Regulation of superoxide production in neutrophils:
role of calcium influx

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Abstract: Upon stimulation, activation of NADPH oxidase complexes in neutrophils produces a burst of superoxide anions contributing to oxidative stress and the development of inflammatory process. Store-operated calcium entry (SOCE), whereby the depletion of intracellular stores induces extracellular calcium influx, is known to be a crucial element of NADPH oxidase regulation. However, the mechanistic basis mediating SOCE is still only partially understood, as is the signal-coupling pathway leading to modulation of store-operated channels. This review emphasizes the role of calcium influx in the control of the NADPH oxidase and summarizes the current knowledge of pathways mediating this extracellular calcium entry in neutrophils. Such investigations into the cross-talk between NADPH oxidase and calcium might allow the identification of novel pharmacological targets with clinical use, particularly in inflammatory diseases. J. Leukoc. Biol. 84: 1223–1237; 2008.

Key Words: store-operated Ca2+ entry · NADPH oxidase · granulocytes

INTRODUCTION

Many cellular signal transduction pathways such as fertilization, proliferation, and development are modulated by a spatial and temporal elevation of the cytosolic-free calcium concentration ([Ca2+]c) [1]. In nonexcitable cells, the [Ca2+]c increase is predominantly a result of a Ca2+-influx from the extracellular medium through the opening of Ca2+-permeable channels subsequent to the emptying of intracellular Ca2+ stores. This store depletion is mediated by the synthesis of inositol 1,4,5-trisphosphate (InsP3), a Ca2+-mobilizing, second messenger, causing the activation of channels located in the endoplasmic reticulum (ER) membranes [2]. The intracellular signal resulting from this mechanism, known as store-operated Ca2+ entry (SOCE), is characterized by the formation of a transient Ca2+-spike (with a substantial amplitude), which returns to a basal level of [Ca2+]c, dependent on the activity of the plasma membrane and sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA) pumps. Over the years, it became clear that Ca2+ mobilization plays an important role in the regulation of superoxide anion secretion by phagocytic cells [3–5]. Originally, it was believed that NADPH oxidase, the most important generator of superoxide anions, was only assembled and activated in the plasma membrane or phagosome. Now, it is well established that this enzymatic complex can be activated in specific granules, and produced oxidants are intracellularly retained (see refs. [6, 7] for review). Evidence for the requirement of extracellular Ca2+ entry for NADPH oxidase activation is supported by a significant decrease of superoxide anion production when extracellular Ca2+ is suppressed or chelated by EGTA [3, 8, 9]. Although oxidants are released from neutrophils upon chemoattractant stimulation, ionomycin-induced oxidant production is largely intracellular [10]. In the latter case, Ca2+ influx through ionophore activity is sufficient to activate NADPH oxidase in the granule fraction [11].

Like Ca2+ ionophores, thapsigargin, an inhibitor of Ca2+ re-uptake by SERCA, permits a slow ER emptying, followed by SOCE [12], which is unable to activate plasma membrane-localized NADPH oxidase in neutrophils. This suggests that NADPH oxidase regulation requires a second signal, Ca2+-independent pathway, acting in synergy with Ca2+ influx from SOCE [3].

During the last decade, SOCE has been studied extensively; many questions remain unanswered about the mechanism linking [Ca2+]c elevation to superoxide anion production. This review summarizes the present knowledge about Ca2+ influx-regulated NADPH oxidase activity induced by external stimuli in neutrophils.

NEUTROPHIL NADPH OXIDASE

Activated state

NADPH oxidase was first described in neutrophils, where it is normally inactive. This complex is a multicomponent enzyme, including cytosolic and membrane-bound proteins, which is unassembled in resting cells. Membrane components include a stable, heterodimeric flavocytochrome b558 composed of two
Subunits: gp91phox (known as Nox2 in the new terminology) and p22phox. Cytosolic components include four soluble factors: p67phox, p47phox, p40phox, and a small G-protein Rac (Rac1 and Rac2 isoforms). Upon cell surface receptor activation by soluble inflammatory mediators (such as fMLF), cytosolic components translocate to the plasma or phagosomal membrane, where NADPH oxidase is assembled (Fig. 1). A large quantity of superoxide anions, which are precursors of a variety of reactive oxygen species (ROS) used for microbial killing, is released. The precise mechanism governing NADPH oxidase assembly has been excellently reviewed previously (see refs. [13–19]) and is therefore not detailed here. Cellular responses induced by opsonized zymosan differ from those induced by chemoattractant-activated G-protein-coupled receptors. Cytosolic components translocate to the membrane upon opsonized particle stimulation, SOCE mediates the translocation of Rac, activates MRP proteins, which enhance the organization and recruitment of cytosolic factors to the membrane-bound flavocytochrome b558, and stimulates protein kinase Cs (PKCs) involved in the phosphorylation of cytosolic phox proteins. Rho-GDI, Rho-GDP inhibitor.

**Primed state**

Complete NADPH oxidase assembly results in a large superoxide anion production, which can be increased in response to a second activating stimulus during a process known as priming [21–24]. Priming agents per se do not cause NADPH oxidase activation. Many studies about different agents able to induce the neutrophil priming suggest that several complementary signal transductions are involved in this mechanism. For example, [Ca\(^{2+}\)], elevation and phosphorylation of p47phox by different protein kinases (PKC, MAPK, PKA, p21-activated kinase) allowing conformational changes of p47phox may promote oxidase activation in response to GM-CSF [25]. Changes in PLC, PLD, PLA2, and phosphoinositide 3-hydroxykinase activity, protein phosphorylation, modulation of the expression of chemotactic peptide receptors, sequential phosphorylation of p47phox and p67phox, and assembly of the NADPH oxidase in lipid rafts (reviewed by Sheppard et al. [26]) also seem to be
implicated in the “priming” phenomenon. Indeed, the great
diversity of substances inducing priming suggests that a variety
of signaling pathways, alone or in combination, is responsible
for NADPH oxidase up-regulation.

It is found that Ca\(^{2+}\) ionophores, such as ionomycin, might be
considered as priming activators and that their effects are
encompassed by [Ca\(^{2+}\)]\(_{c}\) elevation [27]. However, the relation
between [Ca\(^{2+}\)]\(_{c}\) and priming seems to be dependent on the
nature of priming agents. For example, TNF-\(\alpha\) [28] is unable to
mobilize Ca\(^{2+}\) in contrast to lysophosphatidylcholine [29] or
platelet-activating factor (PAF) [30], which can enhance
Ca\(^{2+}\) in response to other agonists. Similarly, several studies
have reported that IL-8 stimulates a [Ca\(^{2+}\)]\(_{c}\) rise in response to
other agonists, as demonstrated by Wozniak et al. [23]. These
authors suggest that the stimulation of neutrophils with IL-8
increases [Ca\(^{2+}\)]\(_{c}\), by mobilizing Ca\(^{2+}\) from internal stores and
by increasing the Ca\(^{2+}\) influx. Our own recent work confirms
that IL-8 regulates extracellular Ca\(^{2+}\) entry and that NADPH
oxidase priming could be mediated by this [Ca\(^{2+}\)]\(_{c}\) elevation
[31]. MacKinnon et al. [32] suggested that [Ca\(^{2+}\)]\(_{c}\) elevation
upon stimulation by priming agents such as PAF or a substance
\(\text{P}\) analog may be dependent on the generation of sphingosine
1-phosphate, described previously as the “calcium influx fac-
tor” by Itagaki and Hauser [33]. Thus, priming agents might be
implicated in sphingosine 1-phosphate synthesis and subse-
quently, through SOCE in the elevation of [Ca\(^{2+}\)]\(_{c}\). However, it
clear that other mechanisms, independent of intracellular
Ca\(^{2+}\) signaling pathways, are required for the priming of the
NADPH oxidase [31].

A signaling role of Ca\(^{2+}\) in priming has been established in
the majority with neutrophils in suspension. However, NADPH
oxidase activation of neutrophils adherent to extracellular matrix
proteins differs considerably from that of neutrophils in
suspension. Effectively, adherence of neutrophils can appar-
ently capacitate them to respond to stimuli that are not effec-
tive when in suspension [34]. For example, although neutro-
phils in suspension show no oxidant production in response to
soluble cytokines such as TNF-\(\alpha\), adhesion of neutrophils to
substrates via leukocyte \(\beta_2\) integrin is able to trigger a massive
oxidative response from neutrophils [34, 35]. As [Ca\(^{2+}\)]\(_{c}\)
elevation is prevented by treatment with cytochalasin B, and
antibodies against the adhesion receptor, \(\beta_2\) integrin-mediated
adhesion is thought to be responsible for [Ca\(^{2+}\)]\(_{c}\) elevation [36, 37].
This conclusion is supported by rapid-time confocal scan-
ing experiments in which Pettit and Hallett [38] observed
multiple elevations in [Ca\(^{2+}\)]\(_{c}\), as a result of Ca\(^{2+}\) influx during
\(\beta_2\) integrin-dependent adhesion. Generation of \(\beta_2\) integrin-
induced Ca\(^{2+}\) influx seems dependent on the formation of
InsP\(_3\)s [39]. Although precise mechanisms remain to be deter-
mained, [Ca\(^{2+}\)]\(_{c}\) elevation may be the link to adhesion-depend-
ent priming [40].

**MECHANISMS OF Ca\(^{2+}\) ENTRY**

**SOCE**

In neutrophils, changes in [Ca\(^{2+}\)]\(_{c}\), are often associated with
InsP\(_3\)-R-mediated, rapid Ca\(^{2+}\) release of intracellular stores
followed by SOCE as a result of the activation of channels in the
plasma membrane [2, 41, 42] (Fig. 1). STIM1 has recently
been identified as the sensor for ER Ca\(^{2+}\) content, which
initiates a process resulting in a signal being sent from the
stores to SOCs [43–45]. Three models have been put forward to
link store-emptying to SOCs activation (reviewed by Putney et
al. [46]): a dynamic, conformational coupling [41] (Fig. 1)
involving a direct protein–protein interaction between
InsP\(_3\)-Rs and SOCs; an unidentified, diffusible messenger,
termed calcium influx factor [33, 46–48], generated and re-
leased in response to store depletion; and a fusion of secretory
vesicles [49, 50] with the plasma membrane causing preformed
SOCs protein insertion into the plasma membrane through
exocytosis or regulatory molecules. Although the conforma-
tional coupling may not apply to all cell types, we cannot rule
out a combination of these different concepts, as considerable
evidence exists to support an involvement of conformational
changes in SOCE.

At this point, it is necessary to introduce proteins that could
be considered as candidates for SOCs in neutrophils. During
the last decade, research focused on mammalian homologs of
Drosophila transient receptor potential canonical (TRPC) that
are activated by an active and passive Ca\(^{2+}\) store depletion.
Mammalian TRPC, closely related to Drosophila TRP, are
divided into several subfamilies: TRPC1, TRPC3-6-7, and
TRPC4-5 based on their sequence homologies and functional
similarities [51]. Human neutrophils are known to express
mRNAs for TRPC1, TRPC3, TRPC4, and TRPC6 [52–54], and
corresponding proteins are found to be expressed in the cell
membrane [53]. First, Itagaki et al. [53] provided evidence that
TRPC proteins might participate in SOCE in human neutro-
phils after internalization of TRPC proteins by calyculin A.
Cytoskeletal reorganization induced by this phosphatase inhib-
itor causes the displacement of TRPC proteins from the cell
surface into a diffuse cytosolic pattern followed by the inhibi-
tion of SOCE induced by physiological and pharmacological
stimuli [53]. Considerable efforts have been devoted to eluci-
date the function of TRPC proteins in SOCE, but few studies
tackled the role that these channels play in neutrophils. Over-
expression of TRPC in other mammalian cells [human embryo
kidney (HEK)-293, DT40 B cells, platelets, salivary gland
cells, adrenal cells] has been reported to result in an enhance-
ment of SOCE, and reduction of TRPC expression using anti-
sense strategies was shown to decrease SOCE. More precisely,
it is generally accepted that TRPC1, TRPC4, and TRPC5 are
activated by Ca\(^{2+}\) store depletion [55–61], and TRPC3,
TRPC6, and TRPC7 are directly activated by diacylglycerol
and its cell-permeant analogs in an InsP\(_3\)-R-independent man-
ner [62–66]. However, some studies have focused on the role
of TRPC3 and TRPC6 as SOCs in human neutrophils. Indeed,
we have found that endogenous TRPC6 channels are sensitive
to Ca\(^{2+}\) store depletion in neutrophil-like, DMSO-differenti-
ated HL-60 cells [67], and TRPC3, in combination with
TRPC1 or TRPC4, might be involved in SOCs formation [53].
TRPC1 seems to be implicated in intracellular Ca\(^{2+}\) signaling
by contributing to functional coupling between the plasma
membrane and the ER. Support for this hypothesis is derived
from information obtained in human platelets and B lympho-
cytes, which suggests that TRPC1 is activated upon interaction with InsP₃-Rs [57] and that it acts not only as a component of SOCs but also as a regulatory subunit of InsP₃-Rs [58]. In addition to these studies, electrotropinjection of a STIM1 antibody into platelets inhibited SOCE by reducing coupling between TRPC1 and InsP₃-Rs [88]. Further, Ca²⁺ store depletion stimulated rapid STIM1 surface expression and its association with TRPC1. However, TRPC channel functions might be dependent on their mutual association in forming heteromeric complexes, a feature that may be typical of certain myeloid lineages.

A novel, essential regulator of SOCE, Orai1, has been identified [69–71]. Mutations of two conserved acidic residues in transmembrane segments of Orai1 [72] as well as RNA interference knockdown [73] result in a decrease of Ca²⁺ influx in mammalian cells leading to three hypothesis: Orai1 is a pore subunit of SOCs; Orai1 forms the channel itself; or Orai1 is an accessory protein of the SOC signaling machinery (plasma membrane acceptor or docking protein, possibly for STIM1) [69]. Experiments of Orai1 and STIM1 overexpression [74–76] provided some evidences for the interaction of STIM1, anchored within ER membranes, with Orai1 after intracellular Ca²⁺ store depletion.

Recent studies provided evidence for the formation of a ternary complex among TRPC, Orai1, and STIM1 (Fig. 2), in which TRPC is the pore-forming component of SOCs and Orai1, the regulatory subunit that confers store depletion sensitivity to TRPC [77, 79]. Despite accumulating data that point to a preponderant role of a ternary complex formed by TRPC/Orai1/STIM1 in the activation of SOCE, conflicting findings exist about the mode of activation of TRPC channels. An expression profile appears to have an influence on the behavior of TRPC channels. To illustrate this hypothesis, it was demonstrated that individual TRPC channels could behave as a SOC or as a receptor-operated channel, depending on their interaction with other TRPC and Orai1 proteins [78, 80]. Although results obtained in other nonexcitable cells might provide an important means to ultimately resolve the molecular mystery of the SOCE pathway in neutrophils, they must be extrapolated with prudence to human neutrophils, and more studies will be necessary in these cells to define the exact arrangement of SOCs and mechanisms leading to their activation.

Non-SOCE

SOCE is considered the prominent mechanism for Ca²⁺ influx into neutrophils after Ca⁴⁺ pool discharge. However, it is becoming increasingly apparent that stimulation of cells not only activates SOCE but also promotes additional Ca²⁺ entry pathways. In addition to the InsP₃ pathway, cyclic ADP-ribose (cADPr) appears to be required for sustained extracellular Ca²⁺ influx in neutrophils stimulated by FMLF [81, 82]. cADPr is formed in neutrophil granulocytes by the transmembrane glycoprotein CD38, which acts as an ADP ribosyl cyclase, converting NAD⁺ into cADPr [83]. Ca²⁺ influx is severely impaired by the specific cADPr antagonist, 8-bromo-cADPr, and in CD38 knockout mice [81]. Based on these observations and additional experiments, Partida-Sanchez et al. [81] postulated that cADPr directly activates Ca²⁺ influx by mobilizing the intracellular Ca²⁺ store through the ryanodine receptor. Later, the same group found functional differences in human neutrophils [82]. The high-affinity, FMLF-binding receptor induces a cADPr-independent Ca²⁺ response, whereas the low-affinity receptor related to the FMLF receptor [84] can trigger a Ca²⁺ response regulated by cADPr, which is primarily a result of extracellular Ca²⁺ influx [82]. Based on functional experiments with neutrophil-like HL-60 cells, another interpretation is provided by our lab: cADPr could support intracellular Ca²⁺ release by regulating extracellular Ca²⁺ entry through non-SOCE [85]. There is no consensus about mechanisms by which cADPr induces Ca²⁺ influx, but the possibility that cADPr is first hydrolyzed to ADPr [86] and then gates channels has recently attracted attention. Patch-clamp experiments in neu-
trophils provided evidence for a channel activation of the TRP family TRPM2 by intracellular ADPr [87, 88]. NAD⁺ is reported as an alternative stimulus for TRPM2 in addition to ADPr [88], where redox state interestingly mediates independent activation of this channel [89].

Other messengers resulting from diacylglycerol metabolism, including arachidonic acid [90], can cause a Ca²⁺ influx clearly distinct from SOCE in some cell types, which involves Orai channels [90]. As it has been demonstrated that diacylglycerol analogs cannot activate TRPC3 and TRPC6 channels through stimulation of PKC, diacylglycerol could itself regulate TRPC3 and TRPC6 directly [91]. Although there are an increasing number of reports supporting significant Ca²⁺ influx via non-SOCE (which are closely related to receptor-operated calcium entry) in neutrophils, intracellular signaling molecules that initiate this response have not formally been identified.

Ca²⁺ SIGNAL-MEDIATED NADPH OXIDASE ACTIVITY

As we described previously, the localization of superoxide anion production induced by antibody-opsonized Escherichia coli or zymosan particles differs from the one induced by G-protein-coupled chemotactrant receptors (iMLF). Although both pathways are Ca²⁺-dependent, the mechanism of Ca²⁺ entry required for intraphagosomal and extracellular superoxide anion production seems different. Complexities to concomitantly measure NADPH oxidase activity and [Ca²⁺], variations with a high resolution make it difficult to analyze the link between both phenomena.

Quantitative measurements of NADPH oxidase activity and Ca²⁺ change

Different methods and instrumentations (spectroscopy, electrophysiology, chemiluminescence, Raman resonance imaging, electron spin resonance) have been developed to detect the production of ROS (see refs. [92–94] for review). Fluorescent dyes have an exquisite spatio-temporal resolution as well as a high dynamic range and are easy to use and cheap. NADPH oxidase activity is routinely measured by the conversion of nonfluorescent compounds to fluorescent counterparts. Dil-hydroethidium (DHR) and more recently, Amplex Red are presented as the most sensitive and stable indicators of ROS production and are often used to assess NADPH oxidase activity. Although Amplex Red is membrane-impermeant, DHR can traverse the membrane and distributes in multiple cellular subcompartments. Although Amplex and DHR allow for reliable measurements of extracellular and global intracellular H₂O₂ production, both dyes are not suitable for measuring ROS formation restricted to phagosomes and their accumulation in granules during phagocytosis [31, 95–98]. Another probe, 2',7'-dichlorodihydrofluorescein (DCDHF), does not redistribute between compartments and therefore, is an effective probe for monitoring kinetics of localized release of oxidative products within the forming phagocytic vacuoles in activated neutrophils [99]. The probe is firmly attached to microorganisms to target it to the specific phagosome. To monitor the oxidative activity during phagocytosis, DCDHF-labeled zymosan has been developed [100]. The major problem with this strategy is that myeloperoxidase, delivered to the phagosome, affects DC-DHF-zymosan oxidation. This effect influences the non-linear relationship between fluorescence in the phagosome and NADPH oxidase activity. To overcome the dependence of peroxidase degragation in the phagosome and to increase the specificity of the assays, measurement of DCDH-zymosan oxidation during phagocytosis in the presence of extracellular peroxidase is required [100].

Similar techniques based on fluorescence indicators are also widely used to measure quantitative changes in [Ca²⁺] [101, 102]. However, these probes cannot be selectively targeted to a specific cellular compartment without microinjection, and [Ca²⁺] elevation can be generated artificially as a result of the rapid diffusion of the probe-Ca²⁺ complex away from a more restricted region of the cytosol. Currently, genetically encoded indicators are developed to measure local Ca²⁺ changes occurring within specific cell compartments. In a detailed review, Demaures [103] discusses design and use of these genetic Ca²⁺ indicators.

Despite some disadvantages characteristic to each probe, fluorescent dyes have remained the most reliable tools to monitor oxidative activity and [Ca²⁺] elevation. In this context, Hallett’s group [100] provided convincing data about temporal and spatial Ca²⁺ signal-regulated phagocytosis in using DCDHF-labeled zymosan and fura-2 microinjection. Furthermore, several reports based on the use of fluorescent dyes underlined the requirement of Ca²⁺ influx in plasma membrane NADPH oxidase activity. Progress in the generation of new indicators will further improve the approach to image Ca²⁺ and NADPH oxidase; this will be ultimately conclusive in deciphering the calcium-related phenomena observed in specific cellular compartments of regions.

Regulation of chemotactrant-induced NADPH oxidase by Ca²⁺ influx

Strong evidence for Ca²⁺ dependence of NADPH oxidase activation came from deprivation of extracellular Ca²⁺ and depletion of intracellular Ca²⁺ stores [3–7]. Although the precise nature of [Ca²⁺], elevation in iMLF-induced NADPH oxidase activation has not been resolved conclusively, evidence, based on the use of pharmacological blockers of SOCE, exists to support a role of SOCE in the oxidative response. Although the specificity of 1-[5-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole hydrochloride (SK&F) 96365 and 2-aminoethyldiphenyl borate (2-APB) remains questionable, N-propargylnitrendipine, MRS1845, represents a promising compound for the development of selective SOCs inhibitors. It possesses micromolar potency at SOCs in HL-60 cells [104] and is far more potent than traditional SOCE inhibitors. Unlike currently used SOC blockers, MRS1845 does not activate intracellular Ca²⁺ release at concentrations required to block SOCE. MRS1845 is able to suppress Ca²⁺ uptake triggered by iMLF and thapsigargin as well as NADPH oxidase activation at concentrations similar to those needed to suppress SOCE [105]. This apparent link between SOCE and NADPH oxidase
is supported by data of Lee et al. [106], relating a modulation of \( \text{Ca}^{2+} \) entry and attenuation of NADPH oxidase activity in neutrophils that have been subjected to MRS1845. NADPH oxidase activity is also inhibited by the pyrazole derivative N-[4-[3,5-bis(trifluoromethyl)-1H-pyrazol-1-yl)(phenyl)-4-methyl]-1,2,3-thiadiazole-5-carboxamide (BTP2), a new inhibitor of SOCE described in neutrophils [107], to the same level as in the absence of extracellular \( \text{Ca}^{2+} \). BTP2 has no effect on PMA-stimulated NADPH oxidase, indicating a specific effect of BTP2 for the \( \text{Ca}^{2+} \) dependence of NADPH oxidase activation. These findings confirm that \( \text{Ca}^{2+} \) uptake via SOCE is required for NADPH oxidase activation.

On the other hand, Itagaki et al. [108] suggest that \( \text{Ca}^{2+} \) influx occurring through a mechanism other than SOCE could be a relevant event to activate the oxidative response. This conclusion is based on multiple observations made in this study. For example, when lysophosphatidic acid is applied to HL-60 cells in \( \text{Ca}^{2+} \)-free medium, no store depletion is observed, but \( \text{Ca}^{2+} \) influx is detected immediately after readdition of external \( \text{Ca}^{2+} \). Further, lysophosphatidic acid has stimulatory effects on NADPH oxidase in a concentration-dependent manner. Taken together, these results provide strong evidence for the involvement of two separate \( \text{Ca}^{2+} \) signaling pathways in NADPH oxidase regulation but no direct correlation between non-SOCE or SOCE, and NADPH oxidase has yet been formally established.

The contribution of transient \( [\text{Ca}^{2+}]_c \) elevation in NADPH oxidase activation varies with applied stimulus classes and within the seven transmembrane-spanning G-protein-coupled receptor family. Activation of neutrophils with PAF and fMLF is accompanied by a transient \( [\text{Ca}^{2+}]_c \) elevation, which is of similar magnitude for each activator. In both cases, the peak of \( [\text{Ca}^{2+}]_c \) is followed by a gradual decrease in \( [\text{Ca}^{2+}]_c \). [109]. Although fMLF and the PAF allow a \( \text{Ca}^{2+} \) mobilization, the functional response elicited by these chemoattractants is distinctly different. Although fMLF provokes NADPH oxidase activation, exposure to PAF does not induce ROS generation. This difference is attributed to the coupling of both chemoattractants to two distinct G proteins and consequently, to different signals of transduction [110]. Therefore, a transient \( [\text{Ca}^{2+}]_c \) elevation is not in any way linked to the generation of a NADPH oxidase-activating signal from G-protein-coupled receptors. An increase of \( [\text{Ca}^{2+}]_c \) is accordingly not sufficient to initiate NADPH oxidase activation in the plasma membrane; an additional signal in combination with \( [\text{Ca}^{2+}]_c \) influx is almost certainly required for NADPH oxidase activation [3].

\( \text{Ca}^{2+} \)-regulated NADPH oxidase during phagocytosis

Sources of \( \text{Ca}^{2+} \) involved in superoxide anion production in the phagosome are not yet determined. As elevation of periphagosomal \( \text{Ca}^{2+} \) is not affected by BTP2, SOCs do not seem to participate in phagocytic stimuli-mediated \( \text{Ca}^{2+} \) influx in contrast to those induced by chemoattractants (Fig. 1).

Through binding to \( \beta_2 \) integrin molecules, complement component, C3bi-opsonized zymosan particles mediate \( \text{Ca}^{2+} \) signals and subsequent NADPH oxidase activation during phagocytosis in neutrophils. The work of Hallett’s group [100] permitted resolving of \( \text{Ca}^{2+} \) signals into two temporally separated phases and clarified the role of [\( \text{Ca}^{2+} \)]c, change in NADPH oxidase activation during phagocytosis. First, the global \( \text{Ca}^{2+} \) signal is occurring during integrin engagement at the point of contact between the particle and the cell and is subsequent to an intracellular \( \text{Ca}^{2+} \) store release near the plasma membrane. Based on their observations, Dewitt et al. [100, 111] proposed that a \( \text{Ca}^{2+} \) influx, triggering a global [\( [\text{Ca}^{2+}]_c \)], change, is responsible for increased mobility of \( \beta_2 \) integrins distant from the contact site, which in turn activates calpain. Subsequently, activated calpain releases distant \( \beta_2 \) integrin from their tethers and allows their diffusion to the contact site to complete the phagocytic event.

Some \( \text{Ca}^{2+} \) stores are located at the periphery of phagocytic vesicles [112] and are distinct from those implicated by G-protein-coupled receptors, such as fMLF [113, 114]. The identity of the intracellular messenger responsible for the release of these peripheral \( \text{Ca}^{2+} \) stores remains unknown. Although Ins\( \text{P}_3 \) can diffuse rapidly in the cytosol [115], it is not certain that this fMLF-mediated phosphoinositide metabolite can trigger integrin-mediated store depletion. A second global [\( [\text{Ca}^{2+}]_c \)]c change is occurring at the time of phagosomal closure and is temporally correlated with the activation of NADPH oxidase. Such a conclusion has been proposed by Dewitt and Hallett [111] following the development of a micromanipulation technique in which C3bi-opsonized particles are presented to neutrophils, coupled to a microscopic detection of intraphagosomal particles labeled with an oxidant-sensitive probe. Activation of the NADPH oxidase is triggered locally in the phagosome, but the second \( \text{Ca}^{2+} \) signal is not restricted to the region of the phagosome. Indeed, localized store depletion may generate a diffusible signal (calcium influx factor), gating \( \text{Ca}^{2+} \) channels in the plasma membrane distant from the initial contact site, and \( \text{Ca}^{2+} \) influx may thus occur across the entire neutrophil surface. In the same study, the authors demonstrated that extracellular Ni\( ^{2+} \), used as a blocker of \( \text{Ca}^{2+} \) influx, and a phosphoinositide-3-kinase inhibitor (LY294002) prevented the \( \beta_2 \) integrin-triggered global \( \text{Ca}^{2+} \) signal. Blockade of \( \text{Ca}^{2+} \) influx is accompanied by a slowed phagocytosis and a decrease of NADPH oxidase activity. Thus, global changes of \( \text{Ca}^{2+} \) resulting from \( \text{Ca}^{2+} \) influx are necessary but insufficient to activate the NADPH oxidase during phagocytosis; other slower events are required, probably including phosphatidylinositol 3,4,5 triphosphate (PIP3) formation in the phagosomal membrane and its binding to the phox homology domain of phox proteins [116]. Furthermore, it is demonstrated that PIP3 accumulation and anchoring at the phagocytic membrane are prerequisites for the generation of \( \text{Ca}^{2+} \) signaling [117]. Exact mechanisms linking PIP3 formation to \( \text{Ca}^{2+} \) signals are not yet known, but PIP3 may activate a PLC isoform triggering Ins\( \text{P}_3 \) production [118]. It is also possible, as described in platelets, that PIP3 regulates a novel pathway of \( \text{Ca}^{2+} \) entry, which is independent of an increase of PLC activity [119] and which would sustain NADPH oxidase activity during phagocytosis.
Potential targets regulated by Ca\(^{2+}\) changes

**Ca\(^{2+}\)-regulated PKC**

PKC, a phospholipid-dependent family of serine-threonine protein kinases, acts in multiple signal transduction pathways including the regulation of the NADPH oxidase. At least 12 different PKC isoforms have been characterized so far, and these can be grouped into the following three subgroups: conventional PKCs (α, βI, βII, and γ); novel PKCs (δ, ε, μ, θ, and η), and atypical PKCs (ζ and ηA), on the basis of their molecular structure and the requirement of Ca\(^{2+}\) for activation (conventional) and diacylglycerol-binding activity (conventional and novel). Conventional and novel PKCs are directly activated by phorbol esters (PMA), potent activators of NADPH oxidase acting as analogs of diacylglycerol, and atypical isoforms are insensitive to PMA [120–123]. Most of the data supports the contention that [Ca\(^{2+}\)]\(_{c}\) does not change during PMA stimulation, and NADPH oxidase activation is independent of the extracellular Ca\(^{2+}\) concentration [124–128]. In disagreement with the fact, largely reported, that PMA stimulates NADPH oxidase independently of Ca\(^{2+}\) influx, some studies demonstrate that PMA-activated NADPH oxidase is decreased significantly in the absence of extracellular Ca\(^{2+}\) [129]. However, this Ca\(^{2+}\) dependency is only observed on adherent neutrophils stimulated at low cell density, reinforcing the plausibility of the granulocyte cellular environment in selecting distinct, Ca\(^{2+}\)-dependent transduction pathways.

In neutrophils and HL-60 cells differentiated into neutrophil-like cells, α, β, δ, and ζ appear to be the main PKC isotypes involved in NADPH oxidase activation by most physiological agonists, as shown by studies using antisense strategy, pharmacological inhibitors, or knockout [105, 123, 122, 130–132]. However, their respective contribution in regulating NADPH oxidase has not been well documented yet. Conventional PKCs are pointed out as participants for regulating NADPH oxidase in neutrophils. By down-regulating the enzyme activity through an antisense oligonucleotide strategy, Korchak et al. [105] established that PKCβ and -α are required for a full FMLF- and PMA-mediated superoxide anion production in neutrophil-like HL-60 cells. Consistent with this assumption, PKCβ antisense inhibits phosphorylation and translocation of p47\(^{phox}\) induced by FMLF, providing evidence for the involvement of PKCβ in the signaling pathway leading to FMLF- and PMA-mediated NADPH oxidase activation. In addition to PKCα, PKCβII expressed in human neutrophils can phosphorylate p47\(^{phox}\) and induce its translocation and NADPH oxidase activation [133]. Thus, downstream effects of conventional, Ca\(^{2+}\)-activated PKCs include a direct phosphorylation of p47\(^{phox}\), which leads to membrane translocation of cytosolic components (Fig. 1).

**Ca\(^{2+}\)-regulated cytosolic PLA\(_2\) (cPLA\(_2\))**

Many agonists that stimulate superoxide anion production in phagocytic cells cause the release of arachidonic acid from membrane phospholipids via the hydrolysis of fatty acids from the sn-2 position of membrane phospholipids by PLA\(_2\) [134–136]. Several PLA\(_2\) isoforms have been described including a cPLA\(_2\), which is activated rapidly by increased [Ca\(^{2+}\)]\(_{c}\). Stimulation of cells induces an immediate and transient translocation of cPLA\(_2\) to nuclear membranes [137]. Evidence that cPLA\(_2\) is recruited at the plasma membrane comes from an intracellular distribution study of cPLA2 in neutrophils and granulocyte-like PLB-985 cells [138]. Although underlying mechanisms are not totally understood, it is known that cPLA\(_2\) is involved in the regulation of phagocytic cell superoxide anion production. In response to a variety of soluble and particulate stimuli, NADPH oxidase activation is impaired in cPLA\(_2\)-deficient, differentiated PLB-985, but addition of arachidonic acid is able to rescue NADPH oxidase activity [139, 140]. Subsequently, Pessach et al. [141] described a normal translocation of oxidase cytosolic components in activated, differentiated PLB-985 cells lacking cPLA\(_2\) or in neutrophils treated with cPLA\(_2\) inhibitors [141]. Thus, cPLA\(_2\) is not required for translocation of cytosolic factors to membranes [139, 140]. Taken together, these data have a substantial implication: cPLA\(_2\) serves a critical role in oxidase activation after the assembly of enzyme complex in neutrophils. Arachidonic acid may mediate structural changes in NADPH oxidase component triggering interaction between oxidase subunits or affecting the function of flavocytochrome b\(_{558}\) [142]. Another alternative idea puts forward the fact that arachidonic acid might be a cofactor enhancing the affinity of the assembled NADPH oxidase for NADPH, probably via induction of structural changes [138].

As cPLA\(_2\) deficiency is associated with profound effects on NADPH oxidase activity, cPLA\(_2\) activation mechanisms by Ca\(^{2+}\) mobilization need to be investigated further. A limited number of studies focused on this signaling pathway in a variety of cells. Indeed, it is shown that translocation of cPLA\(_2\) is dependent on [Ca\(^{2+}\)]\(_{c}\) elevation [143, 144], and PKCα is required for cPLA\(_2\) activity [145], supporting evidence that Ca\(^{2+}\) regulates NADPH oxidase through the PKC/cPLA\(_2\)/arachidonic acid pathway. However, no clear proof exists for such a regulation in neutrophils, and further studies are needed to elucidate the exact involvement of cPLA\(_2\) in NADPH oxidase activity of these cells.

**Ca\(^{2+}\)-regulated S100 proteins**

In the present nomenclature, gp91\(^{phox}\) homologous NADPH oxidase found in non-phagocytic cells is referred to as Nox enzymes (gp91\(^{phox}\) is specified as Nox2). Five Nox proteins are described in humans with distinct tissue distribution. Nox5, essentially expressed in lymphoid tissues and in testis [146], is distinguished from the other Nox proteins by an additional N-terminal extension containing three canonical and one non-canonical EF-hand motifs. These four calcium-binding sites allow Nox5 to be sensitive to the presence of Ca\(^{2+}\). A recent study demonstrated that [Ca\(^{2+}\)]\(_{c}\) elevation triggers a conformational change in the Nox5 N terminus, leading to NADPH oxidase activation through an interaction between the regulatory N terminus and C terminal catalytic domain [147]. In contrast to Nox5, no EF-hand domains have been found in Nox2. However, a potential role of Ca\(^{2+}\) in NADPH oxidase activation via S100A8 and S100A9 has been found in granulocytes [148]. In human neutrophils, Lemarchand et al. [149] described that the translocation of myeloid-related proteins,
Subsequent to Ca2+

system, increased affinity of p67phox for flavocytochrome

Berthier et al. [157] confirmed, in a semirecombinant, cell-free factors and enhance their recruitment to the membrane-bound proteins interact directly with flavocytochrome inferred by S100A8/S100A9. According to these authors, S100 involved in this process [152]. When an extracellular, Ca2+

locates to the plasma membrane, secretory vesicle, and gela-

confocal microscopic analysis established that S100A9 trans-

ates with F-actin, it can be assumed that phosphorylation 

a significant increase in S100A9 localized to the base of S100A8/S100A9 heterodimers appear to interact preferentially 

plasmalipid and as phosphorylated S100A8/S100A9 associ-

ates with F-actin, it can be assumed that phosphorylation results in S100A9 translocation. Biochemical analysis and confocal microscopic analysis established that S100A9 trans-

locates to the plasma membrane, secretary vesicle, and gela-

inase granules dependently on p38 MAPK-mediated phos-

phorylation. Although the mechanism of S100 protein translo-

cation is not yet fully understood, phosphorylation is clearly involved in this process [152]. When an extracellular, Ca2+-

independent stimulus is used, S100A9 proteins, which are marked phosphorylated, and S100A8 proteins, which are phosphorylated weakly, do not translocate [153], indicating that phosphorylation is strictly dependent on Ca2+ influx. Subsequent to Ca2+ fixation, a heterocomplex is formed by a noncovalent association of Ca2+-binding proteins S100A8 and S100A9 [154, 155]. Using an immunofluorescence approach, it has been reported that in bovine neutrophils stimulated by PMA [156], these S100 proteins are concentrated under the plasma membrane with cytosolic phox proteins. A similar observation is made for S100A8 in human neutrophils [155]. S100A8/S100A9 heterodimers appear to interact preferentially with p67phox and might favor the organization of the cytosolic factors and enhance their recruitment to the membrane-bound flavocytochrome b558, acting as scaffold proteins (Fig. 1). Berthier et al. [157] confirmed, in a semirecombinant, cell-free system, increased affinity of p67phox for flavocytochrome b558 inferred by S100A8/S100A9. According to these authors, S100 proteins interact directly with flavocytochrome b558 and mediate its transition from an inactive to an active conformation state, resulting in NADPH oxidase activation. Preincubation of S100A8/S100A9 in the absence of Ca2+ led to an interaction with flavocytochrome b558 but not to a conformational change, supporting the fact that Ca2+, through S100 binding, is required for NADPH oxidase activity [157]. Determination of specific interactions of the S100 complex with flavocytochrome b558 by atomic force microscopy [157] has been confirmed later by the same group using different experimental systems. A combination of ex-vivo and in-vitro methods followed by flavocytochrome b558 purification provides evidence for the activation of flavocytochrome b558 by the recombinant S100 complex in the absence of phox cytosolic factors and arachidonic acid [158]. On the other hand, protein–protein interaction studies based on pull-down assays in a semirecombinant system similar to the one of Berthier et al. [157] reveal that p67phox and Rac proteins might interact with the S100 complex [159]. This controversy about the site of S100 complex interaction could be explained by the use of different neutrophil model systems and different methods to investigate protein interactions. However, the transition of cytochrome b558 to an activated state appears to be a result of the binding of the S100 complex.

NADPH oxidase is also dependent on cPLA2-mediated arachidonic acid formation, which binds reversibly with high affinity to a heterocomplex formed by S100A8 and S100A9 [156]. By adding S100A8/S100A9 complexes to a semi-recombinant system comprising bovine neutrophil membranes, cyto-

solic phox proteins, GTPγS-loaded Rac2, and the optimal amount of arachidonic acid, Kerkhoff’s and Doussièr’s groups [159, 160] demonstrated that binding of arachidonic acid to the S100 heterodimer is a critical step to promote NADPH oxidase activation. Mutant S100A8/S100A9 complexes, unable to bind arachidonic acid, only exhibit a slight variation of oxidase activity in comparison with wild-type recombinant S100 complexes. S100A8/S100A9 complexes facilitate NADPH oxidase activation by transferring arachidonic acid to the NADPH oxidase complex [159]. In fact, S100 proteins binding to arachidonic acid may increase NADPH oxidase activity by decreasing the deactivation rate [160]. Thus, S100 protein translocation to the plasma membrane together with p67phox, p47phox, and Rac supports the hypothesis that these proteins might deliver bound arachidonic acid in a Ca2+-dependent manner to the NADPH oxidase [157].

Ca2+-regulated Rac activation

First, evidence that activation of the monomeric G protein in neuronal cells is dependent on [Ca2+]c elevation induces a temporary increase in Rac translocation to the plasma mem-

brane, emphasizing the fact that this phenomenon is promoted by extracellular Ca2+ entry (Fig. 1). These authors proposed...
that the \([\text{Ca}^{2+}]_e\) rise triggers PKC-dependent Rho-GDI phospho- 
lation, leading to Rac dissociation of the Rac-Rho-GDI complex and sub-sequent translocation of Rac to the plasma 
membrane. Rac translocation is correlated with its activation 
[163], but it appears likely that both events are regulated 
independently. The activation of Rac by a chemoattractant has 
been reported to be independent on \([\text{Ca}^{2+}]_e\) changes, but 
relevant experiments have been performed in \(\text{Ca}^{2+}\)-free buffer 
[164].

Using bone marrow-derived neutrophils from RhoG knock- 
out mice, Condliffe et al. [165] showed a reduction of fMLF- 
duced oxidant production but an unaltered response to PMA 
and opsonized zymosan. This reduction of oxidase activation 
is associated with a partial decrease of Rac protein activation. 
The authors hypothesized that RhoG acts on a subset of Rac 
required for targeted oxidase assembly or for determining its 
precise cellular localization. In spite of this, the precise target 
of calcium ions remains unknown, but it is possible that \(\text{Ca}^{2+}\) 
influx may impact on Rac activity through the regulation of 
RhoG.

REGULATION OF \(\text{Ca}^{2+}\) INFLUX BY 
ACTIVATION OF THE NADPH OXIDASE

Activation of the NADPH oxidase is associated with the 
electrogenic transfer of electrons to molecular oxygen across the 
plasma membrane, generating a decrease in membrane poten- 
tial. Accumulation of negative charges on one side of the 
membrane by activated neutrophils would turn off further 
electron transfer, prematurely interrupting the killing process of 
pathogens. To preserve electroneutrality and to allow for the 
extrusion of the intracellular acid released in the cytosol during 
the hydrolysis of NADPH and its resynthesis by the hexose 
monophosphate shunt, the most efficient mechanism would be 
to extrude H\(^+\) ions from the cells through a proton pathway 
[166, 167]. Proton channels responsible for H\(^+\) efflux were 
originally proposed by the group of Lydia Henderson [168] to 
be contained within the gp91<sub>phox</sub> subunit of NADPH oxidase. 
However, the experimental evidence regarding the H\(^+\) channel 
group of gp91<sub>phox</sub> remains contradictory, and a body of 
evidence indicates that a protein other than the transmembrane 
oxidase subunit gp91<sub>phox</sub> could act as a voltage-gated proton 
channel (for a review, see ref. [169]).

As two depolarizing agents, KCl and a pore-forming iono- 
phore gramicidin D, reduced the \([\text{Ca}^{2+}]_e\), increase caused by 
fMLF, it has been suggested that plasma membrane depolar- 
ization in human neutrophils is a physiological feedback mech- 
anism inhibiting \([\text{Ca}^{2+}]_e\) changes [170]. Later, effects of fMLF 
on SOCE were investigated in neutrophil granulocytes of pa- 

tients suffering from chronic granulomatous disease, who pos- 
sess a deficient NADPH oxidase activity and an attenuation of 
depolarization following fMLF stimulation. By using an indi- 

cert Mn\(^{2+}\)/fura-2 fluorescence-quenching procedure, acceler- 
ated \(\text{Ca}^{2+}\) influx has been observed in these cells, suggesting 
that depolarization impairs uncontrolled \(\text{Ca}^{2+}\) influx in trig- 

gering early attenuation of store-operated \(\text{Ca}^{2+}\) uptake and 
restricting \(\text{Ca}^{2+}\) influx [171, 172]. Failure of depolarization in 
chronic granulomatous disease is associated with \(\text{Ca}^{2+}\) over- 
load as a result of accelerated influx of the cation and hyper- 
activity of several proinflammatory activities of these cells 
[173]. The correlation between inhibition of depolarization and 
\(\text{Ca}^{2+}\) influx through opened SOCs [174] is supported by ex- 
periments in genetically modified neutrophilic cell lines un- 
able to produce superoxide anions but possessing SOCE mech-

More evidence involving NADPH oxidase activation in 
the restriction of SOCE in fMLF-activated neutrophils is de- 

erived from experiments using a selective inhibitor of NADPH oxidase, diphenyleneiodonium. This agent accelerates 
the rates of membrane repolarization and SOCE by preventing plasma 
membrane depolarization [173–175]. Furthermore, \(\text{K}^+\) iono- 
phore valinomycin, which allowed increased charge compensa- 
tion, decreased depolarization-potentiated, chemoattractant- 
mediated \([\text{Ca}^{2+}]_e\). The enhanced \(\text{Ca}^{2+}\) permeability across the 
plasma membrane could be explained again by an increase of 
the driving force for \(\text{Ca}^{2+}\). This notion is supported by exper- 
iments where a decrease in the concentration of external \(\text{Ca}^{2+}\) 
added to thapsigargin-pretreated cells from 500 to 50 \(\mu\text{M}\) 
resulted in a measurable impairment of \(\text{Ca}^{2+}\) entry [171]. The 
general hypothesis is that when the cells are depolarized, the 

driving force for \(\text{Ca}^{2+}\) influx is markedly decreased as a result 
of elimination of the electrical component of the electrochem-

ical gradient for \(\text{Ca}^{2+}\). The driving force for \(\text{Ca}^{2+}\) ions as a 
result of the extensive depolarization following stimulation is 
certainly ample enough to cause detectable diminution of \(\text{Ca}^{2+}\) 
influx by itself.

NADPH oxidase regulates neutrophil \(\text{Ca}^{2+}\) influx not only 
via its electrogenic activity but also as a consequence of 
ROS generation. This phenomenon is well documented by 
the recent work of Tintinger et al. [176], which focused on 
the effects of neutrophil-derived ROS on SOCE. Treatment 
of cells with catalase potentiates the rate and the magnitude 
of store-operated uptake of \(\text{Ca}^{2+}\) [176]. Furthermore, inhibi-
tors of myeloperoxidase (enzyme catalyzing the formation of 
HOCI) shorten the time to onset of \(\text{Ca}^{2+}\) influx, prolong the 
linear phase of influx, and increase the magnitude of store-
operated uptake of \(\text{Ca}^{2+}\). A putative target of derived ROS 
has been identified, linking the redox state to \(\text{Ca}^{2+}\) ho- 

mestasis. The non-selective cation channel TRPM2, a 
member of the TRP family, is recognized to have an oxida- 
tive sensitivity and be positively regulated by \(\text{H}_2\text{O}_2\). In 
TRPM2-transfected HEK-293 cells, \(\text{Ca}^{2+}\) is increased rapid- 
ly by \(\text{H}_2\text{O}_2\) stimulation, suggesting the possibility that 
oxidative stress mediates influx of \(\text{Ca}^{2+}\) through TRPM2 in 
granulocytes [177].

NADPH oxidase also has a physiological relevance in con- 
trolling \(\text{Ca}^{2+}\) influx in neutrophils. It seems to fulfill a physio-

logically important, anti-inflammatory function by preventing 
the \(\text{Ca}^{2+}\) overload and hyper-reactivity of neutrophils. An acti-
vation of NADPH oxidase activity could constitute an interest-
ning pharmacological strategy. However, NADPH oxidase is 
as also potentially involved in the activation of the \(\text{Ca}^{2+}\) pathway, 
and the potentialization of its activity may be accompanied by 
a risk of oxidant-mediated toxicity.
Neutrophilic lung inflammation is an essential component of host defense against various pathogens. During activation, neutrophils release ROS, triggering the inactivation of protease inhibitors, which protect lung tissue from proteolytic damage. Infiltration and persistent presence of neutrophils in response to chronic inflammatory airway diseases, such as chronic obstructive lung disease, asthma, cystic fibrosis, and bronchiolitis, aggravate lung tissue damage observed in these diseases. Few available therapeutic agents efficiently down-regulate neutrophil proinflammatory activity by reducing local neutrophil infiltration. However, neutrophils are reported to be relatively insensitive to such chemotherapeutic strategies, and development of alternative treatment is undoubtedly required.

Given the critical involvement of \([Ca^{2+}]\), elevation in the regulation of ROS generation and thus, in the proinflammatory activities of neutrophils, \(Ca^{2+}\) channel inhibition holds considerable therapeutic promise. TRP and Orai channels, emerging candidates for store-operated and nonstore-operated channels, may be potential drug targets, and their inhibition might offer anti-inflammatory, therapeutical benefits for treating pulmonary diseases responsible for progressive lung degradation (see Tintinger and Steel [178] for review).

A diversity of inorganic channel inhibitors (lanthanides, divalent cations, \(Gd^{3+}\)) or organic SOCE inhibitors, including channel blockers (SK&F 96365, 2-APB), has been described (reviewed by Putney [179]). In most instances, the exact mechanisms by which they interfere with SOCs remain to be established, and nonspecific effects as a result of interference with other mechanisms important for \(Ca^{2+}\) signals, such as \(Ca^{2+}\) pumps, mitochondrial \(Ca^{2+}\) homeostasis, \(Ca^{2+}\) release, and activity of \(K^+\) channels, cannot be excluded. For example, 2-ABP has been shown to potentiate and block \(Ca^{2+}\) entry into cells [180], and it inhibits InsP\(_3\) receptor-mediated \(Ca^{2+}\) release [181]. Similar antagonistic effects are observed with SK&F 96365 [182]; also, an activation of intracellular \(Ca^{2+}\) depletion in various cell types at different concentrations can be observed using those inhibitors [183]. Recently, it was been documented that a pyrazole derivative, named BTP2, inhibits SOCE in human neutrophils [107], potentially by interfering with TRP channels from the extracellular space [184]. Preincubation of BTP2 clearly reduces superoxide anion production, having no effect on phagocytosis or intraphagosomal radical production [107]. These findings make SOCs excellent targets for down-regulating the inflammatory response without impairing bacterial killing. Similarly, MRS1845 seems to represent a new, promising candidate for selective SOC inhibitors and could be used as a lead for drug design [104, 105].

Other pharmacological drugs, such as tenipad [5-chloro-2,3-dihydro-3-(hydroxy-2-thienylmethylene)-2-oxo-1H-indole-1-carboxamide], could also be used as an inhibitor of the \(Ca^{2+}\) influx pathway or channels in nonexcitable cells, as they are known to modify thapsigargin-induced \(Ca^{2+}\) entry across the plasma membrane [185]. Given the importance of \(Ca^{2+}\) influx in NADPH oxidase activation, it is attractive to speculate that tenipad is able to alter the functioning of this enzyme that mediates inflammatory responses. In agreement with this hypothesis, tenipad has been shown to attenuate superoxide anion production by activated neutrophils [186]. However, tenipad has no effect on the generation of superoxide anions by NADPH oxidase reconstituted from fractionated neutrophil lysates; only inhibition of xanthine oxidase is observed [187]. Similar conclusions are provided with the imidazole antmycotic, itraconazole: Interference with SOCE into human neutrophils does not appear to affect the NADPH oxidase [188].

One of the major difficulties for the development of anti-inflammatory drugs targeting TRP channels is linked to the expression of TRPC in many different cell types. Any potential drugs might be associated with undesired effects if not applied strictly, locally. Meaningful progress can be obtained by developing alternative strategies, in which drugs may be developed, which are directed against the activation mechanisms upstream of TRP channels. Some of these mechanisms might be sufficiently unique to allow a specific targeting of neutrophils as observed in TRPM2 activation [189].

**CONCLUSION**

Evidence is provided that SOCE plays a fundamental role in host defense by regulating the oxidative response in human neutrophils. Several targets of \(Ca^{2+}\) ions involved in NADPH oxidase control are resolved, such as PKC\(_{\alpha}\), PKC\(_{\beta}\), S100 proteins, or cPLA\(_2\), but further insight is needed to identify other pathway-activated NADPH oxidase and apparently sensitive \(Ca^{2+}\) influx, particularly in view of the influence of the granulocyte cellular microenvironment. It appears that the latter may selectively modify the transduction pathways associated with NADPH oxidase, although \(Ca^{2+}\) influx may represent the ab initio signal for superoxide anion secretion. The discovery of a selectively targeted transduction intersection in \(Ca^{2+}\)-dependent NADPH oxidase activation might allow for selection of specific inhibitors that modify this signaling process and thus, might be therapeutically useful in chronic and acute inflammatory diseases engendered by an excessive activation of the NADPH oxidase. However, it must be stressed that the mechanism of SOCE is surrounded by multiple contradictions, but some important \(Ca^{2+}\) routes have been resolved, where TRPC, Orai1, and STIM1 are important actors that demonstrated their clear involvement in NADPH oxidase circuious control. However, as a result of some of these inconsistencies, therapeutic approaches could not be exploited adequately for now. Dissection of the mechanism linking \(Ca^{2+}\) store depletion to SOCs channel activation as well as the nature of these \(Ca^{2+}\) channels might help to characterize the regulation of superoxide anion release and serve as targets for pharmacological drug development in inflammatory diseases.

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