Journal of Cellular Biochemistry 78:131–140 (2000)

Regulation of (1-3)-β-Glucan-Stimulated Ca^{2+} Influx by Protein Kinase C in NR8383 Alveolar Macrophages

Ann-Christin Mörk, Xiuhua Sun, Xiao-bing Liu, Denise Rodriguez, J. Ricardo Martinez, Robert Castro, and Guo H. Zhang*

Department of Pediatrics, University of Texas Health Science Center, San Antonio, Texas 78284

Abstract Stimulation of (1-3)-β-glucan receptors results in Ca^{2+} influx through receptor-operated channels in alveolar macrophages (AMs), but the mechanism(s) regulating Ca^{2+} influx is still undefined. In this study we investigated the role of protein kinase C (PKC) regulation of Ca^{2+} influx in the NR8383 AM cell line using the particulate (1-3)-β-glucan receptor agonist zymosan. PKC inhibition with calphostin C (CC) or bisindolymaleimide I (BSM) significantly reduced zymosan-induced Ca^{2+} influx, whereas activation of PKC with phorbol-12-myristate 13-acetate (PMA) or 1,2-dioctanoyl-*sn*-glycerol (DOG) mimicked zymosan, inducing a concentration-dependent Ca^{2+} influx. This influx was dependent on extracellular Ca^{2+} and inhibited by the receptor-operated Ca^{2+} channel blocker SK&F96365, indicating that zymosan and PKC activate Ca^{2+} influx through a similar pathway. NR8383 AMs expressed one new PKC isoform (δ) and two atypical PKC isoforms (ι and λ), but conventional PKC isoforms were not present. Stimulation with zymosan resulted in a translocation of PKC-δ from the cytosol to the membrane fraction. Furthermore, inhibition of protein tyrosine kinases (PTKs) with genistein prevented zymosan-stimulated Ca^{2+} influx and PKC-δ translocation. These results suggest that PKC-δ plays a critical role in regulating (1-3)-β-glucan receptor activated Ca^{2+} influx in NR8383 AMs and PKC-δ translocation is possibly dependent on PTK activity. J. Cell. Biochem. 78:131–140, 2000.

Key words: β-glucan receptor; zymosan; Ca^{2+} influx; PKC activators and inhibitors; PKC isoforms; translocation; protein tyrosine kinases

AMs are the primary resident phagocytes in the lung and play a critical role in the elimination of pathogens from the respiratory tract through phagocytosis and secretion of oxygen metabolites, lysosomal enzymes, and cytokines. One important mechanism activating these processes is through stimulation of the (1-3)-β-glucan receptor on macrophage membrane [Goldman, 1988; Abel and Czop, 1992; Daum and Rohrbach, 1992; Engstad and Robertsen, 1994; Szabó et al., 1995]. Two membrane proteins of 160 and 180 kDa have been isolated and identified as (1-3)-β-glucan receptors in human monocytes and U937 cells [Czop and Kay, 1991]. Both proteins contain a 20 kDa polypeptide that may act as the functional critical structure of this receptor [Szabó et al., 1995]. At present, the intracellular signal transduction mechanism coupled with the (1-3)-β-glucan receptor in macrophages is still not clearly defined.

Mobilization of cytosolic Ca^{2+} is a critical signaling system associated with the ability of macrophages to respond to environmental challenges and infections by regulating the release of inflammatory mediators [Vecchiarelli et al., 1993;

Grant sponsor: NIH; Grant number: DE09270; Grant sponsor: Swedish Medical Research Council; Grant number: K97-04PK-12291.

Abbreviations used: AM, alveolar macrophage; BSA, bovine serum albumin; BSM, bisindolymaleimide I; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_o$, medium Ca^{2+} concentration; CC, calphostin C; ChC, chelerythrine chloride; CR_3, complement receptor type three; DAG, diacylglycerol; DMSO, dimethylsulfoxide; DOG, 1,2-dioctanoyl-sn-glycerol; fura-2/AM; fura-2/acetoxymethylester; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate-13-acetate; PSS, physiological salt solution; PTK, protein tyrosine kinase; SOCE, store-operated Ca^{2+} entry.

Ann-Christian Mörk's present address is Astra, Pain control AB, S-151, 85 Södertäje, Sweden.

J. Ricardo Martinez's present address is Division of Extramural Research, NIDCR, National Institutes of Health, Bethesda, MD 20892.

*Correspondence to: Guo H. Zhang, Ph.D., Department of Pediatrics, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284. E-mail: Zhangg@UTHSCSA.EDU

Received 1 October 1999; Accepted 27 January 2000

This article published online in Wiley InterScience, April 2000.

Bates et al., 1995; Hoyal et al., 1996; Xie et al., 1997; Lehmann and Berg, 1998]. We have previously demonstrated that exposure to particulate (1-3)-β-glucan agonists such as zymosan, induces an increase in $[Ca^{2+}]_i$ in NR8383 AMs [Zhang et al., 1997; Mörk et al., 1998a; Sun et al., 1999]. The (1-3)-β-glucan receptor mediated increase in $[Ca^{2+}]_i$ was due to Ca^{2+} influx via receptor-operated channels since the increase was abolished by the removal of extracellular Ca^{2+} or by SK&F96365, a specific inhibitor of receptor-operated Ca^{2+} channels [Zhang et al., 1997; Mörk et al., 1998a; Sun et al., 1999]. Currently, little information exists on the intracellular signaling pathway regulating the receptor-operated Ca^{2+} influx in AMs.

PKC has been demonstrated to be involved in Ca^{2+} mobilization and the functional regulation of phagocytosis in leukocytes [Zheleznyak and Brown, 1992; Huwiler and Pfeilschifter, 1993; Schondorf et al., 1993; Tuominen et al., 1994; Tapper and Sundler, 1995; Zhu et al., 1995]. PKC activation attenuated intracellular Ca^{2+} release, thereby reducing Ca^{2+} influx and enhancing Ca^{2+} efflux in neutrophils and the murine macrophages cell line PU5-1.8 [McCarthy et al., 1989; Kong et al., 1993]. Furthermore, PKC stimulates Ca^{2+} pump activity altering cytosolic free Ca^{2+} levels in Jurkat T cells [Balasubramanyam and Gardner, 1995]. Activation of the (1-3)-β-glucan receptor and Ca^{2+} influx in NR8383 AMs are not coupled to phospholipase C or the formation of IP_3 and DAG [Zhang et al., 1997]. Since PKC is coupled to the phosphoinositide signaling pathway, it is unknown if PKC is activated by stimulation of (1-3)-β-glucan receptors. Therefore, the purpose of this study was to investigate (1) if stimulation of the (1-3)-β-glucan receptor activates PKC, (2) which PKC isoforms are activated, and (3) whether PKC plays a role in regulating (1-3)-β-glucan-stimulated Ca^{2+} influx in NR8383 AMs.

MATERIALS AND METHODS

Materials

ATP, BSA (type V), digitonin, DMSO, EGTA, HEPES, and ionomycin were purchased from Sigma (St. Louis, MO). Fura-2/AM was from Molecular Probes (Eugene, OR). BSM, CC, ChC, DOG, genistein, and PMA were obtained from Calbiochem (La Jolla, CA). PKC isoform antibodies and proteins (positive controls) were purchased from Transduction Laboratories (Lexington, KY). All other chemicals used were of the highest grade available.

Solutions

PSS used for incubation consisted of (in mM): 110 NaCl, 25 $NaHCO_3$, 20 HEPES (pH 7.4), 10 glucose, 5.4 KCl, 1.2 $CaCl_2$, 0.8 $MgSO_4$, 0.4 KH_2PO_4 and 0.33 NaH_2PO_4. Ca^{2+}-free PSS contained the same component as PSS, except that $CaCl_2$ was omitted.

Cell Culture

The NR8383 AM cell line was established in this laboratory [Helmke et al., 1987, 1989]. These cells were routinely grown in plastic tissue culture flasks in Ham's F12 medium containing 15% fetal bovine serum, 100 μg/ml penicillin and 100 U/ml streptomycin sulfate at 37°C in an atmosphere of 5% CO_2 in air. The medium was changed twice weekly. These cells typically persist as 50% floating and 50% adherent populations. Floating cells were used in the present study. The viability of the AMs was routinely tested by trypan blue exclusion and was consistently greater than 98% (>94% after loading with fura-2/AM for 2 h).

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured using the Ca^{2+} sensitive fluorescent probe fura-2 as previously described [Zhang et al., 1997; Mörk et al., 1998a; Sun et al., 1999]. NR8383 AMs were loaded with fura-2 by incubation with 2 μM fura-2/AM for 20 min at 37°C. Fura-2-loaded AMs were rinsed twice with PSS containing 0.01% BSA and resuspended in the same medium (1.75×10^6 cells/ml). A 2-ml aliquot of fura-2-loaded AMs was centrifuged at 50*g* for 2 min, resuspended in PSS containing 0.01% BSA, and placed in a 4-ml cuvette. $[Ca^{2+}]_i$ was measured using a PTI Deltascan fluorometer (PTI Inc., S. Brunswick, NJ). Excitation wavelengths used were 340 and 380 nm and emission wavelength was 505 nm. Calibration of $[Ca^{2+}]_i$ was performed for each measurement trace as previously described [Zhang et al., 1997; Mörk et al., 1998a; Sun et al., 1999]. Fluorescence ratios of the 340/380 nm excitation and 505 nm emission were converted to $[Ca^{2+}]_i$ according to Grynkiewicz et al. [1985] using 224 nM as the K_d of fura-2 at 37°C for Ca^{2+}.

The initial rate of $[Ca^{2+}]_i$ decline after ionomycin-induced $[Ca^{2+}]_i$ peak was used as an

index of Ca^{2+}-pump function [Balasubramanyam and Gardner, 1995]. Briefly, AMs were loaded with fura-2 as described above, suspended in low-Ca^{2+} medium ($[Ca^{2+}]_o$ was approximately 100 nM), and $[Ca^{2+}]_i$ was monitored. Ionomycin (1 μM) was added to elicit a rapid increase in $[Ca^{2+}]_i$. The initial rate of $[Ca^{2+}]_i$ decline from the ionomycin-elicited peak was calculated using the initial linear portion (first 30 sec) of the decline curve and expressed in nM/min.

Identification of PKC Isoforms by Western Blot Analysis

PKC isoforms were identified as previously described [Chakravarthy et al., 1992; Terzian and Rubin, 1993; Sugita et al., 1999]. In brief, NR8383 AMs were lysed in 50 μl of ice-cold lysis medium containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 mM $NaVO_4$, 1% Triton X-100, 10 μg/ml antipain, and 10 μg/ml leupeptin. The lysed cells were rapidly centrifuged at 4,000 rpm and the supernatants were collected and mixed with 50 μl of sample buffer (2×) containing 0.125 mM Tris-HCl (pH 6.8), 20% glycerol, 10% 2-mercaptoethanol, 4% SDS, and 0.0012% bromphenol blue. The samples and positive controls from rat brain (α, β, δ, γ, ϵ, ι, and λ), or Jurkat cells (θ and μ) (Transduction Laboratories, Lexington, KY) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The stacking and separating gels contained 4 and 10% acrylamide, respectively. The proteins were then transferred electrophoretically onto BA S-83 nitrocellulose membranes (45 μm pore size). The membranes were air dried and blocked with 150 mM NaCl, 10 mM Tris (pH 7.5) and 5% nonfat milk at 4°C overnight, and then washed with 20 mM Tris (pH 7.5), 0.9% NaCl and 0.3% Tween (TBS-T). Membranes were incubated with primary antibodies (Transduction Laboratories, Lexington, KY) to PKC-α (1: 1000), β (1:250), δ (1:500), γ (1:5000), ϵ (1: 1000), ι (1: 250), θ (1: 250), μ (1: 250), and λ (1: 250) in 2 ml of 10 mM Tris (pH 7.5), 150 mM NaCl, and 1% BSA for 2 h at 22°C. After washing, the membranes were incubated with 0.5 μg/ml of anti-rabbit horseradish peroxidase for 1 h and washed 3 times with TBS-T. The signals were detected by chemiluminescence (DuPont NEN, Boston, MA).

Isolation of Cytosolic and Membrane Fractions and Determination of PKC Translocation

Activation of PKC isoforms was examined by determining their translocation according to previously described methods [Terzian and Rubin, 1993]. Cells were stimulated with 200 μg/ml of zymosan for 1 or 5 min, and rapidly centrifuged at 10,000 rpm for 5 sec, and resuspended in 2 ml of ice-cold extraction buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 μg/ml of leupeptin, and 25 μg/ml of soybean trypsin inhibitor. The cell suspensions were homogenized and centrifuged for 10 min at 800*g* at 4°C. The supernatants were collected and centrifuged at 100,000*g* for 60 min at 4°C. The resulting supernatants (cytosol fraction) were stored on ice and the pellets were resuspended and mixed in 2 ml of extraction buffer containing 1% triton X-100, placed on ice for 60 min, and centrifuged at 100,000*g* for 160 min at 4°C. The supernatants (membrane fraction) were collected. Cytosol and membrane fractions were diluted 4:1 (v/v) with 5 × sample buffer (1 × buffer containing 2% SDS, 10% glycerol, 0.0025% bromphenol blue, 62.5 mM Tris-HCl, pH 6.8). Protein concentrations of these samples were measured as described by Smith et al. [1985] using bovine serum albumin as standards. Samples were heated at 90°C for 5 min and 30 μg proteins each from membrane and cytosol fractions were separated on 9% SDS-PAGE and analyzed by Western blotting.

Statistics and Data Presentation

All results are presented as means ± S.E. of at least 5 separate experiments using different cell preparations. Comparisons were made using the unpaired Student's *t*-test or the analysis of variance. P values < 0.05 were considered significant.

RESULTS

Effect of PKC Inhibitors on Zymosan-induced Ca^{2+} Influx

In previous studies we demonstrated that stimulation of (1-3)-β-glucan receptors with particulate (1-3)-β-glucans such as zymosan induces a Ca^{2+} influx through receptor-operated channels in NR8383 AMs [Zhang et al., 1997; Ḿork et al., 1998a]. However, the mechanism regulating this Ca^{2+} influx is still unclear. In

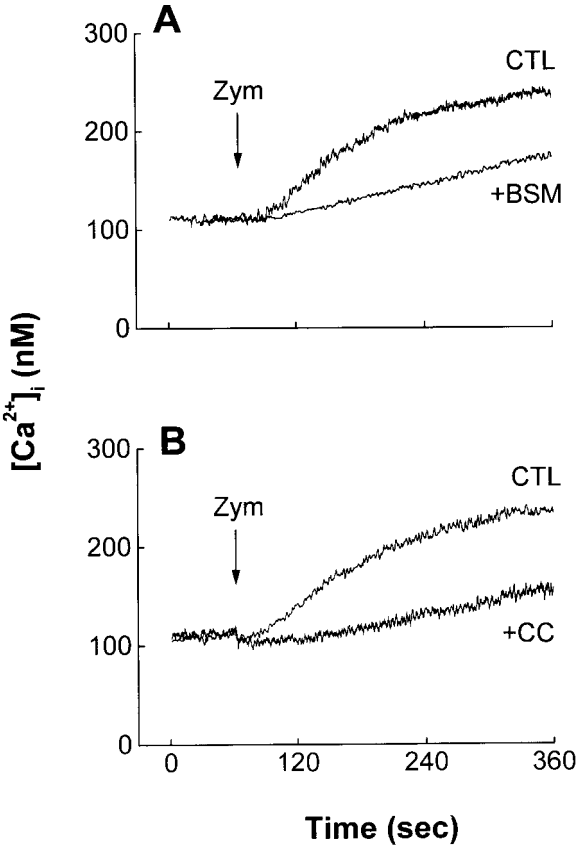

Fig. 1. Effects of PKC inhibitors on zymosan-induced $[Ca^{2+}]i$ Increase. NR8383 AMs were loaded with fura-2, resuspended in PSS (containing 1.2 mM Ca^{2+}), and $[Ca^{2+}]_i$ was measured. **A:** Effect of BSM. AMs were exposed to 100 nM BSM (+BSM) or the same volume of DMSO (CTL) for 30 min and stimulated with 200 μg/ml zymosan (Zym). **B:** Effects of CC. AMs were exposed to 500 nM CC (+CC) or the same volume of DMSO (CTL) for 30 min, then 200 μg/ml zymosan was added. Each trace is a representative response from separate experiments (A: CTL, n = 10; +BSM, n = 5. B: CTL, n = 10; +CC, n = 5).

several cell types, PKC is involved in the regulation of Ca^{2+} influx activated by depletion of intracellular stores [Petersen and Berridge, 1994; Mörk et al., 1998b]. In the present study, the possibility that PKC may participate in the regulation of the zymosan-activated Ca^{2+} influx was examined. As shown in Figure 1, stimulation of AMs with 200 μg/ml of zymosan resulted in a 119 ± 8 nM net increase in $[Ca^{2+}]_i$ at 5 min (n = 10). This increase was abolished by removal of extracellular Ca^{2+} (not shown), indicating the $[Ca^{2+}]_i$ increase is produced by Ca^{2+} influx. Consistent with previous studies, Ca^{2+} influx was also blocked by the receptor-operated Ca^{2+} channel inhibitor SK&F96365. To test whether PKC plays a role in the zymosan-stimulated Ca^{2+} influx, AMs were pre-incubated with PKC inhibitors, CC (500 nM) or BSM (100 nM) for 30 min [Kobayashi et al., 1989; Toullec et al., 1991]. Both treatments significantly reduced zymosan-induced $[Ca^{2+}]_i$ increase. The net increase was 61 ± 26 nM (n = 5; $P < 0.002$) and 54 ± 4 nM (n = 5; $P < 0.001$) at 5 min in BSM- and CC-treated cells, respectively (Fig.1). These results suggest that PKC plays a critical role in regulating zymosan-induced Ca^{2+} influx.

Since activation of PKC stimulates Ca^{2+} pump in Jurkat cells [Balasubramanyam and Gardner, 1995], the effect of BSM on Ca^{2+} pump function was measured in NR8383 AMs by determining the initial rate of $[Ca^{2+}]_i$ decline after ionomycin-induced Ca^{2+} peak. The initial rate was 104 ± 14 nM/min (n = 5) in control (DMSO-treated) AMs and 106 ± 14 nM/min (n = 5) in AMs pretreated with 100 nM BSM for 30 min. These results indicate that inhibition of PKC does not alter Ca^{2+} removal from the cytosol of NR8383 AMs and the decreased zymosan-induced Ca^{2+} influx by PKC inhibitors is independent of Ca^{2+} pump function.

Stimulation of Ca^{2+} Influx by PKC Activators

To confirm the role of PKC in mediating Ca^{2+} influx, PKC was directly activated by two potent membrane-permeable activators, PMA, a phorbol ester and DOG, an analog of DAG [Davis et al., 1985]. Both activators induced a concentration-dependent increase in $[Ca^{2+}]_i$, which was similar to zymosan-induced $[Ca^{2+}]_i$ increase. The net increases were 45 ± 5 nM, 66 ± 6 nM, and 134 ± 17 nM at 5 min when the cells were, respectively, exposed to 100, 200, and 500 nM PMA (all n = 5) (Fig. 2A). This response was abolished when the experiments were conducted in Ca^{2+}-free medium (Fig. 2B), indicating that the increase in $[Ca^{2+}]_i$ was due to Ca^{2+} influx. Exposure of the cells to 10 μM SK&F96365 for 0.5 min significantly reduced the $[Ca^{2+}]_i$ increase induced by 500 nM PMA (59 ± 3 nM at 5 min, n = 5, $P < 0.005$) (Fig. 2B).

Exposure of AMs to DOG activated a similar increase in $[Ca^{2+}]_i$. The net $[Ca^{2+}]_i$ increases were 43 ± 7 nM, 106 ± 12 nM, and 152 ± 12 nM at 5 min when the cells were respectively exposed to 10, 20, and 50 μM DOG (all n = 5) (Fig. 3A). This response was abolished by removal of extracellular Ca^{2+} (Fig. 3B). Pretreatment of the cells with 10 μM SK&F96365

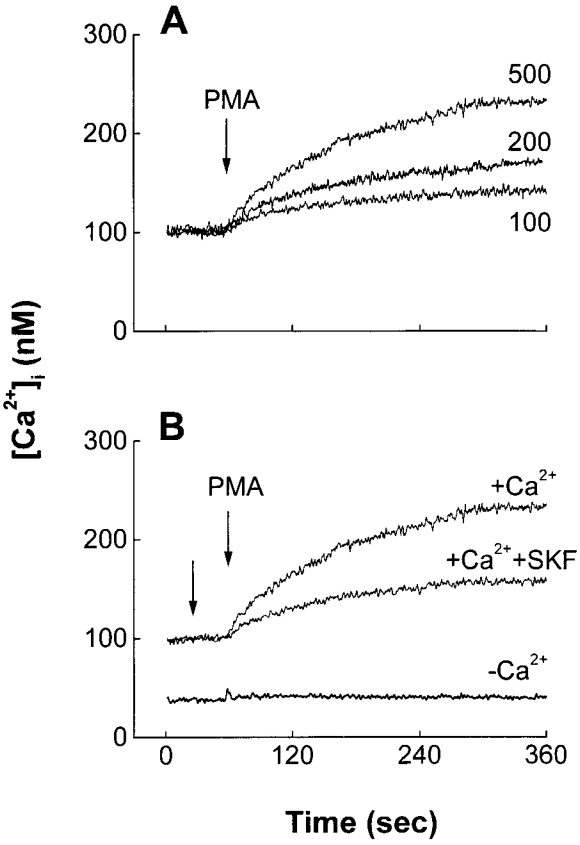

Fig. 2. $[Ca^{2+}]_i$ Changes Induced by PMA. **A:** Fura-2-loaded NR8383 AMs were suspended in PSS and $[Ca^{2+}]_i$ was measured. AMs were exposed to 100, 200, or 500 nM PMA at the time indicated by the arrow. **B:** Fura-2-loaded AMs were suspended in PSS and exposed to either DMSO ($+Ca^{2+}$) or 10 μM SK&F96365 ($+Ca^{2+}$+SKF) at the time indicated by the first arrow and followed by 500 nM PMA at the time indicated by the second arrow. Control cells ($-Ca^{2+}$) in Ca^{2+}-free PSS were stimulated with 500 nM PMA at the time indicated. Each trace is representative of separate experiments (A: 100, 200 and 500 nM PMA, n = 5 each; B: $+Ca^{2+}$, n = 5; $-Ca^{2+}$, n = 5; $+Ca^{2+}$ +SKF, n = 6).

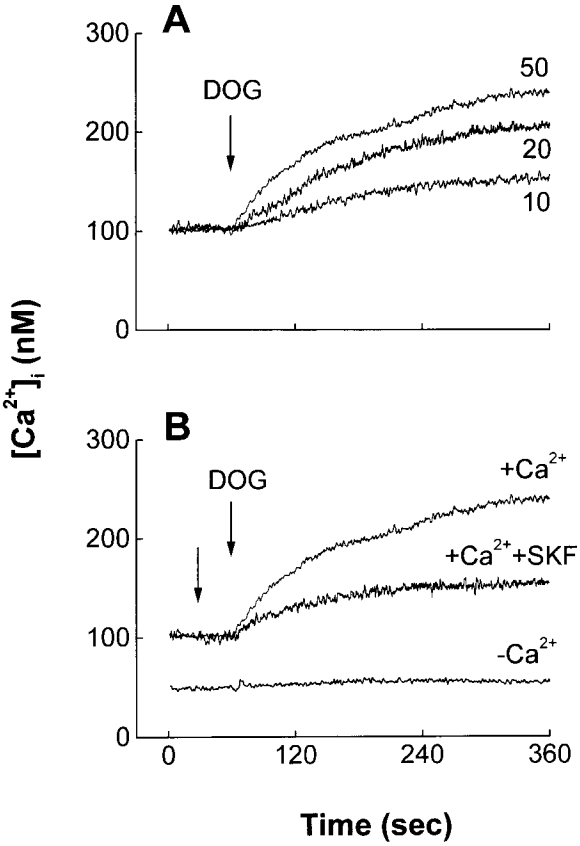

Fig. 3. $[Ca^{2+}]_i$ Changes Induced by DOG. **A:** Fura-2-loaded NR8383 AMs were suspended in PSS and $[Ca^{2+}]_i$ was measured. AMs were exposed to 10, 20, or 50 μM DOG at the time indicated by the arrow. **B:** Fura-2-loaded AMs were suspended in PSS and exposed to either DMSO ($+Ca^{2+}$) or 10 μM SK&F96365 ($+Ca^{2+}$+SKF) at the time indicated by the first arrow and followed by 50 μM DOG at the time indicated by the second arrow. Control cells ($-Ca^{2+}$) in Ca^{2+}-free PSS were stimulated with 50 μM DOG at the time indicated. Each trace is representative of separate experiments (A: 10, 20, and 50 μM DOG, n = 5 each; B: $+Ca^{2+}$, n = 5; $-Ca^{2+}$, n = 5; $+Ca^{2+}$ +SKF, n = 6).

significantly reduced the net $[Ca^{2+}]_i$ increase induced by 50 μM DOG (68 ± 5 nM at 5 min , n = 6; $P < 0.001$) (Fig. 3B).

The effect of PMA and DOG treatment on Ca^{2+} pump function was examined by measuring the initial rate of Ca^{2+} decline. Incubation with 100 nM PMA for 5 min did not affect the initial rate of $[Ca^{2+}]_i$ decline (115 ± 11 nM/min, n = 5; control, 104 ± 14 nM/min, n = 5). Similarly, treatment with DOG for 5 min did not alter the initial rate of Ca^{2+} decline (105 ± 6 nM/min, n = 5). Taken together, these results indicate that the Ca^{2+} influx pathway is activated by induction of PKC in NR8383 AMs.

Effect of PKC Inhibitors on PMA- and DOG-activated Ca^{2+} Influx

To examine whether PMA- and DOG-stimulated Ca^{2+} influx is also blocked by PKC inhibitors, NR8383 AMs were pre-incubated with 100 nM BSM for 30 min and the PMA- and DOG-induced Ca^{2+} influx was measured. The Ca^{2+} influx stimulated by PMA (500 nM) or DOG (50 μM) in control cells treated with vehicle (DMSO) for 30 min was 127 ± 15 nM (n = 5) and 138 ± 12 nM (n = 5) at 5 min, respectively (Fig. 4). Pretreatment with BSM significantly reduced PMA-induced $[Ca^{2+}]_i$ increase (49 ± 9 nM at 5 min, n = 6, $P < 0.002$) (Fig. 4A). Similarly, the

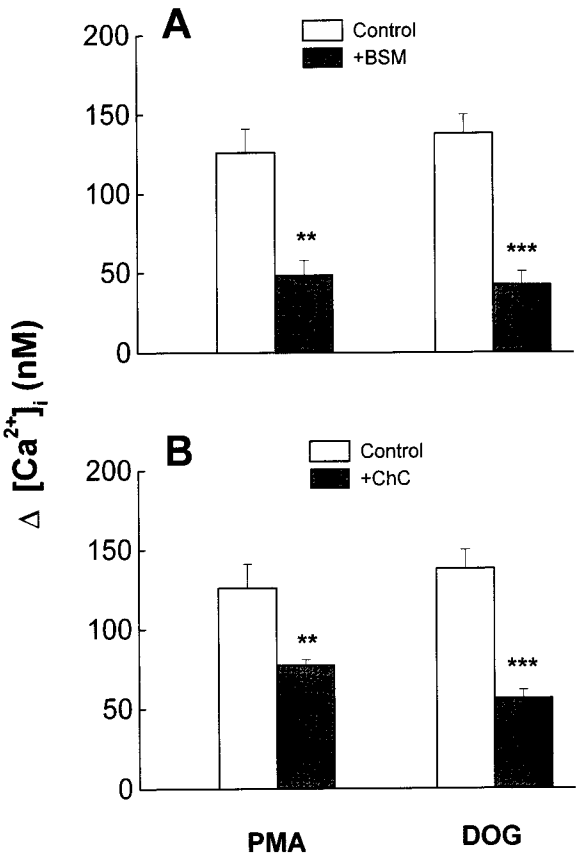

Fig. 4. Effects of PKC Inhibitors on PMA- and DOG-induced $[Ca^{2+}]_i$ Increase. NR8383 AMs were loaded with fura-2, pre-incubated with 100 nM BSM (+BSM) (**A**) or 10 μM ChC (+ChC) (**B**) for 30 min, then exposed to 500 nM PMA or 50 μM DOG for 5 min. Control cells were pre-incubated with the same volume of vehicle (DMSO). The values are means ± S.E. of separate experiments (PMA: control, n = 6; +BSM, n = 6; + ChC, n = 5; DOG: control, n = 5; +BSM, n = 6; +ChC, n = 5).

DOG-induced $[Ca^{2+}]_i$ increase was significantly decreased by BSM (43 ± 8 nM at 5 min, n = 6; $P < 0.001$) (Fig. 4A).

This effect was confirmed using another specific PKC inhibitor ChC [Herbert et al., 1990]. Pretreatment with 10 μM ChC for 30 min significantly attenuated the $[Ca^{2+}]_i$ increase activated by PMA and DOG. The net $[Ca^{2+}]_i$ increase in response to PMA and DOG was 78 ± 3 nM (n = 5, $P < 0.01$) and 57 ± 7 nM (n = 5, $P < 0.001$) at 5 min, respectively (Fig. 4B).

Expression of PKC Isoforms in NR8383 AMs

The expression of PKC isoforms in NR8383 AMs has not been previously studied. We identified the PKC isoforms in this cell line by Western blotting using specific antibodies. As shown in Figure 5, one new PKC isoform (δ) and two atypical PKC isoforms (λ and ι) were present in these cells. On the other hand, no conventional PKC isoforms were expressed. This conclusion was based on the fact that positive controls were clearly shown for each corresponding PKC isoforms in each experiment, but no band was detectable in the samples (Fig. 5).

Translocation of PKC Isoforms

The activation of PKC requires PKC protein translocation from the cytosol to membrane. Therefore, we tested the effects of zymosan on the translocation of the PKC isoforms δ, ι, and λ. Following zymosan stimulation, PKC isoforms λ and ι were not demonstrated in the membrane fraction of NR8383 AMs. In contrast, the amount of PKC-δ in the membrane fraction was increased in a time-dependent manner following zymosan stimulation (Fig. 6). These results indicate that stimulation of the (1-3)-β-glucan receptor activates translocation of PKC-δ isoform and the PKC-dependent Ca^{2+} influx is likely regulated through this PKC isoform.

Effect of Genistein on PKC-δ Translocation

To elucidate the mechanism of PKC activation, we tested the possibility that PTKs activate PKC. As shown in Figure 7, pretreatment of NR8383 AMs for 30 min with 20 μM genistein, a specific inhibitor of PTKs [Akiyama et al., 1987], significantly reduced the zymosan-induced PKC-δ translocation from cytosol to membrane fraction, suggesting that inhibition of PTKs blocks (1-3)-β-glucan-dependent PKC activation.

Similarly, genistein treatment also inhibited Ca^{2+} influx stimulated by zymosan. Zymosan-stimulated Ca^{2+} influx was 118 ± 20 nM at 5 min in control AMs incubated with vehicle (DMSO) for 30 min (n = 6) and 12 ± 4 nM at 5 min in AMs pretreated with 20 μM genistein for 30 min (n = 5, $P < 0.001$).

DISCUSSION

The present study demonstrates that in NR8383 AMs, Ca^{2+} influx activated by (1-3)-β-glucan receptor stimulation is regulated by PKC. We have previously demonstrated that this Ca^{2+} influx is not affected by inhibitors of voltage-gated Ca^{2+} channels including L-, N-,

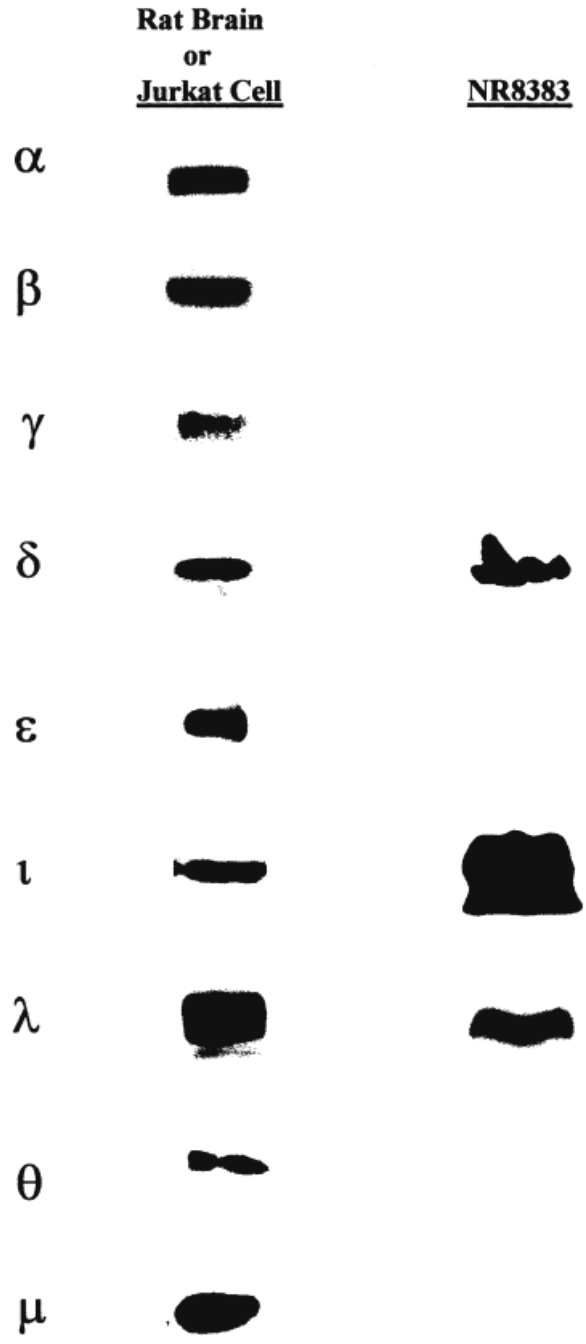

Fig. 5. PKC Isoforms Expressed in NR8383 AMs. NR8383 AMs were lysed and extracted as described in Materials and Methods. The samples were subjected to PAGE-immunoblot analysis using specific antibodies against PKCα, β, γ, δ, ϵ, θ, μ, ι, and λ, separately. Positive controls were from rat brain (α, β, δ, γ, ϵ, θ, and λ) or Jurket cells (θ and μ) (Transduction Laboratories, Lexington, KY).

P-, and T-type voltage-dependent channels [Zhang et al., 1997], but is inhibited by the blocker of receptor-operated Ca^{2+} channels, SK&F96365 [Zhang et al., 1997; Mörk et al., 1998a]. The regulation by PKC is demonstrated by three sets of experiments. First, PKC inhibitors, BSM and CC, significantly inhibited Ca^{2+} influx activated by the (1-3)-β-glucan receptor agonist zymosan. Second, the Ca^{2+} influx induced by PKC activators, PMA and DOG, was comparable to zymosan-induced Ca^{2+} influx. Furthermore, PKC inhibition or Ca^{2+} channel blocker SK&F96365 also inhibited the PMA- and DOG-induced Ca^{2+} influx. Therefore, PKC regulation of Ca^{2+} influx occurs through similar receptor-operated channels and is not mediated by the store-operated Ca^{2+} entry (SOCE) pathway since Ca^{2+} stores were not depleted under these conditions.

Previous studies reported that PKC activation reduced Ca^{2+} influx and enhanced Ca^{2+} efflux in neutrophils and the murine macrophage cell line PU5-1.8 [McCarthy et al., 1989; Kong et al., 1993]. In contrast, the present study demonstrates that activation of PKC is a requisite for the opening of (1-3)-β-glucan-operated Ca^{2+} channels. This discrepancy may be due to different Ca^{2+} channels being investigated in these studies. The Ca^{2+} influx observed by McCarthy et al. [1989] and Kong et al. [1993] was SOCE, activated by depletion of intracellular Ca^{2+} pools [Putney, 1990; Parekh and Penner, 1997]. A regulatory action by PKC on SOCE has been demonstrated in a number of cells [Gaur et al., 1996; Sakai and Ambudkar, 1997; Sugita et al., 1999] through phosphorylation of the SOCE proteins, Drosophila transient receptor potential (Trp) proteins [Huber et al., 1998]. PKC also regulates other types of Ca^{2+} channels in a variety of cells. L-type voltage-dependent Ca^{2+} channels are inhibited by activation of PKC in adrenal chromaffin cells [Sena et al., 1999] and PKC-δ mediates ethanol-induced up-regulation of L-type Ca^{2+} channels in PC12 cells [Gerstin et al., 1998]. Maintenance of Ca^{2+} current through N-type voltage-dependent Ca^{2+} channels in rat dorsal root ganglion neurons also requires endogenous PKC [King et al., 1999]. Therefore, the regulation of Ca^{2+} channels by PKC is cell type- and channel type-dependent.

Expression of PKC isoforms in NR8383 AMs has not been studied previously. Our data provide the first evidence that NR8383 AMs ex-

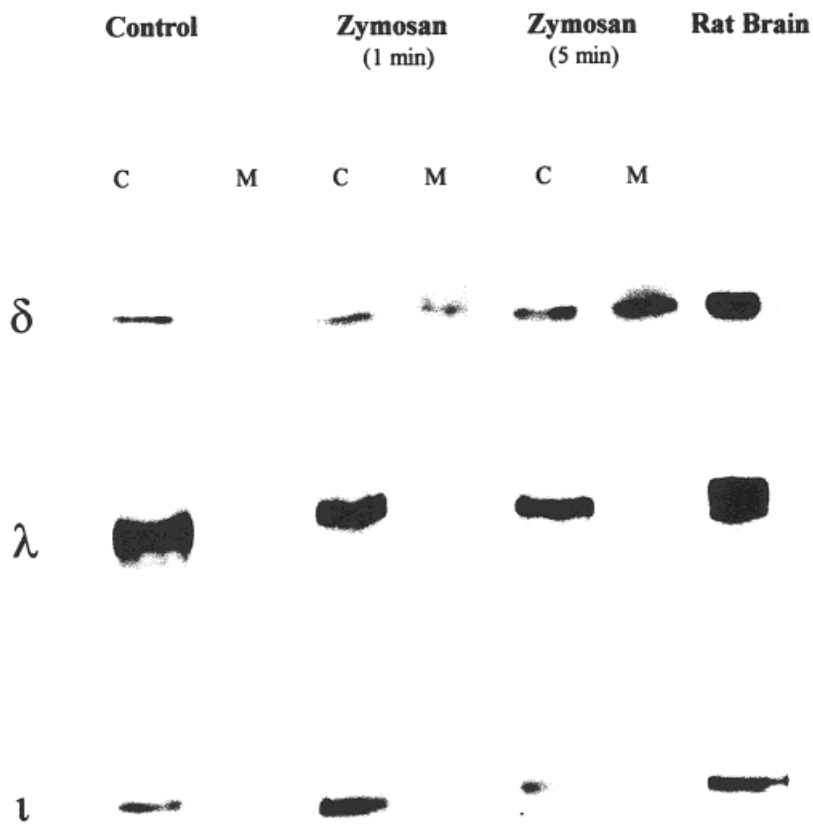

Fig. 6. Zymosan-stimulated Translocation of PKC Isoforms. NR8383 AMs were stimulated with 200 μg/ml of zymosan for 1 and 5 min. Control cells were treated with the same volume of PSS. Cytosol (C) and membrane (M) fractions were isolated and proteins were separated by PAGE. The distribution of PKC isoforms expressed in these cells (δ, ι and λ) in the cytosol and membrane fractions was analyzed by immunoblotting using specific antibodies.

press three PKC isoforms and only PKC-δ is activated by stimulation of (1-3)-β-glucan receptors with zymosan. A translocation of PKC-δ isoform from the cytosol to the membrane fraction occurred after zymosan stimulation, whereas the other two isoforms, PKC-ι and λ were not activated. These results strongly suggest that PKC-δ isoform is functionally associated with (1-3)-β-glucan receptors and this isoform regulates Ca^{2+} influx. Since the other two isoforms (ι and λ) present in NR8383 AMs are classified as atypical PKCs, their function and activation mechanism are still unknown. However, the expression of these atypical PKC isoforms appears not to be Ca^{2+} or DAG-dependent.

The activation of PKC in the NR8383 AM cell line is still not clear. We have shown that PKC-δ translocation is inhibited by genistein (Fig.7), indicating that PKC activation is PTK-dependent. The zymosan-activated Ca^{2+} influx was also significantly reduced by inhibition of PTKs. These results indicate that PTKs are upstream elements in the signal transduction pathway. It has been reported that zymosan stimulation induces tyrosine phosphorylation of endogenous proteins from 28 to 120-kDa, which is reduced by PTK inhibition in human monocytes [Sanguedolce et al., 1993]. Zymosan also activates PKC through activation of PTKs-dependent phospholipase C in murine macrophages [Goldman et al., 1994]. Since zymosan does not activate phospholipase C in NR8383 AMs [Zhang et al., 1997], PKC is probably not activated by DAG produced through the classical phosphoinositide cascade. Two classes of PTKs exist, receptor and non-receptor PTKs. The former is plasma membrane associated and activated by ligation of specific receptors such as growth factor receptors. The latter is comprised of cytosolic enzymes whose activation is still undefined. Obviously, elucidation of the (1-3)-β-glucan-activated PTK forms is of significant importance.

In summary, the present study demonstrates that stimulation of (1-3)-β-glucan receptors on NR8383 AMs activates PKC-dependent Ca^{2+} influx and translocation of the PKC-δ isoform. Ca^{2+} influx and PKC-δ translocation were also found to be PTK-dependent. Although the underlying mechanisms activating PKC and the characteristics of PTKs asso-

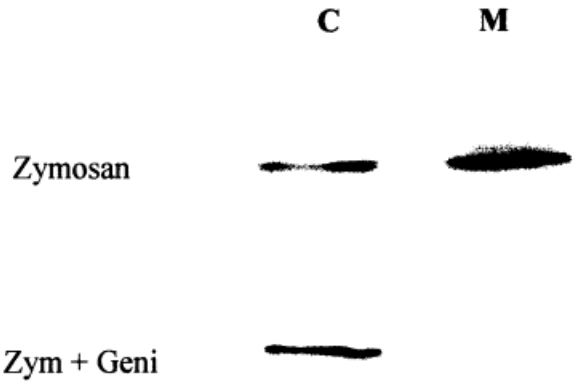

Fig. 7. Effect of Genistein on Zymosan-stimulated PKC-δ Translocation. NR8383 AMs were pretreated with 20 μM genistein (Zym + Geni) or the same volume of DMSO (Zymosan) for 30 min, and then stimulated with 200 μg/ml zymosan for 5 min. The cytosol (C) and membrane (M) fractions were isolated and proteins were separated by PAGE. Translocation of PKC-δ from the cytosol fraction to the membrane fraction was analyzed by Western blotting using specific antibody to PKC-δ.

ciated with (1-3)-β-glucan receptors need further investigation, PKC activation is a critical element in the signal transduction pathway regulating Ca^{2+} influx.

ACKNOWLEDGMENTS

Dr. Ann-Christin Mörk was supported by Swedish Medical Research Council Postdoctoral Fellowship K97-04PK-12291.

REFERENCES

Abel G, Czop JK. 1992. Stimulation of human monocyte β-glucan receptors by glucan particles induces production of TNF-α and IL-1β. Int J Immunopharmacol 14: 1363–1373.

Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe S-I, Itoh N, Shibuya M, Fukami Y. 1987. Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 262: 5592–5595.

Balasubramanyam M, Gardner JP. 1995. Protein kinase C modulates cytosolic free calcium by stimulating calcium pump activity in Jurkat T cells. Cell Calcium 18:526–541.

Bates PJ, Ralston NV, Vuk-Pavlvovic Z, Rohrbach MS. 1995. Calcium influx is required for tannin-mediated arachidonic acid release from alveolar macrophages. Am J Physiol 268:L33–L40.

Chakravarthy BR, Tremblay R, MacDonald P, Krsmanovic V, Whtitfield JF, Durkin JP. 1992. The activation of inactive membrane-associated protein kinase C is associated with DMSO-induced erythroleukemia cell differentiation. Biochim Biophys Acta 1136:83–90.

Czop JK, Kay J. 1991. Isolation and characterization of β-glucan receptors on human mononuclear phagocytes. J Exp Med 173:1511–1520.

Daum T, Rohrbach MS. 1992. Zymosan induces selective release of arachidonic acid from rabbit alveolar macrophages via stimulation of a β-glucan receptor. FEBS Lett 309:119–122.

Davis RJ, Ganong BR, Bell RM, Czech MP. 1985. *sn*-1,2-Dioctanoylglycerol. A cell-permeable diacylglycerol that mimics phorbol diester action on the epidermal growth factor receptor and mitogenesis. J Biol Chem 260:1562–1566.

Engstad RE, Robertsen B. 1994. Specificity of a β-glucan receptor on macrophages from Atlantic salmon (*Salmo salar* L.). Dev Comp Immunol 18:397–408.

Gaur S, Yamaguchi H, Goodman HM. 1996. Growth hormone increases calcium uptake in rat fat cells by a mechanism dependent on protein kinase C. Am J Physiol 270:C1485–C1492.

Gerstin EH Jr, McMahon T, Dadgar J, Messing RO. 1998. Protein kinase C-δ mediates ethanol-induced up-regulation of L-type calcium channels. J Biol Chem 273:16409–16414.

Goldman R. 1988. Characteristics of the β-glucan receptor of murine macrophages. Exp Cell Res 174:481–490.

Goldman R, Ferber E, Meller R, Zor U. 1994. A role of reactive oxygen species in zymosan and β-glucan induced protein tyrosine phosphorylation and phospholipase A2 activation in murine macrophages. Biochim Biophys Acta 1222:265–276.

Grynkiewicz G, Poenie M, Tsien RY. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450.

Helmke RJ, Boyd RL, German VF, Mangos JA. 1987. From growth factor dependence to growth factor responsiveness: the genesis of an alveolar macrophage cell line. In Vitro Cell Dev Biol 23:567–574.

Helmke RJ, German VF, Mangos JA. 1989. A continuous alveolar macrophage cell line: Comparison with freshly derived alveolar macrophages. In Vitro Cell Dev Biol 25:44–48.

Herbert JM, Angereau JM, Gleye J, Maffrand JP. 1990. Chelerythrine is a potent specific inhibitor of protein kinase C. Biochem Biophys Res Commun 172:993–999.

Hoyal CR, Gozal E, Zhou H, Foldenauer K, Forman HJ. 1996. Modulation of the rat alveolar macrophage respiratory burst by hydroperoxides is calcium dependent. Arch Biochem Biophys 326:166–171.

Huber A, Sander P, Bahner M, Paulsen R. 1998. The Trp Ca^{2+} channel assembled in a signaling complex by the PDZ domain protein INAD is phosphorylated through the interaction with protein kinase C (ePKC). FEBS Lett 425:317–322.

Huwiler A, Pfeilschifter J. 1993. A role of protein kinase C-α in zymosan-stimulated eicosanoid synthesis in mouse peritoneal macrophages. Eur J Biochem 217:69–75.

King AP, Hall KE, MacDonald RL. 1999. *kapp*- and *mu*-Opioid inhibition of N-type calcium currents is attenuated by 4β-phorbol 12-myristate 13-acetate and protein kinase C in rat dorsal root ganglion neurons. J Pharmacol Exp Ther 289:312–320.

Kobayashi E, Nakano H, Morimoto M, Tamaoki T. 1989. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 159:548–553.

Kong SK, Choy YM, Lee CY. 1993. Protein kinase C as a multi-targeted feedback inhibitor regulating the Ca^{2+} responses to chemotactic peptide stimulation in the murine macrophage cell line PU5-1.8. Biol Signals 2:84–94.

Lehmann MH, Berg H. 1998. Interleukin-10 expression is induced by increase of intracellular calcium levels in the monocytic cell line U937. Pflügers Arch 435:868–870.

McCarthy SA, Hallam TJ, Merritt JE. 1989. Activation of protein kinase C in human neutrophils attenuates agonist-stimulated rises in cytosolic free Ca^{2+} concentration by inhibiting bivalent-cation influx and intracellular Ca^{2+} release in addition to stimulating Ca^{2+} efflux. Biochem J 264:357–364.

Mörk A-C, Helmke RJ, Martinez JR, Michalek MT, Patchen ML, Zhang GH. 1998a. Effects of particulate and soluble (1-3)-β-glucans on Ca^{2+} influx in NR8383 alveolar macrophages. Immunopharmacology 40:77–89.

Mörk A-C, Zhang GH, Martinez JR. 1999b. Modulation of Ca^{2+} mobilization by protein kinase C in rat submandibular acinar cells. J Cell Biochem 72:47–55.

Parekh AB, Penner R. 1997. Store depletion and calcium influx. Physiol Rev 77:901–930.

Petersen CCH, Berridge MJ. 1994. The regulation of capacitative calcium entry by calcium and protein kinase C in *Xenopus* oocytes. J Biol Chem 269:32246–32253.

Putney Jr JW. 1990. Capacitative calcium entry revisited. Cell Calcium 11:611–624.

Sakai T, Ambudkar IS. 1997. Role for protein kinases in Ca^{2+}-dependent feedback modulation of divalent cation influx internal-Ca^{2+}-store-dependent rat parotid gland cells. Pflügers Arch 433:464–471.

Sanguedolce MV, Capo C, Bouhamdan M, Bongrand P, Huang C-K, Mege J-L. 1993. Zymosan-induced tyrosine phosphorylation in human monocytes. Role of protein kinase C. J Immunol 151:405–414.

Schondorf M, Bidlingmaier F, von Ruecker AA. 1993. Protein kinase C regulates IL-8 and fMLP induced cytoplasmic Ca^{2+} increase in human granulocytes by receptor modulation measurements by flow cytometry. Biochem Biophys Res Commun 197:549–555.

Sena CM, Santos RM, Boarder MR, Rosario LM. 1999. Regulation of Ca^{2+} influx by protein kinase C activator in chromaffin cells: differential role of P/Q- and L-type Ca^{2+} channels. Eur J Pharmacol 366:281–292.

Smith PK, Krohn RI, Hermanson Gtmalla AK, Gartner FM, Provenzamo MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using biconchoninic acid. Anal Biochem 150:76–85.

Sugita K, Mörk A-C, Zhang GH, Martinez JR. 1999. Modulation of Ca^{2+} mobilization by protein kinase C in the submandibular duct cell line A253. Mol Cell Biochem 198:39–46.

Sun XH, Martinez JR, Zhang GH. 1999. Inhibition of Ca^{2+} influx by pentoxifylline in NR8383 alveolar macrophages. Immunopharmacology 43:47–58.

Szabó T, Kadish JL, Czop JK. 1995. Biochemical properties of the ligand-binding 20-kDa subunit of the β-glucan receptors on human mononuclear phagocytes. J Biol Chem 270:2145–2151.

Tapper H, Sundler R. 1995. Protein kinase C and intracellular pH regulate zymosan-induced lysosomal enzyme secretion in macrophages. J Leukoc Biol 58:485–494.

Terzian AR, Rubin RP. 1993. Translocation of the α-isozyme of protein kinase C during stimulation of rat parotid acinar cells by phorbol ester and carbachol. Archs Oral Biol 38:1051–1056.

Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet V, Boissin P, Boursier E, Loriolle F. 1991. The bisindolymaleimide GF 109203 is a potent and selective inhibitor of protein kinase C. J Biol Chem 266:15771–15781.

Tuominen H, Leino L, Akerman KE. 1994. Does protein kinase C regulate receptor agonists-mediated elevation in the cytosolic Ca^{2+} in human neutrophils? Biochem Biophys Res Commun 203:998–1004.

Vecchiarelli A, Dottorini M, Cociani C, Pietrella D, Todisco T, Bistoni F. 1993. Mechanism of intracellular candidacidal activity mediated by calcium ionophore in human alveolar macrophages. Am J Respir Cell Mol Biol 9:19–25.

Xie YC, Schafer R, Barnett JB. 1997. Inhibitory effect of 3,4-dichloropropinaniline on cytokine production by macrophages is associated with LPS-mediated signal transduction. J Leukoc Biol 61:745–752.

Zhang GH, Helmke RJ, Mörk A-C, Martinez JR. 1997. Regulation of cytosolic free Ca^{2+} in cultured rat alveolar macrophages (NR8383). J Leukoc Biol 62:341–348.

Zheleznyak A, Brown EJ. 1992. Immunoglobulin-mediated phagocytosis by human monocytes requires protein kinase C activation. Evidence for protein kinase C translocation to phagosomes. J Biol Chem 267:12042–12048.

Zhu Z, Bao Z, Li J. 1995. MacMARCKS mutation blocks macrophage phagocytosis of zymosan. J Biol Chem 270: 17652–17655.