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Store-Operated Calcium Entry in Human Neutrophils Reflects Multiple Contributions from Independently Regulated Pathways

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Human polymorphonuclear neutrophil (PMN) responses to G protein-coupled chemoattractants are highly dependent upon store-operated Ca$^{2+}$ entry (SOCE). Recent research suggests that SOCE currents can be mediated by a variety of related channel proteins of the transient receptor potential superfamily. SOCE has been regarded as a specific response to depletion of cell calcium stores. We hypothesized that net SOCE might reflect the contributions of more than one calcium entry pathway. SOCE was studied in normal human PMN using Ca$^{2+}$ and Sr$^{2+}$ ions. We found that PMN SOCE depends on at least two divergent cation influx pathways. One of these was nonspecific and Sr$^{2+}$ permeable; the other was Ca$^{2+}$ specific. The two pathways show different degrees of dependence on store depletion by thapsigargin and ionomycin, and differential sensitivity to inhibition by 2-aminopyridine and gadolinium. The inflammatory G protein-coupled chemoattractants fMLP, platelet-activating factor, and IL-8 elicit unique patterns of Sr$^{2+}$ and Ca$^{2+}$ influx channel activation, and SOCE responses to these agonists displayed differing degrees of linkage to prior Ca$^{2+}$ store depletion. The mechanisms of PMN SOCE responses to G protein-coupled chemoattractants are physiologically diverse. They appear to reflect Ca$^{2+}$ transport through a variety of channels that are independently regulated to varying degrees by store depletion and by G protein-coupled receptor activation. The Journal of Immunology, 2002, 168: 4063–4069.

Polymorphonuclear neutrophils (PMN) are the central effectors in human innate immune responses to inflammatory stimuli. Clinically, inappropriate PMN activation is associated with organ failure, whereas suppression of PMN function is associated with septic complications. Therefore, understanding the regulation of PMN stimulus-response coupling in inflammation is of great importance. Inflammatory processes expose PMN to a wide variety of agonists that can attract, prime, or activate PMN via heterotrimeric G protein-coupled receptors. PMN G protein-coupled receptors typically signal by increasing cytosolic calcium concentration ([Ca$^{2+}$]i) in addition to activating other second messengers. Increases in [Ca$^{2+}$]i have profound effects on PMN, including the initiation of cytoskeletal changes, degranulation, presentation of adhesion molecules, and oxidative burst. The magnitude and duration of [Ca$^{2+}$]i signals responses to G protein-coupled chemoattractants are clearly important (1), but the mechanisms by which the magnitude and duration of [Ca$^{2+}$]i responses are regulated in vivo are incompletely understood.

When agonists bind to the receptors, inositol 1,4,5-triphosphate (InsP$_3$) interaction with its receptors in the endoplasmic reticulum (ER) results in rapid Ca$^{2+}$ release from ER stores. Such calcium store release depletes ER Ca$^{2+}$ stores and subsequently activates Ca$^{2+}$ influx across the plasma membrane by mechanisms generally referred to as store-operated (or capacitative) calcium entry (SOCE) (2). Our clinical experience (3) suggested that PMN dysregulation due to injury and inflammation involved abnormally enhanced SOCE, and other authors have demonstrated that SOCE is required for the activation of PMN functions seen in inflammatory surroundings (4–6).

However, the exact mechanisms of SOCE continue to be a subject of great controversy (7). Visual phototransduction in Drosophila uses a G protein-coupled receptor mechanism linked to SOCE. After cloning of the Drosophila transient receptor potential gene (trp), in vitro expression studies indicated that TRP was a cationic influx channel activated by Ca$^{2+}$ signaling from cell stores (8, 9). Multiple isoforms of trp have subsequently been cloned based on data from Drosophila, and recent work has implicated calcium channel proteins of the TRP superfamily in the mediation of human SOCE (10–16).

Thus, the human genome is known to contain many candidate SOCE channel proteins. Consequently, cells may express them individually or in combination (16), and such expression profiles may play a role in achieving phenotype-specific calcium entry responses. However, no studies exist evaluating whether SOCE in human cells is a unitary process or reflects the summated contributions of combined calcium entry pathways. Therefore, we examined the hypothesis that net PMN SOCE after inflammatory stimulation might reflect the contribution of multiple pathways, rather than a single calcium entry pathway.

Materials and Methods
Neutrophil preparations

Freshly withdrawn healthy human blood was used to prepare PMN samples. A detailed protocol is described elsewhere (17). Briefly, heparinized whole blood (10 U/ml) was centrifuged at 150 × g for 10 min. The plasma and platelets were discarded. The buffy coat and RBC were layered onto
Polymorphoprep (Robbins Scientific, Sunnyvale, CA), followed by a 30-min centrifugation at 300 × g. The PMN layer was collected and diluted with an equal volume of 0.45% NaCl to restore osmolality. The suspensions were then washed in RPMI 1640 and centrifuged at 150 × g for 10 min. Neutrophil pellets were suspended in 2 ml HEPES buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, and 0.1% BSA, pH 7.4). PMN were counted on a flow cytometer and kept on ice until dye-loaded for study.

**Dye loading and pretreatment**

PMN were incubated in 1 μM fura-2 AM (Molecular Probes, Eugene, OR) at 37°C for 30 min in the dark. Specimens were divided into aliquots of 2 × 10⁶ cells and placed on ice in the dark until ready for use. Just before each experiment, individual aliquots were incubated at 37°C for 5 min. Cells were then pelleted by centrifugation at 4500 rpm for 5 s in a programmable microcentrifuge and resuspended in 3 ml HEPES in the cuvette. Experiments were generally begun in nominally calcium-free medium containing 0.3 mM EGTA. The sole exceptions were the experiments involving cat-ionic inhibitors (Gd³⁺, La³⁺, etc.). These were performed in calcium-free medium without EGTA to avoid chelation of the inhibitors.

**Divalent cation measurements by spectrofluorometry**

Intracellular calcium was monitored by measuring fura fluorescence at 505 nm, using a 340/380-nm dual wavelength excitation in a fluorometer (Jobin Yvon-Spex, Edison, NJ). Cuvette temperatures were kept at 37°C with constant stirring. Calibration was performed at the end of each experiment by the addition of 100 μM digitonin (Molecular Probes) for Rₘₐₓ and then 15 mM EGTA for Rₘᵟᵣₙ. The autofluorescence of a sample cell suspension treated with 100 μM digitonin and 2 μM MnCl₂ was subtracted from total fluorescence. The [Ca²⁺]ₗ was then calculated from the 340:380 nm fluorescence ratio (Kᵦ = 220 μM) as per the methods of Grynkiewicz et al. (18). Dye leakage is trivial and has no influence on [Ca²⁺]ₗ calculations using our methods. The order of study of isolates was alternated to avoid bias related to duration of dye loading or time of cell study.

Modifications of these methods were used to assess the apparent influx of calcium ions ([Ca²⁺]ₗ). Sr²⁺, has a lesser affinity for fura than does Ca²⁺; and its binding causes less fluorescence, but its isobestic point and 340:380 ratio profile are very similar (19). However, we noted in preliminary studies that [Ca²⁺]ₗ store release measurements were unaffected by the presence of Sr²⁺ in the medium at the time of cell lysis for calibration of Rₘₐₓ and Rₘᵟᵣₙ (Fig. 1A). Thus, we found we could quantitatively assess Ca²⁺ store release and qualitatively assess relative Sr²⁺ and Ca²⁺ entry into PMN in the same experiments by serial addition of Sr²⁺ (to 1 mM) and then Ca²⁺ (to 1 mM) to the medium after resolution of the store release transients. As seen in Fig. 1B, this method allows us to assess the area under the curve (AUC) for Ca²⁺ release in Ca²⁺/Sr²⁺-free medium, as well as the relative magnitude of Sr²⁺ and Ca²⁺ entry as those ions are added to the medium (see below). Thus, initial [Ca²⁺]ₗ traces from experiments in which Sr²⁺ is added later are both quantitatively and qualitatively correct. However, because of its higher Kᵦ for fura and the potential for interactions, [Sr²⁺]ₗ, is always reported as apparent [Sr²⁺]ₗ. Similarly, [Ca²⁺]ₗ measurements made after addition of Sr²⁺ may be inexact, and are therefore treated as apparent. However, in all cases only experimental responses obtained under identical conditions are compared.

**Data analysis**

Cytosolic [Ca²⁺]ₗ responses, apparent [Ca²⁺]ₗ responses, after Sr²⁺ influx, and apparent [Sr²⁺]ₗ responses were measured and recorded both as the peak and (Δ[Ca²⁺]ₗ measured in nanomoles × seconds) as the integrated AUC (measured in nanomoles × seconds) for the 100 s after stimulation with the agonist in question, or in arbitrary units of concentration × seconds after the addition of external Sr²⁺ or Ca²⁺. Both the peak influx and the duration of influx signals may be of importance in determining specific cell responses to calcium. We elected to combine these parameters by assessing the AUC. This pharmacologic approach creates a quantitative and reproducible assessment of total influx. AUC measurements diminish the effect of artifacts on assessments while avoiding the use of curve-smoothing programs. The AUC does not address differences in peak and duration of response individually. These may have considerable physiologic significance, but no changes in the morphology of SOCE currents were seen in these studies to suggest that peak and duration of influx changed independently of one another. Integration using a concentration of 0.3 mM EGTA (Fig. 1B) was performed using an automated software package (GRAMS/32; Galactic Industries, Salem, NH). Data analysis was done using SigmaPlot and SigmaStat software (SASS, Chicago, IL).

**FIGURE 1. A.** [Sr²⁺]ₗ, and [Ca²⁺]ₗ, calculations. These experimental traces were derived from identical PMN aliquots stimulated with IL-8 in a Ca²⁺-free medium. In one case, Ca²⁺ only was added (this curve was displaced rightward for clarity) in the other Sr²⁺, and then Ca²⁺ was added. F340/380 and apparent [Ca²⁺]ₗ were calculated for each trace using its unique Rₘᵟᵣₙ and Rₘₐₓ value after digitonin and EGTA were added. Basal [Ca²⁺]ₗ, and the store release transients (obtained when only intracellular Ca²⁺ was present) are identical, showing that [Ca²⁺]ₗ, calculations are unaffected by the subsequent addition of Sr²⁺. Also, note the final [Ca²⁺]ₗ value after Ca²⁺ only, and the apparent [Ca²⁺/Sr²⁺]ₗ, value after Sr²⁺ and then Ca²⁺ appear identical. IL-8 elicits no measurable Ca²⁺ entry (see text), and the Ca²⁺ and Sr²⁺ entry seen here after additions to the medium are quantitatively identical to the leak current seen when unstimulated PMN are exposed to Sr²⁺ or Ca²⁺ (data not shown). The [Ca²⁺]ₗ values calculated after Sr²⁺ entry are arbitrary measurements, because the relative contribution of Sr²⁺ and Ca²⁺ is not defined. **B.** Quantifying Sr²⁺ and Ca²⁺ entry. The AUC in nanomoles × seconds for the 100 s following agonist (in this case, PAF)-induced Ca²⁺ store depletion was used as a measure of store release (SR). Subsequent addition of 1 mM Sr²⁺ and then 1 mM Ca²⁺ to the medium was used to assess the store-operated Sr²⁺ entry (SrE) and Ca²⁺ entry (CaE) into the PMN. SrE and CaE are calculated in arbitrary units of concentration × seconds. AUC for each event is integrated as the area between the baseline value before addition of agonists and the trace, as shown (see Materials and Methods).

Where mathematical curve fitting was performed to assess the relationships between store release and Sr²⁺ or Ca²⁺ influx, each curve-fit analysis was performed using two different software packages (SigmaStat and 2-D Curve-fit; SPSS, Chicago, IL). The function with the highest R value was accepted as most representative of the form of the relationship. A p value ≤ 0.01 was required before accepting that a curve fit was indicative of a significant relationship.

**Results**

**PMN SOCE entry channels show differential ionic selectivity**

Prior studies have shown that cells transfected with TRP3 had enhanced permeability to strontium ions (Sr²⁺) after store depletion (20). Others, however, have suggested that SOCE occurs through channels that are calcium selective (1). We examined this question by stimulating PMN with 100 nM fMLP. Addition of Sr²⁺ (1 mM) to the medium after resolution of the store depletion transient resulted in a brisk influx event. Nonetheless, subsequent addition of Ca²⁺ (1 mM) resulted in a further influx event (Fig. 2A). This influx was Ca²⁺ specific, because Sr²⁺ influx was saturated at 1 mM and showed no further entry at higher concentrations (Fig. 2C). Direct addition of 1 mM Ca²⁺ to the medium after
store depletion yields an influx response (Fig. 2B) similar to that seen when Ca\(^{2+}\) was applied after Sr\(^{2+}\). In the presence of extracellular Ca\(^{2+}\), neither Sr\(^{2+}\) (Fig. 2B) nor further Ca\(^{2+}\) (to 2 mM) increased apparent [Ca\(^{2+}\)] (Fig. 2D).

These events are consistent with the concept that calcium store depletion by fMLP activates two different divalent cation entry pathways in PMN. The more calcium specific of these pathways yields higher influx, and thus Sr\(^{2+}\) entry was not seen in the presence of extracellular Ca\(^{2+}\). Sr\(^{2+}\) entry through the nonspecific pathway appears to be inadequate to shut down the calcium-specific entry mechanism. Thus, SOCE influx can still occur after store-operated Sr\(^{2+}\) influx, and the total magnitude of calcium entry is independent of the presence of Sr\(^{2+}\) at the time of Ca\(^{2+}\) addition (Fig. 2, A and B).

**Dependence of Sr\(^{2+}\) and Ca\(^{2+}\) entry on store release**

Our initial results suggested that PMN calcium entry integrates multiple SOCE pathways, which show differential cation permeability. Because influx in these studies was initiated by a G protein-coupled mediator (fMLP), an argument could be made that some portion of the cation entry seen might have occurred through receptor-operated mechanisms. We therefore studied whether store emptying per se elicited similar patterns of Sr\(^{2+}\) and Ca\(^{2+}\) entry in the PMN. To do this, we stimulated PMN with ionomycin at very low doses (100 nM). This depletes calcium stores, and allows study of subsequent Sr\(^{2+}\) and Ca\(^{2+}\) entry. In addition, it has been suggested that the opening of some store-operated calcium channels by store depletion requires a physical coupling between InsP\(_3\) receptors and the channels themselves (21). Therefore, we used the same system to evaluate whether blockade of the InsP\(_3\) receptors (using 2-aminoethoxydiphenyl borane (2-APB)) would differentially affect ionomycin-induced store-operated PMN Ca\(^{2+}\) and Sr\(^{2+}\) entry. We found that isolated store depletion by ionomycin led to responses very similar to those elicited by fMLP in that Ca\(^{2+}\) still enters the PMN after store-operated Sr\(^{2+}\) entry is complete (Fig. 3, upper line). However, we found that Sr\(^{2+}\) entry was almost totally inhibited by 2-APB, whereas Ca\(^{2+}\) entry was relatively unaffected (Fig. 3, lower line). These data further support the concept that calcium store depletion activates two different divalent cation entry pathways in PMN. The Sr\(^{2+}\)-permeable mechanism is 2-APB inhibitable. The second, more Ca\(^{2+}\)-specific pathway allows greater Ca\(^{2+}\) influx and is relatively 2-APB resistant.

**Inhibitory profiles of Ca\(^{2+}\) and Sr\(^{2+}\) entry**

One of the accepted characteristics of SOCE is that it is inhibited by gadolinium (Gd\(^{3+}\)) at low concentrations (22). Therefore, we studied the inhibitory effects of Gd\(^{3+}\) on PMN Ca\(^{2+}\) and Sr\(^{2+}\) entry after both G protein-coupled chemoattractant store depletion (using fMLP) and direct store depletion using the Ca\(^{2+}\)-ATPase inhibitor thapsigargin (TG). We found that Gd\(^{3+}\) inhibited Sr\(^{2+}\) entry at far lower concentrations than it inhibited Ca\(^{2+}\) entry. This was true for both G protein-coupled and direct store depletion-initiated cation entry. PMN Sr\(^{2+}\) and Ca\(^{2+}\) entry at Gd\(^{3+}\) concentrations from 1 nM to 10 \(\mu\)M was then assessed and curve fit over its linear range (Fig. 4). The EC\(_{50}\) of Gd\(^{3+}\) for inhibition of Sr\(^{2+}\) entry was 48 nM for fMLP-mediated entry and 81 nM for TG-mediated entry. In contrast, the EC\(_{50}\) of Gd\(^{3+}\) for inhibition of Ca\(^{2+}\) entry was 2291 nM for fMLP-mediated entry and 1019 nM for TG-mediated entry. Thus, after both types of store depletion, G protein mediator dependent and direct store depletion dependent, Gd\(^{3+}\) was 10- to 20-fold more potent as an inhibitor of Sr\(^{2+}\) than of Ca\(^{2+}\) entry. However, in all cases the inhibitory Gd\(^{3+}\) concentrations were well within those generally used to inhibit SOCE (22). Interestingly, the inhibition of Sr\(^{2+}\) entry by Gd\(^{3+}\) found in this study was very similar to that found in studies of
Having determined that both Sr\textsuperscript{2+} and Ca\textsuperscript{2+} entry could be elicited by store depletion, we assessed their dependence upon the degree of store depletion. PMN were exposed to TG for varying lengths of time in divalent cation-free medium (Fig. 5) and then exposed either to 1 mM Sr\textsuperscript{2+}, or to 1 mM Ca\textsuperscript{2+} in the presence of 500 nM Gd\textsuperscript{3+} (thus blocking all influx through Sr\textsuperscript{2+}-permeable pathways). Because TG depletes ER calcium stores passively by blocking reuptake, depletion is progressive over time. We noted that both the rate of rise and the maximal extent of Sr\textsuperscript{2+} influx became maximal early in the course of store depletion. Both the rate of rise and the maximal extent of entry through the calcium-specific pathway were more dependent upon the completeness of store emptying, taking over three times as long to achieve maximal influx. Again, these findings demonstrate marked differences in cation influx through the two mechanisms: the nonspecific Sr\textsuperscript{2+}-permeable pathways appear to allow brisk early responses that are relatively independent of the degree of store depletion; the Ca\textsuperscript{2+}-specific influx mechanism is more regulated by the degree of store depletion, and may allow for later entry of larger amounts of calcium.

**G protein-coupled chemoattractants differentially regulate PMN Ca\textsuperscript{2+} entry**

In vivo, PMN SOCE may occur when calcium stores are mobilized by a wide variety of G protein-coupled chemoattractants. We hypothesized that PMN stimulation at specific G protein-coupled receptors might elicit unique SOCE responses. We therefore compared Sr\textsuperscript{2+} and Ca\textsuperscript{2+} entry responses with Ca\textsuperscript{2+} store depletion elicited by three different G protein-coupled chemoattractants.
Each of these is physiologically important in human PMN function, but elicits different PMN functions as well as unique \([\text{Ca}^{2+}]_i\) transient morphology (3). Platelet-activating factor (PAF) is a lipid autacoid crucial to many inflammatory processes. PMN calcium entry after PAF stimulation occurs exclusively via SOCE (17). fMLP activates PMN via receptors for formylated (bacterial) peptides. IL-8 is a chemokine with important roles in PMN chemotaxis and priming. IL-8 acts via two receptors (CXCR1 and CXCR2). In these studies, therefore, we blocked CXCR2 with a specific mAb (gift of J. Bussiere, Genentech, South San Francisco, CA) to isolate the actions of IL-8 at CXCR1. In each of these studies, PMN were exposed to ascending agonist doses from zero to the EC100 for \([\text{Ca}^{2+}]_i\) store depletion in calcium-free medium. Each point in Figs. 6A, 7A, and 8A represents the mean ± SE of 6–10 experiments. As expected, PMN Ca2+ store release increases in a dose-dependent fashion when PMN are stimulated by each of the agonists tested. However, store-operated Sr2+ and Ca2+ entry responded in different and quite unique ways after each specific agonist.

After PAF stimulation, store release Sr2+ and Ca2+ entry events were very similar in their apparent magnitude (Fig. 6A). Each was maximal after 1 nM PAF and none increased at higher agonist doses. In contrast, when PMN were treated with fMLP (Fig. 7A), store release always exceeded Ca2+ entry, which always exceeded Sr2+ entry. Moreover, even though store release was maximal at all fMLP concentrations ≥1 nM, Sr2+ and Ca2+ entry responses continued to increase with increasing fMLP concentration. IL-8 stimulation of CXCR1 (Fig. 8A) resulted in significant store release at doses ≥10 nM. However, Sr2+ and Ca2+ entry after IL-8 were never greater than the nonspecific Sr2+ and Ca2+ entry (leak current) that is seen when nonstimulated PMN in calcium-free medium are exposed to Ca2+ or Sr2+. Thus, after IL-8, no Sr2+ or Ca2+ entry was seen even at levels of receptor activation and store release that were associated with brisk Sr2+ and Ca2+ entry after PAF or fMLP.

The implications of these findings are as follows: 1) as seen with IL-8, not all G protein-coupled Ca2+ store depletion events activate divalent cation entry channels, and 2) the differential

![FIGURE 6. PAF regulation of store-operated Sr2+ and Ca2+ entry. A. The x-axis represents the concentrations of PAF applied to the PMN, and the y-axis represents the AUC value (mean ± SE) for the corresponding store release (SR), calcium entry (CaE), and strontium entry (SrE) events. All flux events were maximal at 1 nM PAF and above. Each point represents at least 6 (typically 8–10) experiments. B. The CaE and SrE found in these experiments were subjected to curve-fitting analysis as a function of the magnitude of the initiating SR event. Both CaE (△) and SrE (▼) are highly correlated to the magnitude of SR in a linear fashion.](image)

![FIGURE 7. fMLP regulation of store-operated Sr2+ and Ca2+ entry. A. The x-axis represents the concentrations of fMLP applied to the PMN, and the y-axis represents the net AUC value (mean ± SE) for the corresponding store release (SR), calcium entry (CaE), and strontium entry (SrE) events. Each point represents at least six (typically 8–10) experiments. B. CaE (△) and SrE (▼) after fMLP were subjected to curve-fitting analysis. CaE and SrE were highly correlated to SR in an exponential fashion.](image)

![FIGURE 8. IL-8 regulation of store-operated Sr2+ and Ca2+ entry. A. The x-axis represents IL-8 applied to the PMN, and the y-axis again represents net AUC value for store release (SR), calcium entry (CaE), and strontium entry (SrE) events. Each point represents at least six experiments. SR becomes substantial above 10 nM IL-8, but CaE and SrE remain insignificant. When CaE and SrE after IL-8 are subjected to curve-fitting analysis (B), no significant relationship is found to the magnitude of SR.](image)
responses to fMLP and PAF stimulation confirm that radically different relationships can exist between entry events and store release of similar magnitude elicited by different receptors. These findings demonstrate that events other than InsP₃ production and associated store release are involved in the regulation of PMN SOCE after G protein-coupled receptor stimulation. In addition, because no SOCE is seen after CXCR1 stimulation despite significant store release, it suggests that G protein-coupled receptor activation may result in events that suppress as well as activate SOCE.

To further analyze these relationships, we curve-fit the store release Sr²⁺ and Ca²⁺ entry data for each agonist. Highly linear relationships were noted (R > 0.99 and p < 0.001) between store release and Sr²⁺ and Ca²⁺ entry after PAF exposure (Fig. 6B). Thus, after PAF, both Sr²⁺ and Ca²⁺ influx appear to be direct linear functions of store release. This suggests that calcium store release is the major determinant of both nonspecific and Ca²⁺-specific divalent cation entry after PAF stimulation. In contrast, fMLP stimulation (Fig. 7B) yields an exponential relationship (R > 0.82 and p < 0.001) between store release and divalent cation entry. Both nonspecific and Ca²⁺-specific divalent cation entry became less dependent upon store depletion and more dependent on agonist concentration at increasing fMLP concentrations. Thus, after fMLP, store release leads to SOCE, but this SOCE is modulated by other secondary mechanisms. No significant cation entry was seen after IL-8 stimulation. Thus, no meaningful mathematical relationship could be found between store release and cation entry under these conditions (R < 0.10 and p > 0.5 for all equations tested; Fig. 8B).

Thus, the mechanisms linking store release and calcium entry vary markedly with the specific G protein-coupled receptor activated. Furthermore, it appears that although calcium entry into the PMN may be initiated by store depletion, it can be quantitatively regulated by other factors that are determined by the initiating G protein complex as well as by the degree of store depletion. Moreover, the initiating G protein receptor complex appears to regulate the relationships between store release and divalent cation entry both through the Sr²⁺-permeable and the Ca²⁺-selective PMN calcium entry pathways in ways that are roughly parallel.

Discussion

Increasing cytosolic calcium is a major cell signaling pathway by which G protein-coupled inflammatory mediators activate PMN, and SOCE is a crucial contributor to such stimulus-response coupling (6). The process is not fully understood, but it is of enormous potential importance. Moreover, the mechanisms of SOCE in PMN as well as other nonexcitable cells have remained controversial, and the physiology of the TRPs and related proteins that are the candidate channels for SOCE has been studied almost exclusively in heterologous expression systems rather than in native cells. The present data confirm that PMN SOCE responses to clinically relevant agonists occur through multiple pathways. The prolongation of elevated [Ca²⁺]ᵢ, levels in PMN after G protein-coupled receptor stimulation represents the summation of at least two such contributions. These pathways fulfill the usual definitions of SOCE both in that they are activated by passive store depletion and by virtue of their ionic inhibitor profiles. Yet the two pathways differ in the magnitude and time course of their contributions to calcium flux, the EC₅₀ of inhibition by Gd³⁺, and their inhibition by the InsP₃ receptor inhibitor 2-APB. Most importantly, though, these influx pathways demonstrate differential regulation when activated by different G protein-coupled receptor agonists, thus suggesting an entirely novel mechanism by which [Ca²⁺]ᵢ and downstream cellular function can be regulated in nonexcitable cells.

The contributions of TRP channels to SOCE are still controversial, but work in both expression and wild-type systems shows that TRP channels can participate in SOCE (21, 25–27) as well as respond to G protein signaling complex-generated mediators such as diacylglycerol (28–30). The divalent cation entry pathways we find in human PMN also appear to have some characteristics usually attributed to receptor operated as well as to SOCE. Similar complexity in the relationships between store-emptying and TRP activation has been noted by others (31). Parekh et al. (32) found that I₄[,RAC] currents were nonlinearly activated in RBL cells by G protein-coupled muscarinic receptors. We noted exponentially increasing activation of Ca²⁺ influx at high doses of fMLP. In contrast, PAF mobilized cation influx linearly over a broad range of store release. IL-8 acting at CXCR1 failed to elicit SOCE. This might reflect a failure to achieve a trigger concentration of InsP₃ and store release. Conversely though, because high doses of IL-8 do achieve store release equivalent to mid-dose PAF, this could imply the generation of a secondary SOCE-suppressive event by CXCR1 stimulation.

Such complexity has also been hypothesized to suggest the possible formation of heteromultimeric TRP channels (30, 33). Based on their primary sequences, TRP proteins are predicted to have six transmembrane segments similar to those found in voltage-dependent Ca²⁺, Na⁺, and K⁺ channels (34). Because such Na⁺ and Ca²⁺ channel proteins form pores using four repeated six-transmembrane domains, it is probable that TRP channels also function as homo- or heterotetramers of TRP proteins (1, 34). Such heteromultimeric channels could incorporate multiple control mechanisms inherent to each of their component subunits.

In other studies (data not shown), we have noted that four distinct TRP channels can be found in circulating PMN. If indeed TRP channel proteins do mediate SOCE in PMN, those data would imply that either multiple channel types or heteromultimeric channels must exist. Thus, our findings may also suggest that the control of calcium entry in wild-type nonexcitable cells like PMN may depend on the relative expression of channel proteins and on their assembly into functional channels as well as on the characteristics of the individual channel proteins. The involvement of multiple component pathways will complicate attempts to understand the role and regulation of SOCE in inflammatory responses. However, such diversity also suggests that the molecular control of SOCE may play a role in conferring stimulus-response specificity to PMN-mediated inflammation as well as to other [Ca²⁺]ᵢ-driven immune responses.

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