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Cytoskeletal Reorganization Internalizes Multiple Transient Receptor Potential Channels and Blocks Calcium Entry into Human Neutrophils[1]

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Store-operated calcium entry (SOCE) is required for polymorphonuclear neutrophil (PMN) activation in response to G protein-coupled agonists. Some immunocytes express proteins homologous to the *Drosophila* transient receptor potential gene (*trp*) calcium channel. TRP proteins assemble into heterotetrameric ion channels and are known to support SOCE in overexpression systems, but the evidence that TRP proteins support SOCE and are functionally important in wild-type cells remains indirect. We therefore studied the expression and function of TRP proteins in primary human PMN. TRPC1, TRPC3, TRPC4, and TRPC6 were all expressed as mRNA as well as membrane proteins. Immunofluorescence microscopy demonstrated localization of TRPC1, TRPC3, and TRPC4 to the PMN cell membrane and their internalization after cytoskeletal reorganization by calyculin A (CalyA). Either TRPC internalization by CalyA or treatment with the inositol triphosphate receptor inhibitor 2-aminoethoxydiphenyl borane resulted in the loss of PMN SOCE. Cytochalasin D (CytoD) disrupts actin filaments, thus preventing cytoskeletal reorganization, and pretreatment with CytoD rescued PMN SOCE from inhibition by CalyA. Comparative studies of CytoD and 2-aminoethoxydiphenyl borane inhibition of PMN cationic entry after thapsigargin or platelet-activating factor suggested that SOCE occurs through both calcium-specific and nonspecific pathways. Taken together, these studies suggest that the multiple TRPC proteins expressed by human PMN participate in the formation of at least two store-operated calcium channels that have differing ionic permeabilities and regulatory characteristics. ***The Journal of Immunology*****, 2004, 172: 601–607.**

Homologues of the *Drosophila* transient receptor potential (*trp*)[4] channel gene are widely distributed in mammalian tissues. These genes encode a superfamily of 20 or more proteins that are believed to assemble into a wide variety of ion channels. Although TRP proteins probably serve many functions in different tissues, their specific physiological roles in individual tissues are generally unknown. Nonetheless, the TRP channel proteins (TRPC) are widely assumed to be critically involved in the generation of capacitative or store-operated calcium entry currents (SOCE) in electrically nonexcitable cells (1–4). The evidence linking TRP genes to SOCE is generally derived from systems in which the heterologous expression of TRPC has increased secondary calcium entry into cells either in response to G protein-coupled (GPC) agonists or to direct (pharmacologic) store depletion (5–9). Overexpressed TRPC proteins can overwhelm endogenous channel function however, so expression systems cannot prove that native SOCE depends on TRPC proteins.

Antisense strategies have also been used to evaluate the role of TRPC proteins in mammalian SOCE (5, 6). Decreased expression of TRPC1 is associated with diminished calcium entry in salivary gland cells and B cells (9, 10). Treatment with TRPC4 antisense cDNA reduced both native TRPC4 protein and endogenous SOCE in bovine adrenal cortical cells (11). Genetic disruption, however, is typically only practical in cell lines, and native cells may express multiple channel proteins. Also, in some cases antisense strategies may decrease inositol triphosphate (InsP3)-mediated Ca^{2+} store depletion from the endoplasmic reticulum (ER) (10), which would also decrease SOCE. Last, studies have compared the characteristics of calcium entry into native calls and expression systems. For example, TRPC6 is present in T cells that have a calcium entry current that is activated by diacylglycerols and has many characteristics of SOCE (12, 13). Nonetheless, the functional importance of such findings is still unknown.

Thus, although many cell types, including immunocytes, express TRP proteins, it has not been established whether TRP proteins are the functional agents of SOCE in native immunocytes. Also, the arrangement of TRPC proteins into hetero-oligomeric ion channels is widely thought to be a key determinant of calcium signal regulation (3). Thus, the channel proteins expressed by any one cell type are likely to be important determinants of that cell's calcium signaling as well as its susceptibility to pharmacologic control.

A wide variety of human polymorphonuclear neutrophil (PMN) responses to inflammation require calcium entry. Studies using ^{45}Ca show that calcium entry into PMN occurs subsequent to store depletion (14), and patch-clamp experiments confirm that PMN calcium influx after fMLP is store-operated rather than voltage-gated (15). Moreover, we and others have shown that PMN calcium entry in response to other GPC agonists occurs predominantly via SOCE (14–

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[4] Abbreviations used in this paper: TRP, transient receptor potential; 2-APB, 2-aminoethoxydiphenyl borane; AUC, area under the curve; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CalyA, calyculin A; CytoD, cytochalasin D; ER, endoplasmic reticulum; GPC, G protein-coupled; InsP3, inositol triphosphate; PAF, platelet-activating factor; PLC, phospholipase C; PMN, polymorphonuclear neutrophil; SOCE, store-operated calcium entry; TG, thapsigargin; TRPC, TRP channel protein; I_{CRAC}. calcium, release-activated calcium entry.

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16), and we have shown that injury and clinical hyperinflammatory states are characterized by aberrant PMN calcium signaling (17–19). Moreover, we have shown that GPC chemoattractant stimulation of PMN is linked to SOCE PMN by multiple pathways that involve sphingolipid second messengers (20, 21). Little is known, however, about the expression of TRP in primary PMN, and although TRP transcripts have been detected (22), nothing is known about PMN TRP protein expression or function. We therefore examined human PMN, seeking evidence of their expression of TRP channel proteins and evidence that such TRP proteins might participate in SOCE.

Materials and Methods

Neutrophil preparations

Neutrophil (PMN) samples were prepared from volunteer human blood samples. Details of our protocol were previously published (16, 20). Briefly, heparinized whole blood (20 U/ml) is centrifuged at 150 × *g* for 10 min. The platelet-rich plasma layer is discarded. The cells are layered onto Polymorphoprep (Robbins Scientific Corp., Sunnyvale, CA) and centrifuged for 30 min at 300 × *g*. The neutrophil layer is then collected and diluted 1/1 with 0.45% NaCl to restore osmolarity. After a 5-min rest, the PMN suspensions are washed in RPMI 1640 and pelleted by centrifugation at 150 × *g* for 10 min. The PMN are then suspended in 2 ml of HEPES buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, and 0.1% BSA, pH 7.4), counted on a flow cytometer, and kept on ice until prepared for further study. PMN purity using this method is >98%. Using our methods, flow cytometric studies with annexin and propidium iodide demonstrate a uniform PMN population with total cell viability >99% and early apoptotic changes seen in ~5% of the PMN (23).

RT-PCR analysis

Neutrophils were isolated by our usual methods and then further purified by a second gradient centrifugation. Total RNA was extracted from these twice-purified neutrophils using the TRIzol reagent method (Life Technologies, Gaithersburg, MD). cDNA was prepared by SuperScript II (Life Technologies) and used for PCR with *Taq* polymerase (Life Technologies). Two pairs of specific primers each were used to detect TRPC1, TRPC3, and TRPC4. One primer pair was used for TRPC5, and three pairs were used for TRPC6. These primers were a gift from Dr. J. Putney, Jr. (National Institute of Environmental Health and Sciences, Research Triangle Park, NC). The sequences of the primer pairs used along with the predicted size of their expected fragments (shown in parentheses in base pairs, are as follows: TRPC1, 5′-cttcctctccatcctcttcc-3′ and 5′-gtttctgacaaccgtagtcc-3′ (273 bp); 5′-ttctgtggattattgggatga-3′ and 5′-cagaacaaagcaaagcaggtg-3′ (505 bp); TRPC3, 5′-gcga
attgttaactttcccaaatgc-3′ and 5′-tcttccaaaagttcataacgaaggc-3′ (300 bp); 5′-atgct gcttttaccactgtag-3′ and 5′-tgagttagactgagtgaagag-3′ (449 bp); TRPC4, 5′-ttgc ctctgaaagacataacataag-3′ and 5′-ctactaacacacattgttcactgag-3′ (300 bp); 5′-cct ggacattttgaagtttc-3′ and 5′-ctgcatggtcagcaatcag-3′ (339 bp); TRPC5, 5′-tgcattgctctatgccatacgcaag-3′ and 5′-cctctgaactagacacacactccac-3′ (265 bp); and TRPC6, 5′-cagcacagataatgcaaaacag-3′ and 5′-atgatgctctgggctttg-3′ (249 bp); 5′-ctgagctgttccagggccat-3′ and 5′-ctcttgatttggttccatg-3′ (427 bp); 5′-aca gataatgcaaaacagctg-3′ and 5′-atgatgctctgggctttg-3′ (244 bp). After PCR amplification, the reaction mixtures were applied to 1% agarose gel for electrophoresis, and DNA fragments were detected by ethidium bromide staining.

Western blot analysis

Crude membranes were prepared from pooled human neutrophil samples. Details of the membrane preparation were previously reported (24). Briefly, after preparation of the membranes and assay for protein content with Coomassie reagent, 50 μg of membrane protein was diluted with ImmunoPure Lane Marker Reducing Sample Buffer (Pierce, Rockford, IL), boiled for 5 min, then applied to a 3–8% NuPAGE minigel (Invitrogen, Carlsbad, CA) for electrophoresis. After separation, proteins were transferred to nitrocellulose paper, and the transfer efficiency was checked using Ponceau S stain. Blots were blocked with SuperBlock (TBS) Blocking Buffer (Pierce), followed by a second block using 5% nonfat milk in TTBS (20 mM Tris-HCl, (pH 7.5), 68.5 mM NaCl, and 0.1% Tween 20). A 1-h incubation with the primary Abs listed below was followed by several washes and then a 1-h incubation with secondary Abs (anti-rabbit IgG for TRPC1, TRPC4, and TRPC6 and anti-chicken IgG for TRPC3). After subsequent washes, the blots were stained using West Pico Chemiluminescent Substrate (Pierce) for 5–30 min at room temperature. The nitrocellulose paper was then used to expose X-OMAT film (Eastman Kodak, Rochester, NY) for the detection of proteins. The Abs used for TRPC1 and the HSG cells used as positive controls for that Ab were gifts from Dr. I. Ambudkar (National Institutes of Health, Bethesda, MD) (25). Abs to TRPC3 (26) were gifts from Dr. C. Montel (The Johns Hopkins University School of Medicine, Baltimore, MD). Abs to TRPC4 (11) and a partial (43-kDa) TRPC4 peptide used as a positive control were gifts from Dr. V. Flockerzi (Institut fur Pharmakologie und Toxikologie der Universitat des Saarlandes, Homburg, Germany). TRPC6 Abs were purchased from Alomone Laboratories (Jerusalem, Israel).

Spectrofluorometry

PMN were incubated in 1 μM fura-2/AM (Molecular Probes, Eugene, OR) at 37°C for 30 min in the dark. PMN specimens were divided into aliquots of 2×10^6 cells and placed on ice in the dark until ready for use. The individual aliquot to be used was incubated at 37°C for 5 min before each experiment. Cells were pelleted by centrifugation at 4500 rpm for 5 s and then resuspended in 3 ml of HEPES in the cuvette. Experiments were begun in nominally calcium-free medium containing 0.3 mM EGTA. Intracellular calcium was monitored by measuring fura fluorescence at 505 nm, using 340/380 nm dual wavelength excitation in a Fluoromax-2 spectrofluorometer (Jobin Yvon-Spex, Edison, NJ). Cuvette temperatures were kept at 37°C with constant stirring. Individual experiments were calibrated by adding 100 μM digitonin (Molecular Probes) for the R_{max} and then 15 mM EGTA for the R_{min}. The autofluorescence of a sample cell suspension treated with 100 μM digitonin and 2 μM $MnCl_2$ was subtracted from the total fluorescence. The intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was then calculated from the 340/380 nm fluorescence ratio (K_d = 220 nM) according to the methods of Grynkiewicz et al. (27). Dye leakage is trivial and has no influence on $[Ca^{2+}]_i$ calculations using these methods. The order of study of isolates was alternated to avoid bias related to duration of dye loading or time of cell study. The entry of calcium into PMN after stimulation by GPC chemoattractants typically occurs uniquely via store-operated calcium channels (14–16). Calcium entry currents are measured by the readdition of 1 mM external calcium to cells stimulated in a nominally calcium-free environment (extracellular $[Ca^{2+}]$, ~50 nM).

We used modifications of these methods to assess the relative influx of strontium ions ($[Sr^{2+}]_i$). Sr^{2+} entry has been widely used as a marker for calcium entry via channels formed from expressed TRP proteins (28–30), and we have shown brisk Sr^{2+} entry into PMN in response to store depletion by GPC agonists as well as by ionomycin (20). Although Sr^{2+} has less affinity for fura than does Ca^{2+}, and its binding causes less fluorescence, its isobestic point and 340/380 ratio profile are very similar (31). Thus, relative Sr^{2+} entry can be accurately assessed as the area under the Sr^{2+} entry curve (AUC; see *Data analysis* below). We have also previously shown that assessments of $[Ca^{2+}]_i$ store release in Ca^{2+}-free medium are unaffected by subsequent addition of Sr^{2+} to the medium before calcium addition and cell lysis for calibration of R_{max} and R_{min} (20). Because of its uncertain K_d for fura and the potential for interactions, however, $[Sr^{2+}]_i$ is always reported as apparent $[Sr^{2+}]_i$. Similarly, $[Ca^{2+}]_i$ measurements made after the addition of Sr^{2+} may be inexact and are therefore treated as apparent. In all cases, however, only experimental responses obtained under identical conditions are compared.

Immunofluorescence microscopy

Freshly prepared PMN were allowed to adhere to a chamber slide coated with poly-L-lysine. Detailed methods were previously reported (24). Briefly, adherent PMN were incubated with 100 nM calyculin-A (CalyA) or vehicle for 20 min at room temperature. Cells were then fixed with 3% paraformaldehyde and permeabilized with methanol at −70°C on dry ice. The PMN were treated with the primary Abs described above. In addition, we used commercial Abs from Sigma-Aldrich (St. Louis, MO) for TRP1, -3, and -4 for the immunohistochemistry experiments, which yielded indistinguishable results. The anti-TRPC6 Abs used were not recommended for immunohistochemistry. The dilution factors used were: anti-ezrin, 1/100; anti-TRPC1, 1/50; anti-TRPC3, 1/50; and anti-TRPC4, 1/50. The secondary Abs used were linked with FITC. Confocal images were collected using an MRC 1024 krypton/argon laser scanning confocal system (Bio-Rad, Hercules, CA) equipped with an Optihot II photomicroscope (Nikon, Melville, NY).

Data analysis

SOCE responses were measured and assessed quantitatively as the integrated $[Ca^{2+}]_i$ AUC (in nanomoles per liter times seconds) for the 100 s (AUC_{100}) after stimulation with the agonist in question, followed by readdition of a divalent cation (either calcium or strontium) to the medium (Fig. 1). AUC was measured in arbitrary units of apparent $[Sr^{2+}]_i$ per

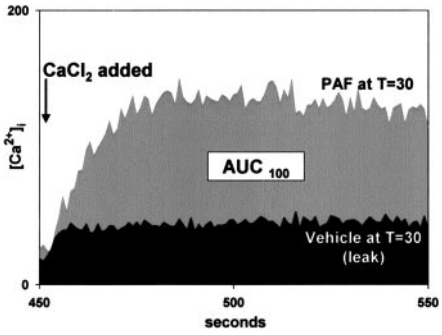

FIGURE 1. Quantitative measurement of store-operated cation entry. PMN are stimulated (in this case with PAF) at 30 s in calcium-free medium. After resolution of the store depletion (at 450 s), $CaCl_2$ is added to bring the Ca^{2+} concentration to 1 mM. $[Ca^{2+}]_i$ is assessed over the next 100 s, and the $[Ca^{2+}]_i$ AUC is calculated by a computer algorithm. The AUC for nonspecific calcium entry (leak) into an identical aliquot of cells is calculated in the same way and subtracted from the total to yield the AUC_{100}. Identical operations are performed when $SrCl_2$ is added, recognizing that the $[Sr^{2+}]_i$ measurements are relative rather than absolute.

second or of $[Ca^{2+}]_i$ per second after the addition of external Sr^{2+} and Ca^{2+}, respectively. This approach renders measurements less dependent on momentary peaks and therefore more reproducible. Also, specific SOCE currents will often have specific morphology over time that may be ignored if peak values alone are used (20). The AUC_{100} values reported are further corrected to yield agonist-specific entry by subtracting the AUC_{100} for unstimulated cation entry (leak) into an identical aliquot of untreated PMN from the same isolation studied under identical conditions. The integration of concentration curves was performed using an automated software package (GRAMS/32, Galactic Industries Corp., Salem, NH). Data analysis was performed using SigmaPlot and SigmaStat software (SPSS, Chicago, IL). Data were evaluated using one-way ANOVA with Tukey's all-pairwise comparisons or unpaired or paired *t* tests as indicated. In all cases, significance accepted at $p \leq 0.05$.

Results

Trp *expression in PMN*

Multiple specific primer pairs were used to screen for TRPC1-TRPC6 gene products in highly purified human PMN. In all cases except TRPC5, at least two primer pairs were available for each assay. Although some primer pairs worked better than others, at least one appropriate band was detected, confirming the presence of TRPC1, TRPC3, TRPC4, and TRPC6 mRNA in PMN (Fig. 2). No evidence was found of TRPC5 gene product, and no assay was performed for TRPC2, which is a known human pseudogene. Similar to the findings of Heiner et al. (22), PMN were also found to express message for CaT1 (TRPV6; data not shown). The finding of multiple *trp* gene products by RT-PCR suggested the presence of multiple TRP proteins in human PMN and presumably their involvement in calcium signaling. Further, although circulating PMN have a very short life span, the active synthesis of *trp* gene products suggests that TRP channel protein synthesis may contribute to the regulation of PMN calcium signaling and cell function in circulating PMN, as has been found in other cell types (32).

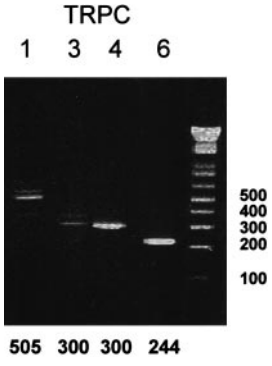

FIGURE 2. Expression of *trp* genes in human neutrophils. Specific RT-PCR products for each TRP amplified from human neutrophil total RNA are indicated by the arrows, with their expected size in base pairs listed below. See *Materials and Methods* for details of the primers and conditions used.

Immunoblotting for PMN TRP proteins

To confirm the expression of the TRP proteins, we assayed crude membrane preparations from freshly isolated human PMN using Western blot techniques. Specific Abs for TRPC1, TRPC3, TRPC4, and TRPC6 all revealed strong bands in the 90–105 kDa range (Fig. 3). Thus, human PMN synthesize and express at least four distinct TRP channel protein isoforms. The molecular structure of TRP proteins implies that they assemble into tetramers to act as calcium channels (1). Thus, the present findings support the contention that SOCE may represent the contributions of a variety of entry pathways in the same cell (20, 33) and that a variety of TRP-based calcium entry channels may be the basis of SOCE in human PMN.

Cytoskeletal redistribution of neutrophil TRP proteins

In *Drosophila*, TRP channels form a supramolecular complex with multiple other signaling and accessory proteins (34, 35). In mammalian expression systems, cell surface supramolecular complexes containing TRP proteins have been shown to be internalized under the influence of the phosphatase inhibitor CalyA. CalyA reorganizes existing filamentous actin without inducing new actin polymerization (4, 36) We therefore examined the effects of CalyA on the distribution of TRPC1, TRPC3, and TRPC4 in PMN by confocal immunofluorescence microscopy. We found that all TRP proteins examined were initially localized to the cell surface in a specked distribution (Fig. 4, *b–d*). After exposure to CalyA, the TRP proteins were redistributed into a diffuse cytosolic pattern. (Fig. 4, *f–h*).

Cytoskeletal reorganization and SOCE

Internalization of TRP proteins by CalyA attenuates cationic influx in response to GPC agonists in expression systems (36). We examined PMN SOCE currents in response to the GPC chemoattractant platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). PAF activates store-operated calcium and strontium influx in PMN (16, 20). After exposure to CalyA, PAF-initiated Sr^{2+} influx into PMN was almost completely (95%) abolished (Figs. 5 and 7*A*). Total store-operated Ca^{2+} influx was markedly (83%) attenuated (Figs. 6 and 7*A*). Similar studies performed using fMLP to stimulate SOCE (Fig. 8) showed that CalyA inhibits SOCE initiated by a variety of GPC receptors that act through different G proteins. Thapsigargin (TG) inhibits ER Ca^{2+} uptake

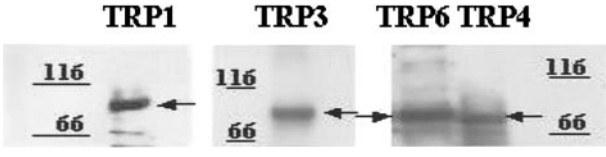

FIGURE 3. Western blots. Western blotting of crude neutrophil membrane preparations stained with specific Abs for TRPC1, TRPC3, TRPC4, and TRPC6 (see *Materials and Methods*) revealed strong bands in the expected locations.

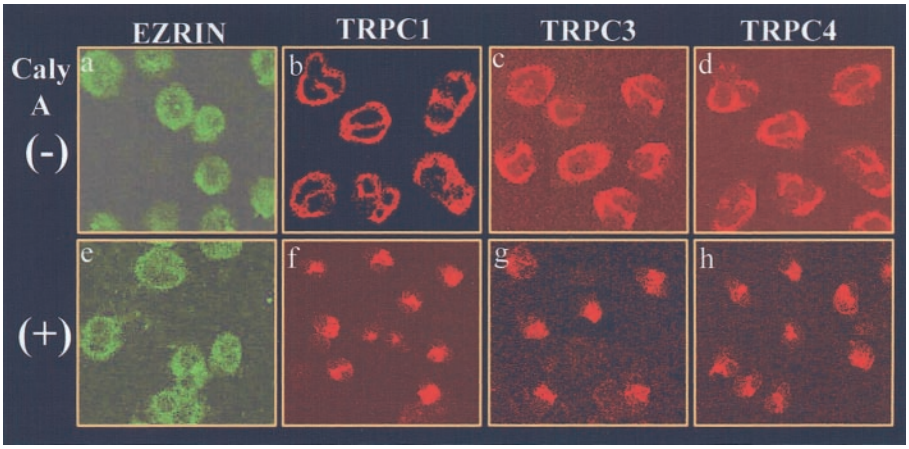

FIGURE 4. The effects of CalyA on TRP protein localization in PMN. PMN were permeabilized and stained for TRPC1, TRPC3, and TRPC4 with or without prior treatment with CalyA. TRP proteins are normally localized to the cell surface in a speckled pattern (*upper panels*; *b–d*) suggestive of signaling complexes. Treatment with CalyA (*lower panels*; *f–h*) resulted in redistribution of the stain to the cell interior in a diffuse pattern. Control experiments (*a* and *e*) revealed no significant changes in the distribution of staining for ezrin after CalyA. The experiments shown are representative of three different cell preparations.

directly. It therefore elicits SOCE independent of GPC receptors. Stimulation by this mechanism typically elicits more potent SOCE than does stimulation by GPC agonists. Again, however, we found that treatment of PMN with CalyA significantly attenuated store-operated calcium entry in response to TG (Fig. 7*B*). The entry of strontium into PMN after TG was much less susceptible to CalyA, although it did show a trend ($p = 0.08$) toward inhibition.

Rescue of SOCE by cytoskeletal disruption

Taken together, the above findings suggest that SOCE inhibition by CalyA depends on actin reorganization and calcium channel internalization rather than upon altered afferent signaling between GPC receptors and ER calcium stores. Nonetheless, CalyA is a phosphatase inhibitor and might have other, nonspecific effects on cell signaling. We therefore evaluated the degree to which CalyA suppression of SOCE was specific for its effects on the cytoskeleton. Cytochalasin D (CytoD) acts directly in PMN to cap actin (37, 38). Thus, it disrupts actin polymerization and prevents cytoskeletal rearrangement by CalyA. We found that pretreating PMN with CytoD significantly rescued fMLP-initiated SOCE from CalyA inhibition (Fig. 8, *A* and *B*). In identical studies, CalyA decreased PAF-mediated Ca^{2+} entry and again, CytoD restored CalyA inhibited SOCE from 5055 ± 721 to 7400 ± 943 nM·s ($p < 0.01$; $n = 6$ isolates), where AUC_{100} for PAF-mediated Ca^{2+} entry in 10 volunteer PMN isolates was 9320 ± 463 nM·s.

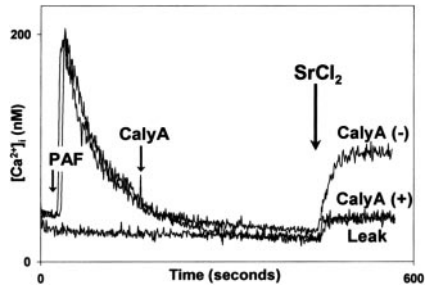

FIGURE 5. The effects of CalyA on Sr^{2+} entry after PAF. PAF (100 nM in ethanol) was applied at 30 s, and CalyA (50 nM in DMSO) was added at 150 s. $SrCl_2$ (1 mM) was added at 450 s. To assess PAF-independent leak current, equal volumes of the vehicles were applied before Sr^{2+}. $CaCl_2$ was added before cell permeabilization. $[Ca^{2+}]_i$ values before the addition of Sr^{2+} are quantitatively correct (20). The traces after addition of Sr^{2+} indicate relative Sr^{2+} flux, but they cannot be assigned an absolute concentration value and are treated as apparent $[Ca^{2+}]_i$. Each trace shown is representative of six to eight experiments. The PAF and CalyA trace is displaced 5 s to the *left* for clarity. Sr^{2+} entry after PAF is inhibited to the level of Sr^{2+} leak by CalyA. Thus, CalyA abolishes Sr^{2+} entry into PMN through nonspecific store-operated channels regulated by PAF.

Interactions with InsP3 receptors

TRPC3, -4, and -6 have C-terminal binding sites for ER InsP3 receptors (39), and CalyA has been shown to cause redistribution of TRP proteins in colocalization with InsP3 receptors in expression systems (36). We therefore evaluated the effects of the InsP3 receptor inhibitor 2-aminoethoxydiphenyl borane (2-APB) on SOCE in PMN. 2-APB has been shown to inhibit SOCE entry through TRPC3-derived channels after receptor activation (40) and to inhibit calcium release-activated calcium entry (I_{CRAC}) as well as store-dependent CaT1-derived currents (41). We had previously shown that 2-APB inhibits PAF-initiated calcium entry in PMN (16). We found in this study that 2-APB caused almost complete suppression of both the calcium-specific and the strontium-permeable SOCE mechanisms in PMN treated by PAF (Fig. 9*A*). We also found that 2-APB caused a high degree of SOCE inhibition in PMN subjected to direct store depletion by TG (Fig. 9*B*).

Discussion

The equivalence of TRP gene products to the channel-forming proteins responsible for SOCE in mammalian cells has not been fully established. To date, assumptions that TRP genes encode SOCE channels is based on the ability of expressed TRP proteins to produce *trans*-membrane cation currents, rather than upon evidence of TRP function in wild-type cells. Moreover, many wild-type cells express a range of TRP proteins that may confer functionally specific calcium signaling to that cell type. Thus, genetic

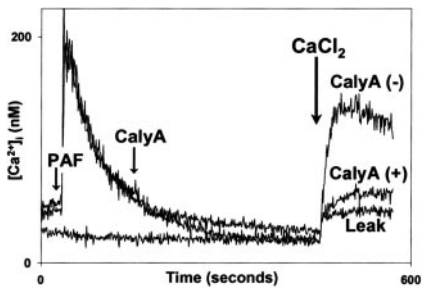

FIGURE 6. The effects of CalyA on Ca^{2+} entry after PAF. Again, 100 nM PAF was applied at 30 s, and CalyA was added at 150 s. $CaCl_2$ (1 mM) was added at 450 s. Leak currents were again assessed after vehicle only. The traces shown are representative of seven or eight experiments. PMN store-operated Ca^{2+} entry after PAF is strongly, but not completely, inhibited.

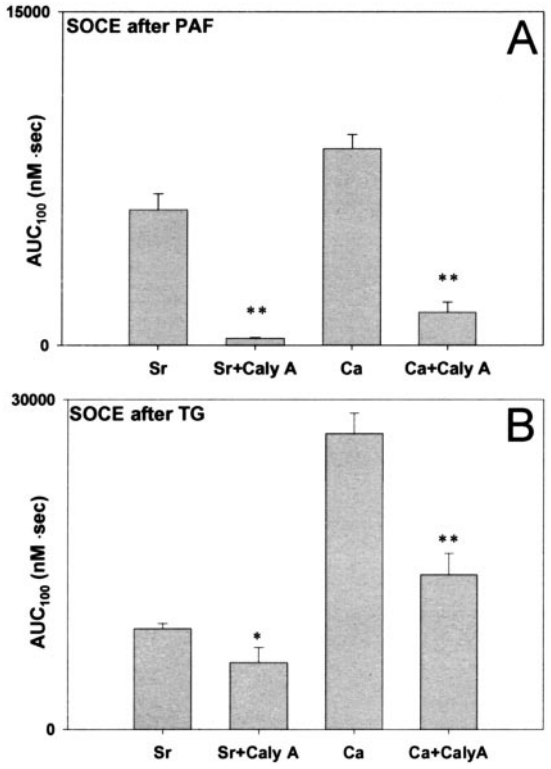

FIGURE 7. PAF-initiated SOCE with and without CalyA. The effects of CalyA on PMN SOCE. In the *upper panel* (*A*), SOCE was stimulated by direct, pharmacologic store depletion using TG. In the *lower panel* (*B*), SOCE was stimulated by activation of the GPC PAF receptor. PMN were suspended in cuvettes under nominally calcium-free conditions. TG (500 nM), PAF (100 nM), or their respective vehicles were added at 30 s (see Figs. 4 and 5). CalyA (50 nM) or vehicle (DMSO) was added at 150 s. $SrCl_2$ or $CaCl_2$ (1 mM) was added at 450 s, and the area under the cation influx curve was measured for the next 100 s (AUC_{100}; nanomoles per liter times seconds). The AUC_{100} for spontaneous Sr^{2+} or Ca^{2+} entry into an untreated identical aliquot of PMN is subtracted from each result to assess agonist-specific cation entry. CalyA inhibited Ca^{2+} entry by 83% and inhibited Sr^{2+} entry by 95%. *, $p = 0.08$; **, $p < 0.01$. $n = 4$ for *A*; $n = 6–8$ for *B*.

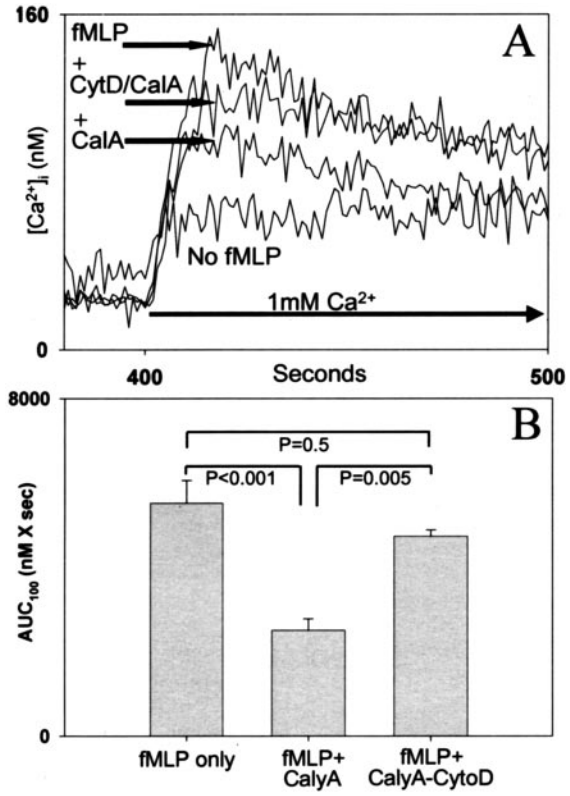

FIGURE 8. *A*, Rescue of SOCE by CytoD. Representative traces depict the readdition of calcium (e.g., as in Fig. 1) after stimulation with 100 nM fMLP in the absence of inhibitors, the presence of CalA, or the presence of CalA after pretreatment with CytoD. The timing of drug administration is the same as in Figs. 5 and 6. Where CytD is present, it was given 2 min before fMLP. In all cases the store release transient is essentially identical. The *lowermost trace* represents calcium entry in the absence of prior agonist, i.e., the nonspecific leak. The results of these experiments are summarized in *B* (*lower panel*). Cytoskeletal rearrangement by CalyA strongly inhibited SOCE initiated by fMLP (bar 1 vs 2, $p < 0.001$). Pretreatment of the PMN with CytoD disrupted the actin cytoskeleton and thus rescued SOCE from the effects of CalyA (bar 2 vs 3, $p = 0.005$). SOCE thus rescued by CytoD was indistinguishable from the control (bar 1 vs 3, $p = 0.5$). Treatment with CytoD alone had no effect ($0.1 < p < 0.2$) on fMLP-initiated SOCE (not shown). $n = 4$ for all groups. All statistical values are the result of one-way ANOVA, followed by Tukey's test.

disruption of TRP protein production may change, rather than abolish, SOCE.

Our initial studies found that TRPC1, TRPC3, TRPC4, and TRPC6 were expressed as mRNA in PMN (Fig. 2). These findings are somewhat similar to those of Heiner et al. (22), although by screening with single primer pairs those authors only found expression of TRPC3 and TRPC6 in PMN. They also, however, found evidence of TRPV1, -2, -5, and -6 expression. We also found evidence of TRPV6 (CaT1) message expression (not shown). Thus, although some differences exist between these datasets, it is clear that PMN express a broad range of TRP channels likely to be involved in ionic signaling.

Subsequent to the finding of TRPC message, we have demonstrated that TRPC1, TRPC3, TRPC4, and TRPC6 proteins are normally expressed on the PMN cell membrane. We also show that cytoskeletal reorganization causing displacement of TRPC1, -3, and -4 from the cell surface to the cytosol inhibits SOCE whether due to receptor stimulation by PAF or fMLP or to direct Ca^{2+} store depletion by TG. Taken together, these data suggest that TRPC-based channels are important contributors to the induction of SOCE in normal human PMN by both physiologic and pharmacologic stimuli.

The findings are important in several other ways. The expression of multiple TRP proteins is widely thought to lead to the formation of hetero-oligomeric channels, where each involved TRP may contribute to the regulation and conductance characteristics of the channel. Evaluation of TRP proteins in expression systems suggests that they can form calcium influx channels that are regulated both by the depletion of intracellular calcium stores and by upstream G protein and phospholipase C (PLC)-coupled mechanisms. Moreover, combinations of TRP proteins into store-operated heteromultimeric channels are likely to result in channels with novel and specific properties (42). We have previously suggested that SOCE in PMN is comprised of at least two divalent cation entry pathways (20). The current results confirm that TRP proteins form nonspecific (Sr^{2+}-permeable) and Ca^{2+}-specific channels that are inhibited by CalyA to differing extents depending upon whether the cells are stimulated by GPC agonists or by direct store depletion. Isolated store depletion by TG resulted in 2- to 3-fold more SOCE than did store depletion by GPC agonists. Both the nonspecific and Ca^{2+}-specific channels were activated by PAF and

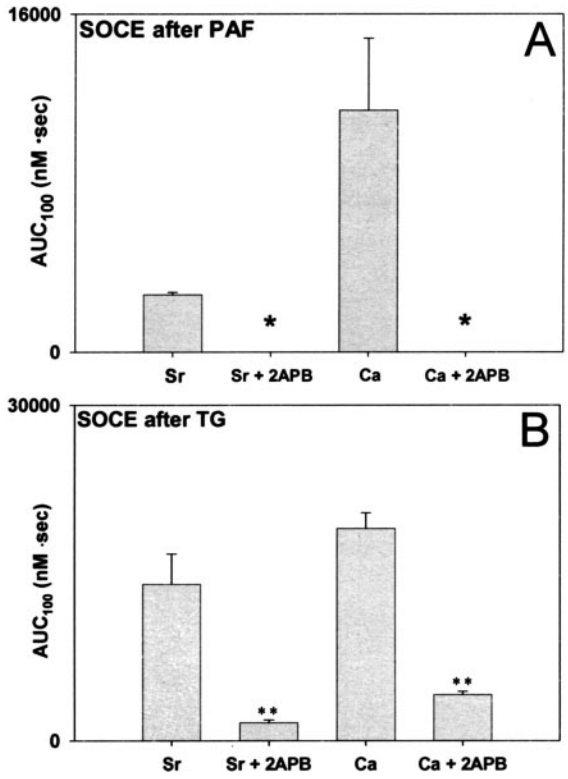

FIGURE 9. The effects of 2-APB on SOCE. In the *upper panel* (*A*), SOCE was stimulated by activation of the GPC PAF receptor. In the *lower panel* (*B*), SOCE was stimulated by direct, pharmacologic store depletion using TG. PMN were suspended in cuvettes under nominally calcium-free conditions. TG (500 nM) or PAF (100 nM) was added at 30 s. 2-APB (75 μM) or vehicle was added at 200 s. Sr^{2+} or Ca^{2+} was added at 300 s, and the AUC_{100} was measured. 2-APB caused complete suppression of both calcium and strontium entry after PAF (*A*). 2-APB caused potent, but incomplete, suppression of calcium and strontium entry after TG (*B*). *, $p < 0.05$; **, $p < 0.01$ (by ANOVA and Tukey's test). $n = 6–8$ experiments for each condition.

fMLP as well as by TG. These pathways open with slightly different time courses (20), and PMN calcium entry in vivo is likely to reflect their combined actions, with each pathway regulated by both G proteins and the status of Ca^{2+} stores.

Although we have been able to show to date that PMN express at least four TRP membrane proteins and that at least three of these are internalized by CalyA, the exact molecular compositions of two SOCE channel mechanisms identified to date remain to be determined. Prior work, however, suggests several likely possibilities. Both TRPC3 and TRPC4 acting alone in expression systems can form nonspecific cation channels responsive to GPC agonists that act via G_q-coupled receptors and PLC (28, 29, 43–45); PAF is known to signal through $G_{q/11}$ (46). TRPC6 is also activated by diacylglycerol rather than store depletion (12, 13). Thus, TRPC3, TRPC4, or TRPC6 could be implicated as a molecular component of an Sr^{2+}-permeable, G protein-regulated PMN SOCE channel.

Divalent cation entry into PMN after PAF exposure was essentially completely blocked by CalyA. Direct store depletion by TG produced more Ca^{2+} influx than PAF, but CalyA inhibition of Ca^{2+} influx after TG was only partial. Moreover, Sr^{2+} entry after TG was relatively resistant to inhibition by CalyA, whereas Sr^{2+} entry after PAF was completely inhibited. These findings suggest that pure store depletion in the absence of GPC receptor stimulation may activate influx mechanisms that are negatively regulated by GPC receptors. This again points to the multiplicity and complexity of SOCE mechanisms in PMN, and the individual specificity of their regulation. Also, specific TRP proteins can display sensitivity to physical and chemical stimuli, such as temperature, osmolality, and cellular deformation. Thus, the differential sensitivity of SOCE pathways to cytoskeletal reorganization may also reflect the participation of specific TRP proteins.

PMN Ca^{2+} influx is therefore likely to be supported by store-operated channels composed of TRP proteins. These may be activated by store depletion, by both store-dependent and store-independent (i.e., GPC) mechanisms, or by physical and chemical stimuli, or may be activated by store depletion and regulated by GPC mechanisms. In this respect, prior work suggests that Ca^{2+} entry through purely TRPC1-based channels would be store depletion dependent (47). TRPC3-based channels can also respond to store depletion (48), and TRPC3/TRPC1 hetero-oligomers have been shown to be regulated by both Ca^{2+} and by PLC in coexpression systems (30). Similarly, TRPC1 and TRPC4 proteins are normally coexpressed in bovine adrenal cortical cells, and this combination is associated with an I_{CRAC}-like influx current (11). Thus, TRPC1, either alone or in combination with TRPC3 or TRPC4, may also be implicated in the Ca^{2+}-specific SOCE found in PMN. Other combinations of the TRP proteins we found may exist. The vanilloid TRP CaT1 (TRPV6) has been shown to conduct calcium-specific influx in other systems (49). Thus, if CaT1 protein is present, it might also play a role in Ca^{2+}-specific SOCE in PMN. Clearly, a functional understanding of PMN store-operated Ca^{2+} entry channels will require much further study.

2-APB is a competitive inhibitor of ER receptors for InsP3 and can inhibit the binding of these receptors to TRPC proteins (36). Such binding is considered a necessary step in the conformational coupling model of SOCE (2). Previous work (29, 50) has shown that 2-APB can prevent both receptor-induced and store-induced SOCE. 2-APB has been shown to inhibit both receptor-induced SOCE through expressed TRPC3 channels (50) and I_{CRAC}-like conductances through expressed CaT1 channels (41). The present data similarly show that 2-APB strongly inhibits the entry of both Sr^{2+} and Ca^{2+} into PMN exposed to TG. TRP proteins are known to be cointernalized along with InsP3 receptors and $G_{q/11}$ (which associates with the PAF receptor) after CalyA (36). Inhibition by 2-APB therefore further suggests that PMN SOCE via both specific and nonspecific mechanisms may be based upon TRP channels internalized by CalyA. The extent to which SOCE depends upon physical coupling of TRPC and InsP3 receptors remains unclear, however. We have recently shown that SOCE can also be initiated by sphingosine-1-phosphate acting as a second messenger (21). Thus, the mechanisms by which SOCE is initiated and modulated appear to include both changes in cellular architecture and the generation of soluble messengers.

The properties of TRP-based SOCE channels in PMN are likely to be of considerable importance in both neutrophilic inflammation and in the understanding of calcium influx-mediated activation of other immunocytes. Several members of the TRP family are expressed in human B cells (51). Human monocytes, T cells, and platelets all express mRNA for TRP1, -3, -4, and -6, and cell maturation has been associated with both increased transcript expressions and increased SOCE (12, 52). Thus, specific clusters of TRP protein expression may be typical of certain immunocyte or hemopoietic lineages and may be important determinants of immune responses that require prolonged elevations of cell calcium concentration. Moreover, the development of pharmacologic agents specific for molecular species involved in SOCE might lead to novel and highly specific forms of immune modulation effected through the control of cellular calcium entry.

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