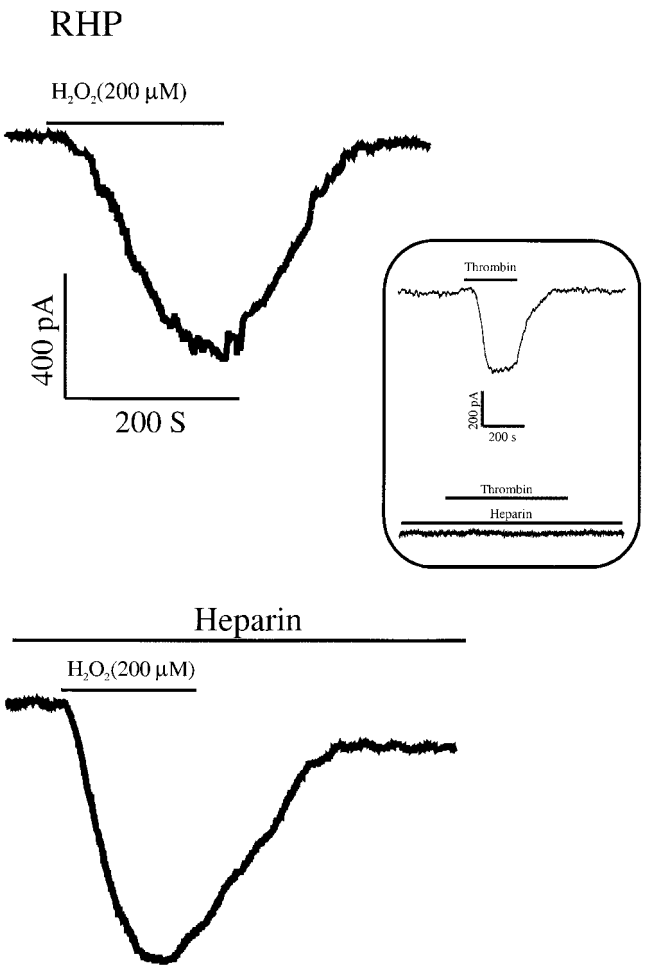

Figure 6. **Activation of the PECAM-1–regulated NSC current is IP_3 independent.** RHP cells and HUVEC (unpublished data) dialyzed with the IP_3 receptor inhibitor, heparin sulfate, through the patch pipette (5 mg/ml heparin in CsCl) were voltage clamped at a holding potential of –60mV and puffed with 200 μM H_2O_2 for 5 min. Currents after dialysis (B) were identical to those of typical PECAM-1–dependent responses (A). Identical results were obtained in RHP and HUVEC puffed with 400 μM H_2O_2 (unpublished data). In RHP cells analyzed with nonheparin containing pipette solution, thrombin treatment (1.5 U/ml) yielded similar large, prolonged inward currents with a Vrev = 0 and an ohmic I/V relationship. However, in heparin-dialyzed RHP cells (inset) and HUVEC (unpublished data) puffed with thrombin (1.5 U/ml), no current was detected.

current is activated by an alternate signaling mechanism and through channels that are distinct from those mediating capacitative calcium entry (O'Brien et al., 2001).

The PECAM-1 cytoplasmic domain is necessary and sufficient for current activation

In order to specifically examine the role of the PECAM-1 intracellular domain, REN cells stably transfected with a panel of PECAM-1 mutant constructs were examined in a series of patch clamp studies (Fig. 7). The first construct, PITC, consists of the PECAM-1 extracellular domain fused to the nonhomologous ICAM-1 transmembrane and cytoplasmic domains. This fusion protein does not elicit cation current after antibody engagement (O'Brien et al., 2001), but supports homophilic interaction and localizes to the cell–cell border in confluent monolayers (unpublished data). No currents were observed in voltage-clamped PITC cells puffed with 200 μM H_2O_2 (n = 7), suggesting that the PCD, and possibly the transmembrane domain, are necessary for current activation. A second construct, IL2PCD, was utilized to evaluate the role of the isolated PCD in current regulation. IL2PCD contains the nonhomologous interleuken (IL)-2 receptor (CD25) extracellular and transmembrane domains fused to an intact PECAM-1 cytoplasmic domain that can serve as a substrate for H_2O_2-induced tyrosine phosphorylation (unpublished data). In voltage-clamped IL2PCD cells puffed with 200 or 400 μM H_2O_2, cation currents with kinetics identical to full-length PECAM-1 control cells were observed in 14/15 (93%) cells (Fig. 7). These findings indicate that the PECAM-1 cytoplasmic domain is necessary and sufficient for H_2O_2 current activation.

Src family kinase activity is required for PECAM-1–regulated H_2O_2-activated cation current signals

In EC and RHP cells, PECAM-1 engagement yields calcium transients mediated by a NSC channel that is Src tyrosine kinase-dependent (Gurubhagavatula et al., 1998; O'Brien et al., 2001). Therefore, we sought to determine the relationship of tyrosine kinase activity to the PECAM-1–dependent H_2O_2 current. RHP cells (n = 8) and HUVEC (n = 5) pretreated for 1 h with 10 μM genistein and purified with 200 μM H_2O_2 failed to activate a current, suggesting a similar role for tyrosine kinase activity in regulation of this channel. To further refine this observation, voltage-clamped HUVEC and RHP cells were dialyzed through the patch pipette with 10 μg/ml Sc-18, an Src-neutralizing rabbit polyclonal antibody specific for the Src family nonreceptor kinases (Marrero et al., 1995). In HUVEC and RHP cells dialyzed with Sc-18, attenuated current was observed in 2/10 (20%) and 3/11 (27%) of cells exposed to 200 μM H_2O_2, whereas H_2O_2 failed to elicit any current in the remaining cells. By contrast, exposure of HUVEC and RHP cells dialyzed with an irrelevant rabbit polyclonal control antibody (anti-HSVTK) to H_2O_2 evoked currents identical to nondialyzed cells in 10/12 (83%) and 4/5 (80%) of cells tested, respectively (Fig. 8). These findings confirm that tyrosine kinase activation, including Src-family nonreceptor tyrosine kinases, is required for activation of PECAM-1–dependent cation signaling.

The PECAM-1 Y663/Y686 motif is selectively tyrosine phosphorylated after H_2O_2 exposure and is required for H_2O_2-activated, PECAM-1–dependent cation currents

To determine whether PECAM-1 is a substrate for H_2O_2-induced tyrosine phosphorylation, we exposed REN, RHP, and REN cells stably transfected with Y663F/Y686F, a full-length PECAM-1 construct with two Y-to-F point mutations (Fig. 7) known to disrupt PECAM-1 tyrosine phosphorylation and phosphotyrosine-mediated protein–protein association (Newman 1999), to 0.5 mM H_2O_2 for 10 min. Anti–PECAM-1 immunoprecipitation and phosphotyrosine analysis of these lysates revealed that PECAM-1 is heavily ty-

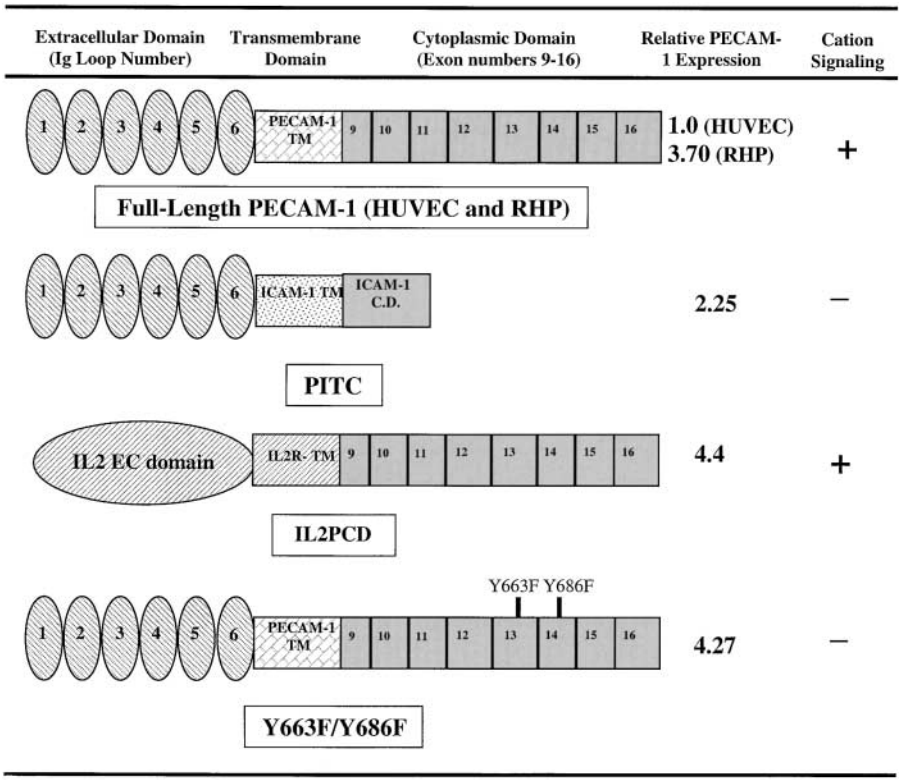

Figure 7. **The PECAM-1 cytoplasmic domain is necessary and sufficient for current activation.** Schematic representation of wild-type PECAM-1 (expressed endogenously in HUVEC) and mutant PECAM-1 constructs stably transfected into the REN cell line. The extracellular domain containing six Ig-like loops is represented as filled ovals 1–6, the transmembrane domain as a rectangle, and the cytoplasmic domain as a series of boxes representing cytoplasmic exons 9–16. PITC is a chimera containing the intact PECAM-1 extracellular domain fused to the nonhomologous ICAM-1 transmembrane and cytoplasmic domains. IL2PCD is comprised of the intact PECAM-1 cytoplasmic domain fused to the nonhomologous IL2 receptor extracytoplasmic and transmembrane domains. Y663F/Y686F contains dual Y-to-F point mutations in the cytoplasmic Y663/Y686 SH2-interaction motif. Surface PECAM-1 expression relative to HUVEC is shown. Construct ability to mediate H_2O_2-activated cation signals is indicated.

rosine phosphorylated in RHP and HUVEC after H_2O_2 exposure, predominantly on the Y663/Y686 cytoplasmic motif (Fig. 9 A). Subsequently, we analyzed phosphorylation patterns in REN, RHP, and PITC under conditions identical to the patch clamp and Ca^{2+} imaging experiments. After a 10-min H_2O_2 exposure at 200 μM, near the threshold dose required for cation signaling, full-length PECAM-1 phosphorylation was increased by 40% above expected baseline phosphorylation (Lu et al., 1996, 1997), as seen by densitometric normalization to PECAM-1 levels (Fig. 9 B). Notably, this concentration of H_2O_2 appears to represent the threshold dose for PECAM-1 phosphorylation, as increasing H_2O_2 doses yielded increasingly intense PECAM-1 tyrosine phosphorylation (Fig. 9, A and B). No phosphotyrosine signal was detected in PITC or untransfected REN cells (Fig. 9 B), confirming that the intact PECAM-1 cytoplasmic domain may serve as a substrate for H_2O_2-induced tyrosine phosphorylation.

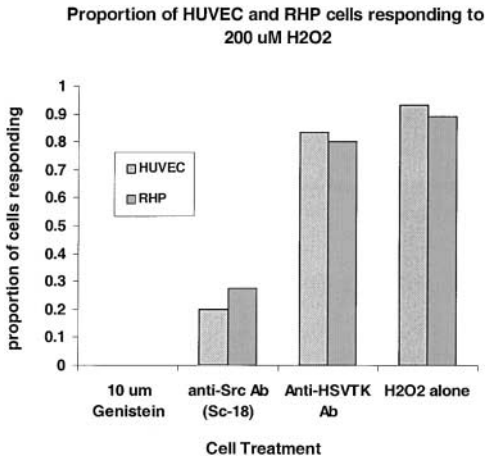

Figure 8. **H_2O_2-activated cation current signals require Src family tyrosine kinase activity.** Proportion of voltage-clamped HUVEC (hatched) and RHP cells (spotted) responding to 200 μM H_2O_2 after 1 h pretreatment with 10 μM genistein or intracellular dialysis with 10 μg/ml Sc-18, an inactivating polyclonal antibody directed against p60 Src tyrosine kinase. Results are compared with nondialysis and anti-HSV TK antibody dialysis controls. Notably, of cells dialyzed with Sc-18, those responding to H_2O_2 manifested markedly attenuated current amplitude compared with nondialysis and anti-HSV TK controls.

To determine whether this motif represents a critical element of the PECAM-1 cytoplasmic domain for cation current signaling, we conducted patch clamp studies on the Y663F/Y686F cells. No cation currents were observed in voltage-clamped Y663F/Y686F cells puffed with 200 μM H_2O_2 ($n = 8$), suggesting that the PECAM-1 cytoplasmic Y663/Y686 SH2-interaction domain is necessary for current activation. Notably, Y663F/Y686F and PITC also serve as transfection controls demonstrating that current activation is not merely an artifact of transfection, selection, or heterologous protein expression.

Discussion

Reactive oxygen species such as hydrogen peroxide are critical mediators of physiologic and pathologic vascular responses after inflammatory, ischemic, and hyperoxic insults. Central to these effects are changes in EC $[Ca^{2+}]_i$, although the molecular interactions underlying H_2O_2 increases in $[Ca^{2+}]_i$ are not known. Here we report that physiologically relevant concentrations of H_2O_2 activate Ca^{2+}-permeant plasmalemmal cation channels and that this ionic signaling

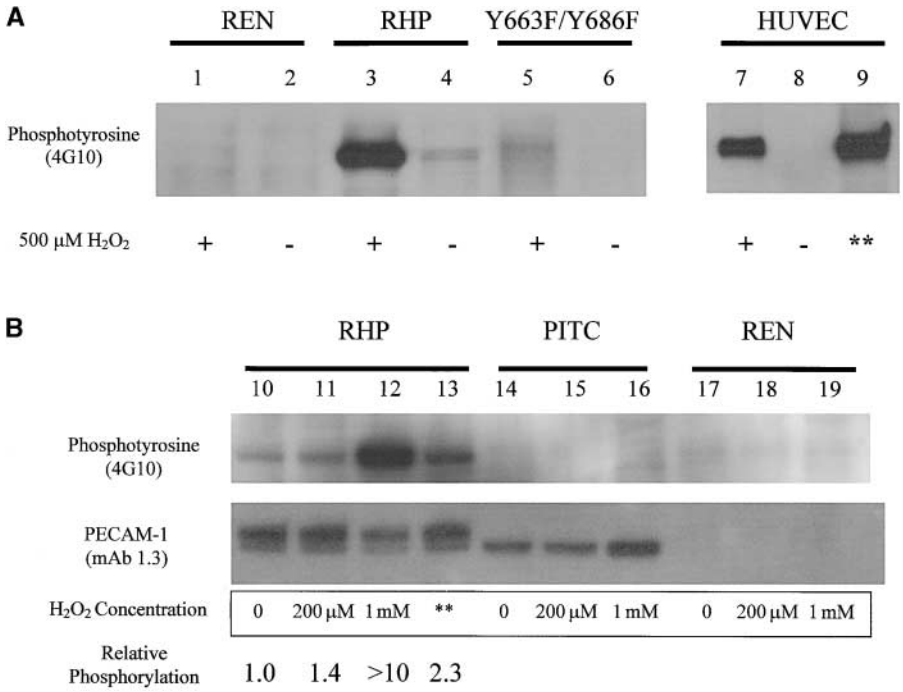

Figure 9. **The PECAM-1 cytoplasmic Y663/Y686 motif is a substrate for selective H_2O_2-mediated tyrosine phosphorylation.** (A) REN, RHP, Y663F/Y686F, and HUVEC were treated for 10 min with vehicle (lanes 2, 4, 6, 8), 0.5 mM H_2O_2 (lanes 1, 3, 5, 7) in low serum conditions, or 0.5 mM vanadate (3 h) as a positive control (**). PECAM-1 phosphotyrosine signals were detected by immunoblot with antiphosphotyrosine antibody (mAb 4G10) on anti–PECAM-1 immunoprecipitates (mAb 4G6). (B) REN, RHP, and PITC in low serum conditions were treated with buffer (lanes 10, 14, 17), 200 μM (lanes 11, 15, 18), or 1 mM H_2O_2 (Lanes 12, 16, 19), for 10 min or 0.5 mM vanadate for 3 h (**). PECAM-1 phosphotyrosine signals were detected with antiphosphotyrosine antibody on anti–PECAM-1 immunoprecipitates. Relative PECAM-1 levels were detected by stripping the membrane and reprobing with mAb 1.3 (anti–PECAM-1) (bottom). PECAM-1 phosphorylation was normalized to PECAM-1 content and compared with baseline phosphorylation (set at 1.0) to yield relative phosphorylation.

requires PECAM-1 expression. Activation of this current requires the PECAM-1 cytoplasmic Y663/Y686 motif and Src family nonreceptor tyrosine kinase activity, but is independent of IP_3 receptor activation. This is a previously unknown EC function for PECAM-1 that may represent a widespread mechanism linking neutrophil–EC interactions and EC response in the context of oxidant injury and inflammation.

H_2O_2 has been proposed to function as an endothelial signal transduction intermediate at low micromolar concentrations and as a mediator of cell response to pathologic processes at higher concentrations. Although the signaling processes underlying these actions are not well understood, H_2O_2 is known to induce tyrosine phosphorylation through activation of tyrosine kinases and inhibition of protein tyrosine phosphatases (Barchowsky et al., 1995; Carbajal and Schaeffer, 1998), and can activate a range of $[Ca^{2+}]_i$ signal responses (Suzucki et al., 1997; Yang et al., 1999; Lounsbury et al., 2000). We observed consistent activation of PECAM-1–dependent cation currents at concentrations of H_2O_2 in excess of 100 μM. At H_2O_2 levels below 100 μM, minimal Ca^{2+} signaling has been reported, whereas sublethal concentrations exceeding 100 μM manifest prolonged, low-amplitude $[Ca^{2+}]_i$ transients involving influx of extracellular Ca^{2+} (Bowles et al., 2001). Cytotoxic concentrations exceeding 500 μM result in the activation of phospholipases and release of Ca^{2+} ions from internal calcium stores (Schilling and Elliott, 1992; Natarjan et al., 1993; Barchowsky et al., 1994; Doan et al., 1994; Siflinger-Birnboim et al., 1996; Yolk et al., 1997; Hu et al., 1998; Min et al., 1998). Hydrogen peroxide levels between 200 and 500 μM, although not cytolytic (Shasby et al., 1988), may yield substantial cell toxicity by inducing signal transduction pathways that ultimately lead to apoptosis (Barchowsky et al., 1994; Suhara et al., 1998). Our findings indicate that PECAM-1, a protein highly expressed in ECs, functions as a critical regulator of plasmalemmal ion channels that result in depolarization and Ca^{2+} influx during sublethal hydrogen peroxide exposure. Although PECAM-1 is essential in regulating plasmalemmal cation influx, our results do not exclude other Ca^{2+}-mobilizing actions of H_2O_2, particularly at higher H_2O_2 concentrations. We did not observe consistent PECAM-1–dependent cation signaling or tyrosine phosphorylation at H_2O_2 concentrations below 100–200 μM, suggesting that this level of H_2O_2 represents a threshold for PECAM-1–mediated events. Thus, low levels of H_2O_2, such as those endogenously produced as signaling intermediates during ICAM-dependent EC responses to leukocytes (Wang and Doerschuk, 2000), may be PECAM-1 independent. Instead, PECAM-1 appears to function as a calcium-regulating protein at the moderate H_2O_2 levels (100–400 μM) that are generated during EC–neutrophil interaction in the context of inflammation (Test and Weiss, 1984). However, an important caveat in interpreting experiments evaluating H_2O_2 cellular responses is the substantial variability in effective dosage associated with media conditions, particularly serum concentration. For example, it has been reported that H_2O_2 concentration–response relationships may be decreased tenfold relative to serum-free conditions that may support EC morphologic changes after exposure to H_2O_2 levels as low as 20 μM (Bradley et al., 1995).

Although we demonstrate that PECAM-1 is required for H_2O_2 ionic signaling, the identity of the channel and potential linking molecules remain unknown. As the current kinetics, ionic selectivity, and associated $[Ca^{2+}]_i$ transients in ECs are indistinguishable when activated by H_2O_2 or PECAM-1 engagement (Gurubhagavatula et al., 1998; O'Brien et al., 2001), it is likely that these phenomena are associated

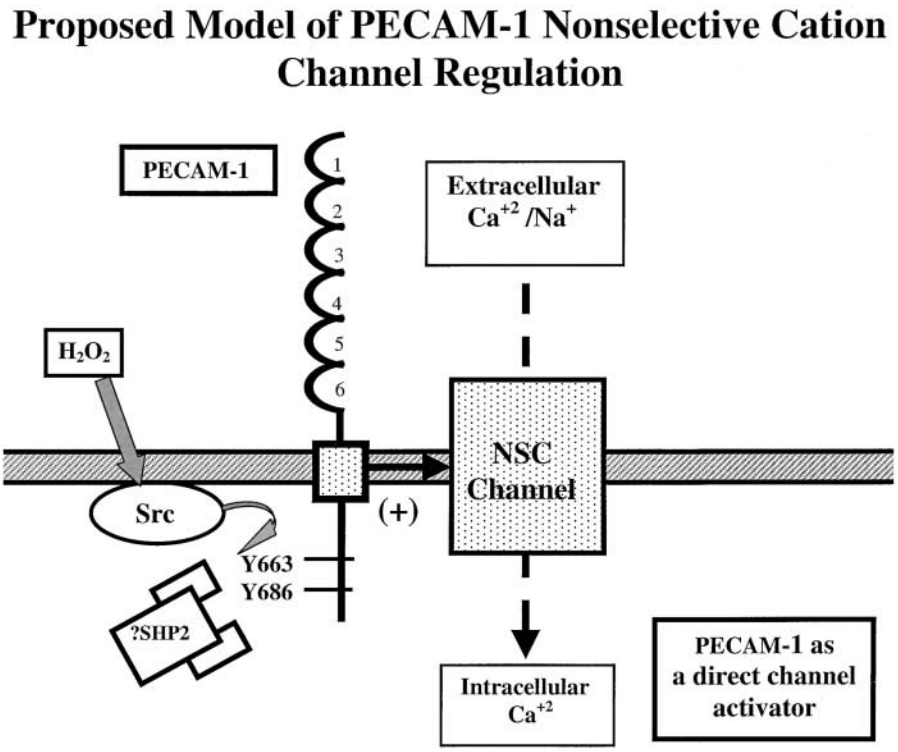

Figure 10. **Moderate dose H_2O_2 mediates EC calcium influx through a PECAM-1–regulated NSC channel activated via a tyrosine kinase-dependent, phosphoinositide-independent signaling pathway.** After moderate dose hydrogen peroxide (100–400 μM), the PECAM-1 cytoplasmic domain and (most likely) other central downstream components undergo tyrosine phosphorylation, possibly by Src family tyrosine kinases. The tyrosine-phosphorylated PECAM-1 intracellular Y663/Y686 motif then interacts either directly with a plasmalemmal NSC channel or through one or more intermediaries. Alternatively, phosphorylated PECAM-1 may inhibit or sequester a channel suppressor, thus indirectly activating the channel. The PECAM-1–regulated channel is independent of calcium store release or IP_3 receptor activation, manifests only partial trivalent cation sensitivity, and is neither $[Ca^{+}2]_i$ nor voltage gated, suggesting a calcium signaling pathway distinct from capacitative calcium entry mechanisms. Once activated, this PECAM-1–regulated plasmalemmal channel conducts a prolonged NSC current that mediates the calcium transients observed in ECs and PECAM-1–transfected REN cells following moderate level (100–400 μM) hydrogen peroxide exposure.

with activation of the same channel(s). Similarly, the failure of PECAM-1 to reconstitute cation signaling in certain cell types suggests that PECAM-1 is not, itself, a channel component (O'Brien et al., 2001). Recently, attention has been directed toward mammalian homologues of the *Drosophila* transient receptor potential and transient receptor potential–like proteins as possible components in multimeric channels activated by Ca^{2+} store depletion (Xu et al., 1997; Moore et al., 1998; Groschner et al., 1999). As we have previously reported (O'Brien et al., 2001), there is a distinct similarity between PECAM-1–dependent currents and nonspecific cation currents observed in ECs after depletion of Ca^{2+} stores (Vaca and Kunze, 1995; Parekh and Penner, 1997; Kamouchi et al., 1999). Although the H_2O_2-activated current is kinetically similar to store activated currents (Groschner et al., 1999), differences in La^{3+} sensitivity, lack of gating associated with Ca^{2+} depletion, and independence of IP_3 receptor activation (Nilius et al., 1997; Kamouchi et al., 1999) suggest important differences in channel permeability and gating.

Our results are similar to those describing an H_2O_2-activated, store release–independent, plasmalemmal calcium–conducting channel in EC that is gated through an unknown mechanism (Bowles et al., 2001). They are also consistent with reports of a tyrosine kinase–dependent NSC channel activated through engagement of basic FGF (bFGF) or insulin-like growth factor–1 receptors in ECs (Munaron and Pla, 2000). As tyrosine kinase activity is required for both receptor-dependent (bFGF or insulin-like growth factor–1) and independent (H_2O_2 and PECAM-1 ligation) EC Ca^{+2} signaling, it is likely that the tyrosine kinase substrate(s) responsible for channel activation are downstream of specific receptors, which are phosphorylated by either mechanism (Fleming et al., 1996). Exposure to phosphatase inhibitors such as vanadate, pervanadate, and H_2O_2 is known to yield selective tyrosine phosphorylation of the PECAM-1 cytoplasmic domain (Sagawa et al., 1997). After tyrosine phosphorylation, PECAM-1 can interact with several SH2 domain–containing proteins, including SHP2, PI3kinase, and PLC-γ (Newman, 1999), though the physiologic relevance of these interactions in EC is not certain. Similarly, PECAM-1 can function as a reservoir for transcription-regulating proteins such as β and γ-catenin in a phosphorylation-dependent manner (Ilan et al., 2000).

Utilizing endothelia-like REN cells stably transfected with wild-type and mutant PECAM-1 constructs, we have begun to conduct a detailed analysis of PECAM-1 structure–function relationships that would be otherwise impossible in ECs. Where possible, we have confirmed these findings in HUVEC. Our data demonstrate that PECAM-1 and likely other participating proteins serve as substrates for H_2O_2-induced tyrosine phosphorylation, that the PECAM-1–regulated current requires Src family nonreceptor tyrosine kinase activity, and that the PECAM-1 cytoplasmic domain (and its incorporated Y663/Y686 phosphotyrosine motif) is necessary and sufficient for H_2O_2-induced current activation (Fig. 10). In confluent cells, full-length PECAM-1, PITC, and Y663F/Y686F localize predominantly to cell–cell borders, whereas IL2PCD is diffusely distributed (Sun et al., 2000). However, the techniques employed in this study do not allow conclusions to be drawn regarding PECAM-1 cell localization requirements for current regulation in confluent monolayers. Similarly, although H_2O_2 activates tyrosine ki-

nases including p60src in ECs (Barchowsky et al., 1995; Carbajal and Schaeffer, 1998), the specific tyrosine kinase(s) mediating H_2O_2-induced PECAM-1 phosphorylation remains an important unresolved question. Interestingly, preliminary data, beyond the scope of this manuscript, suggest that the PECAM-1 transmembrane domain may also play a role in modulating PECAM-1–dependent ionic signals in the absence of an intact cytoplasmic domain (unpublished data). This is consistent with findings that the isolated PECAM-1 transmembrane and cytoplasmic domains function in signal transduction after matrix metalloproteinase–induced extracellular domain cleavage (Ilan et al., 2001). The identification of proteins that have a functional association with PECAM-1 in this response, as well as further delineation of specific tyrosine kinase subtypes underlying H_2O_2-induced PECAM-1 phosphorylation, will be important steps in further defining this unique signaling pathway.

Modulation of endothelial $[Ca^{2+}]_i$ is critical to a range of EC processes including neutrophil transmigration (Huang et al., 1993), a key EC function regulated by PECAM-1. Exogenous H_2O_2 stimulates neutrophil extravasation in vivo, which is blocked by anti–PECAM-1 antibodies (Scalia and Lefer, 1998). Similarly, anti–PECAM-1 antibodies block neutrophil transmigration after inflammatory stimuli known to induce generation of reactive oxygen species (Vaporciyan et al., 1993; Chosay et al., 1998). Our finding that the PECAM-1 extracellular domain, a critical element in EC–neutrophil interaction during transmigration (Nakada et al., 2000), is not involved in channel regulation, suggests that H_2O_2 ionic signaling may not be sufficient to induce leukocyte transmigration. Thus, PECAM-1 may serve dual functions of adhesion (extracellular domain) and ionic signaling (cytoplasmic domain), both of which may be required to recapitulate PECAM-1–dependent transmigration. The role of PECAM-1–mediated $[Ca^{2+}]_i$ signaling and the involvement of specific PECAM-1 domains in the regulation of neutrophil–endothelial interaction and in endothelial response to oxidant injury remains an important area for future investigation.

Materials and methods

Antibodies, reagents, immunoprecipitation, and Western blotting

Antibodies used include the following: mAb 4G6, a murine immunoglobin (IgG) directed against the PECAM-1 extracellular Ig loop six domain (Yan et al., 1995); mAb 1.3, a murine IgG directed against the PECAM-1 extracellular domain, a gift of Dr. Peter Newman (Blood Center of Southeastern Wisconsin, Milwaukee, WI); PCD, a rabbit polyclonal antibody directed against the PECAM-1 cytoplasmic domain; Sc-18 (Santa Cruz Biotechnology), a rabbit polyclonal antibody found to specifically inhibit Src family kinases Src, Yes, Fyn, and Fgr (Marrero et al., 1995); anti-HSVTK, rabbit polyclonal anti-HSVTK antibody (Elshami et al., 1996); anti-IL2 receptor (CD25) mAb 3G10 (Boehringer Mannheim); and 4G10, an anti-phosphotyrosine murine mAb (Upstate Biotechnology). Purified antibodies were obtained by protein G affinity chromatography of hybridoma supernatants or serum. Active binding of antibodies was confirmed by flow cytometry. Reagent grade hydrogen peroxide (Sigma-Aldrich) was diluted in isotonic PBS or media on the day of experiments as indicated. PECAM-1 immunoprecipitation was performed on REN cell lysates incubated with mAb 4G6 or anti-CD25 mAb (Il2PCD) followed by incubation with protein A Sepharose (Amersham Pharmacia Biotech). Lysates were separated on 10% SDS polyacrylamide reducing gels (Invitrogen). Membranes were probed with anti-phosphotyrosine antibody (4G10), counterstained with HRP-conjugated donkey anti–mouse IgG (Cappel), and signals were visualized with ECL (New England Nuclear). Membranes were then stripped per the manufacturer (NEN Life Science Products) and reprobed with mAb 1.3 or PCD and counterstained with donkey anti–mouse or donkey anti–rabbit HRP-conjugated secondary antibody (Cappel). Signals were again detected with ECL. Image capture and densitometry analysis was performed on a Kodak Image Station as per the manufacturer.

Cell lines and mutant PECAM-1 constructs

HUVEC (Clonetics) were cultured in M199 medium (Mediatech, Inc.) supplemented with 15% FBS, 100 μg/ml EC growth factor (Clonetics), 100 ug/ml Heparin (Elkins-Sinn), and 2 mM L-glutamine (GIBCO BRL). Only cells of passage six or less were used. PECAM-1 mutant constructs IL2PCD (Sun et al., 2000), PITC, and Y663F/Y686F, a gift of Dr. P. Newman (Fig. 7) were constructed with the sequence overlap extension technique as previously described (Sun et al., 2000; O'Brien et al., 2001). All full-length and mutant PECAM-1 constructs were subcloned into the pcDNA-neo vector. REN cells, a human mesothelioma cell line previously isolated in our laboratory (Smythe et al., 1994), were grown in RPMI media (Mediatech) supplemented with 10% FBS and 2 mM L-glutamine (R10 media). Stable polyclonal populations of REN cell PECAM-1 transfectants were generated by magnetic bead sorting (Dynal, Inc.) and selected in G418 (0.5 mg/ml) supplemented R10 media as previously described (Gurubhagavatula et al., 1998). Expression was subsequently confirmed by flow cytometry using an EPICS Elite Flow cytometer (Coulter Corporation) as described (O'Brien et al., 2001). All stably transfected cell lines uniformly expressed PECAM-1 at high levels relative to HUVEC (Fig. 7).

Patch clamp recording

Cells were prepared for patch clamp experiments on round 12-mm glass coverslips (Fisher) coated with fibronectin (GIBCO BRL) or 1% gelatin (HUVEC). Treated coverslips were placed in 6-well plates and seeded for 18–24 h with 100,000 cells/well in 3 ml media. Whole-cell currents were recorded utilizing standard whole-cell or perforated patch clamp methods as described previously (O'Brien et al., 2001). Coverslips were transferred to a temperature-controlled chamber maintained at 35°C (Brook Industries), and superfused at 1 ml/min with either HBSS (123 mM NaCl, 2 mM $CaCl_2$, 1 mM KCl, 25 mM Hepes, and 15 mM glucose, pH 7.4), high-calcium solution (100 mM $CaCl_2$, 10 mM glucose, 10 mM Hepes, pH 7.4), CsCl solution (130 mM CsCl, 10 mM glucose, 10 mM Hepes, pH 7.4), or Tris-Cl solution (123 mM Tris-HCl, 2 mM $CaCl_2$, 1 mM KCl, 25 mM Hepes, and 15 mM glucose, pH 7.4) as indicated. Borosilicate glass electrodes (resistance 3–5 Mohm) were filled with internal solution containing 130 mM CsCl, 1.2 mM $MgCl_2$, 0.075 mM EGTA, 1 mM Mg-ATP, and 10 mM Hepes, except for asymmetric anion experiments in which 130 mM CsAcetate was substituted for CsCl as indicated. For perforated patch experiments, pipettes were dipped 1–2 s in pipette solution then backfilled with pipette solution containing 200 mg/ml nystatin. After seal formation and establishment of whole-cell recording configuration, cells were voltage clamped at –60 mV (Axopatch 200B; Axon Instruments). Records were sampled at 1 kHz and filtered at 100 Hz. Current reversal potentials were measured either by step or ramp (−60 up to +100 mV applied every 30 s) depolarization protocols. Only current traces returning to baseline were considered. Hydrogen peroxide solution was puffed directly onto cells through puffer pipettes (Picospritzer; General Valve). EGTA, heparin, and antibody dialysis experiments were conducted with 10 mM EGTA (Sigma-Aldrich), 5 mg/ml heparin sulfate, or 10 μg/ml of PCD, Sc-18, or anti-thymidine kinase antibody added to the pipette solution. $LaCl_3$ inhibition experiments were performed with varying concentrations of $LaCl_3$ (0–100 μM) in HBSS extracellular solution puffed directly onto cells after agonist stimulation with H_2O_2 or thrombin. Cells were equilibrated for 10 min after break-in before initiating experimental protocols (Davis and Sharma, 1997; Wang and Kotlikoff, 1997).

Calcium measurements

Cells were incubated with 10 μM Fluo-4 AM (Molecular Probes) for 10 min at room temperature in a recording chamber mounted on an inverted microscope (TE300; Nikon), and washed with HBSS extracellular solution for 30 min. Fluo-4 fluorescence was recorded using a laser scanning confocal head (Radiance 2000; Bio-Rad Laboratories) attached to an inverted microscope with a plan-apo 60× water immersion objective (1.2 n.a.; Nikon). Cells were excited with 488 nm light from a krypton/argon laser and x-y images (128 × 30 pixels) recorded using Lasersharp software (Bio-Rad Laboratories) at a 44-ms interval. Images were analyzed using LaserPix version 4.2 software (Bio-Rad Laboratories). Fluorescence profiles were obtained by computing the mean fluorescence from a region of the cell (F) for

each image and dividing this by the mean fluorescence of the cell prior to stimulation (F_o).

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