Phorbol Ester-Induced Actin Assembly in Neutrophils: Role of Protein Kinase C

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Abstract. The shape changes and membrane ruffling that accompany neutrophil activation are dependent on the assembly and reorganization of the actin cytoskeleton, the molecular basis of which remains to be clarified. A role of protein kinase C (PKC) has been postulated because neutrophil activation, with the attendant shape and membrane ruffling changes, can be initiated by phorbol esters, known activators of PKC. It has become apparent, however, that multiple isoforms of PKC with differing substrate specificities exist. To reassess the role of PKC in cytoskeletal reorganization, we compared the effects of diacylglycerol analogs and of PKC antagonists on kinase activity and on actin assembly in human neutrophils. Ruffling of the plasma membrane was assessed by scanning EM, and spatial redistribution of filamentous (F)-actin was assessed by scanning confocal microscopy. Staining with NBD-phallacidin and incorporation of actin into the Triton X-100–insoluble ("cytoskeletal") fraction were used to quantify the formation of (F)-actin. [^{32}P]ATP was used to detect protein phosphorylation in electroporated cells. Exposure of neutrophils to 4β-PMA (an activator of PKC) induced protein phosphorylation, membrane ruffling, and assembly and reorganization of the actin cytoskeleton, whereas the $4a$-isomer, which is inactive towards PKC, failed to produce any of these changes. Moreover, 1,2-dioctanoylglycerol, mezerein, and 3-(N-acetylamino)-5-(N-decyl-N-methylamino)-benzyl alcohol, which are nonphorbol activators of PKC, also promoted actin assembly. Although these effects were consistent with a role of PKC, the following observations suggested that stimulation of conventional isoforms of the kinase were not directly responsible for actin assembly: (a) Okadaic acid, an inhibitor of phosphatases 1 and 2A, potentiated PMA-induced protein phosphorylation, but not actin assembly; and (b) PMA-induced actin assembly and membrane ruffling were not prevented by the conventional PKC inhibitors 1-(5-isoquinolinesulfonyl)-2-methylpiperazine, staurosporine, calphostin C, or sphingosine at concentrations that precluded PMA-induced protein phosphorylation and superoxide production. On the other hand, PMA-induced actin assembly was inhibited by long-chain fatty acid coenzyme A esters, known inhibitors of nuclear PKC (nPKC). We conclude that PMA-induced actin assembly is unlikely to be mediated by the conventional isoforms of PKC, but may be mediated by novel isoforms of the kinase such as nPKC.

E XPOSURE of neutrophils to a variety of agents including chemotactic factors and phorbol esters initiates a coordinated series of biological responses. Several of these responses, including shape change, projection of membrane ruffles and pseudopodia, cell motility, and phagocytosis, are dependent on the mechanical displacement of part or all of the cell. This in turn requires the assembly and reorganization of cytoskeletal microfilaments, which are composed primarily of actin (reviewed by Stossel, 1989). Actin also plays a major structural role in resting and activated neutrophils (Worthen et al., 1989). Despite the importance of actin-containing microfilaments, the molecular basis of the processes that signal their assembly and reorganization

remains to be clarified (see reviews by Korn, 1985; Omann et al., 1987; Sha'afi and Molski, 1988).

It is known that actin assembly induced by receptor-mediated stimuli such as N-formyl-methionyl-leucyl-phenylalanine (fMLP)[1] and leukotriene B$_4$ is transduced through GTP-binding regulatory proteins (G-proteins): Pretreatment with pertussis toxin inhibits ligand-induced actin assembly (Yassin et al., 1985) and direct activation of G-proteins by GTPγS or fluoroaluminate in permeabilized cells results in actin assembly (Downey et al., 1989; Therrien and Naccache, 1989; Bengtsson et al., 1990). By comparison, the nature of the

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1. *Abbreviations used in this paper:* DAG, diacylglycerol; DiC$_8$, 1,2-dioctanoylglycerol; KRPD, Kreb's Ringer's Phosphate Dextrose buffer; F-actin, filamentous actin; fMLP, N-formylmethionyl-leucyl-phenylalanine; H7, 1-(5-isoquinolinesulfonyl)-2-methylpiperizine; nPKC, novel PKC; PKC, protein kinase C; RFI, relative fluorescence index.

events that follow activation of the G-proteins is much less clear. Membrane phosphoinositides can interact directly with actin-binding and sequestering proteins such as profilin (Lassing and Lindberg, 1985; Stossel, 1989) and gelsolin (Janmey and Stossel, 1989), and it has been postulated that this interaction leads to the dissociation of free actin monomers, which can then assemble into microfilaments (reviewed by Stossel, 1989). However, other studies have questioned this hypothesis (Bengtsson et al., 1988). Thus, the nature of the events that signal actin assembly and reorganization remains to be more precisely defined.

In activated neutrophils, protein kinase C (PKC) is stimulated by accumulation of diacylglycerol (DAG), formed upon breakdown of membrane phospholipids by phospholipase C (Nishizuka, 1984) or by the sequential effects of phospholipase D and phosphatidate phosphohydrolase (reviewed by Billah and Anthes, 1990). PKC stimulation follows activation of G-proteins by the receptor–ligand complex and is thought to be involved in various aspects of neutrophil responsiveness, including superoxide generation by the NADPH oxidase (Castagna et al., 1982), release of enzymes from granules (White et al., 1984), and activation of the Na^+/H^+ antiporter (Grinstein and Furuya, 1986). The evidence for involvement of PKC in these latter pathways stems mainly from their susceptibility to activation by exogenous DAG and phorbol esters such as PMA, and their inhibition by agents such as staurosporine and 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) (Dewald et al., 1989; Bass et al., 1987; however, see Badwey et al., 1989, and Sha'afi et al., 1988, for alternative views), which are potent PKC activators and inhibitors, respectively. The role of PKC activation in signaling changes in the microfilament lattice is less clear. It is known that exposure of cells to phorbol esters or DAG results in pronounced alterations in the content and organization of filamentous actin (F-actin) in a variety of cell types (Grant and Aunis, 1990; Miegs and Wang, 1986; Roos et al., 1987; Sheterline et al., 1986). In leukocytes, for example, exposure to phorbol esters induces characteristic membrane ruffling, which is thought to be dependent on changes in the underlying microfilaments, and an increase in the amount and redistribution of F-actin (Howard and Wang, 1987; Phatak et al., 1988; Sheterline et al., 1986; Roos et al., 1987; Yassin et al., 1985; Apgar, 1991).

The well-documented elevation in the cellular level of DAG upon stimulation, together with the ability of phorbol esters to induce actin assembly, strongly suggests that microfilament remodeling in activated cells may be mediated by PKC. Recent observations, however, have clouded this interpretation. Sha'afi et al. (1988) observed that PMA-induced actin assembly in neutrophils was not inhibited by the PKC inhibitor H7, raising the possibility that PMA was acting through a pathway not involving PKC. However, it has become apparent recently that multiple isoforms of PKC exist that have different substrate specificities and are differentially regulated. The predominant isoform of PKC present in neutrophils is β-PKC, which is known to be sensitive to the conventional inhibitors of PKC, including staurosporine and H7. However, neutrophils also contain nPKC, an isoform that is calcium independent (Majumdar et al., 1991) and insensitive to the conventional PKC inhibitors (Gschwendt et al., 1989). Furthermore, the β- and n-isoforms of PKC have different substrate specificities (Majumdar et al., 1991), rais-

ing the possibility that divergent signaling pathways may be affected by selective activation of the various isoforms. We therefore considered the possibility that the reported effects of phorbol esters on the actin cytoskeleton may be mediated by activation of one of the novel isoforms of PKC, such as nuclear PKC (nPKC), or by mechanisms independent of PKC.

To clarify the relationship between stimulation of PKC, protein phosphorylation, and the assembly and spatial reorganization of the actin cytoskeleton, we have reexamined the effects of phorbol esters in intact and electropermeabilized human neutrophils. Permeabilized cells were used in order to ensure that PKC inhibitors had access to the cytosol and to enable the use of [^{32}P]ATP for the study of protein phosphorylation, a procedure that greatly reduces background phosphorylation. Electroporation was chosen as the permeabilization method because cells subjected to this procedure retain responsiveness to physiological ligands such as chemoattractants (Grinstein and Furuya, 1988; Downey and Grinstein, 1989). We report that phorbol esters and related activators induce actin assembly in human neutrophils, and that these alterations in the actin cytoskeleton may be mediated by one of the novel PKC isoforms such as nPKC.

Materials and Methods

Reagents

Percoll was obtained from Pharmacia Chemicals (Montreal, P.Q., Canada). Reagents for Krebs Ringer's phosphate dextrose buffer were obtained from Mallinckrodt Inc., (Paris, KY). Superoxide dismutase, ferricytochrome c, EGTA, fMLP, Hepes and ATP (K^+ salt), coenzyme A, were obtained from Sigma Chemical Co. (St. Louis, MO). NBD-phallacidin and rhodamine-phalloidin were obtained from Molecular Probes (Eugene, OR), lyso-phosphatidylcholine from Avanti Polar Lipids (Pelham, AL); H7, staurosporine, DiC$_8$, L-sphingosine, and diisopropylfluorophosphate from Calbiochem (San Diego, CA); calphostin C from Kamiya Biomedical Company (Thousand Oaks, CA), mezerein, 3-(N-acetylamino)-5-(N-decyl-N-methylamino)-benzyl alcohol (ADMB), 4α- and 4β-PMA from LC Services Corp. (Woburn, MA); palmitic acid, myristic acid, oleic acid, palmitoyl-CoA, oleoyl-CoA, myristoyl-CoA, from Sedary Research Laboratories Inc. (London, Ontario, Canada); [γ-^{32}P]ATP from Amersham Canada Ltd. (Oakville, Ontario).

Cell Isolation

Human neutrophils (>98% pure) were isolated from citrated whole blood obtained by venipuncture, using dextran sedimentation and discontinuous plasma–Percoll gradients, as previously described in detail. The separation procedure required 2 h, and the cells were used immediately after isolation for the experiments described. The functional integrity and nonactivated state of neutrophils isolated in this manner have been extensively validated in previous publications (Haslett et al., 1985).

Permeabilization Procedure

Neutrophils were permeabilized by electroporation essentially as described (Grinstein and Furuya, 1988). Briefly, 10^7 cells were sedimented and resuspended in 1 ml of ice-cold permeabilization medium (140 mM KCl, 1 mM MgCl$_2$, 10 mM glucose, 1 mM ATP, 10 mM Hepes, pH 7.0, 1 mM EGTA, and 0.193 mM CaCl$_2$ to give a final free Ca^{2+} concentration of 100 nM, calculated as described by Fabiato and Fabiato (1979). Aliquots of this suspension (0.8 ml) were transferred to a Pulser cuvette (BioRad, Mississauga, Ontario) and subjected to two discharges of 2 kV from a 25-μF capacitor using the Bio-Rad Gene Pulser. The cells were quickly sedimented and resuspended in fresh ice-cold permeabilization medium between pulses. Finally, the cells were equilibrated for 30 s in the indicated medium at 37°C prior to stimulation and measurement of actin assembly.

Measurement of F-Actin

Neutrophil content of polymerized F-actin was determined by NBD-phallacidin staining using the methanol extraction method of Howard and Oresajo (1987). For cells that were electropermeabilized, the method of Howard and Meyer (1984) using flow cytometry was utilized, as we have found (data not shown) that the methanol extraction method is less sensitive under these conditions. These fluorescence methods have been shown to correlate well with biochemical measurements of F-actin (Howard and Meyer, 1984; Wallace et al., 1984). All data are reported as the ratio of the fluorescence of the stimulated cell population to that of the control cells, or the relative fluorescence index (RFI).

Superoxide Production

Production of superoxide was measured spectroscopically by the superoxide dismutase–inhibitable reduction of ferricytochrome c (e.g., Dewald et al., 1989), using an extinction coefficient of 21.1 mM^{-1} cm^{-1}.

Triton X-100 Extraction, SDS-PAGE, and Immunoblotting

For Triton X-100 extraction (White et al., 1983), 2×10^6 cells were sedimented and resuspended in Triton-PHEM buffer (0.75% Triton X-100, 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$) containing 1 mM phenylmethylsulfonylfluoride, 20 μg/ml leupeptin, 80 μg/ml aprotinin, and 156 μg/ml benzamidine at 4°C, and allowed to incubate on ice for 20 min. Next, the insoluble fraction was sedimented in a microfuge by spinning at 12,000 g for 5 min. The supernatant (soluble fraction) was removed, and the pellet resuspended in 150 μL of boiling 2% SDS sample buffer, boiled for an additional 10 min, and analyzed by SDS-PAGE. 15 μL of each sample and molecular weight standards were then subjected to PAGE in the presence of SDS on 10% gels by the method of Laemmli (1970) using the LKB Midget Gel electrophoresis system. Gels were stained using Coomassie blue and dried overnight using Bio-Gel Wrap (BioDesign Inc., NY). For immunoblotting, following electrophoresis the samples and the molecular weight standards were electrophoretically transferred onto nitrocellulose paper for 40 min at 0.8 mA/cm^2 using the LKB Novablot Transfer System. The blot was incubated for 2 h at 4°C with blocking solution containing 5% skim milk powder and 0.05% Tween-20 in TBS. Next, the blot was incubated overnight at 4°C on a shaker with 10 ml of blocking solution containing 2 μg of murine monoclonal anti-actin antibody (kindly provided by Dr. J. Lessard, The University of Cincinnati, OH). The blot was then washed three times with blocking solution and incubated with goat anti-mouse antibody covalently linked to alkaline phosphatase (Bio-Rad), and staining was performed as recommended by the manufacturer.

[32P]ATP Labeling

Prior to study, neutrophils were incubated in the presence of 5 mM diisopropylfluorophosphate for 30 min at 37°C to inactivate proteases and thus minimize protein degradation (Rotrosen and Leto, 1990). Cells were permeabilized as above except that 100 μM cold ATP was used (to maximize sensitivity for the detection of phosphorylation), 10 μCi of [γ-^{32}P]-ATP added, and the suspension incubated for 5 min at 37°C. Following addition of the appropriate stimulus, cells were incubated for a further 5 min and the reaction stopped by sedimenting the cells and resuspending the pellet in 150 μl of boiling SDS sample buffer, followed by boiling for an additional 10 min. SDS-PAGE was then carried out using a 4–20% gradient gel (Novex Experimental Technology, San Diego, CA), with the gels stained using Coomassie blue and dried overnight as specified above. The gels were then placed in a cassette with Kodak X-OMAT AR film (Eastman-Kodak Co., Rochester, NY) and exposed for 72 h at −70°C prior to development.

Electron Microscopy

Neutrophils in suspension were prefixed with 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide, dehydrated in a series of graded ethanol baths (50-70-80-90-100%), critical-point dried, mounted, and coated by gold sputtering. Samples were examined using a Hitachi S-4000 field emission scanning electron microscope at 10 kV, and micrographs were recorded.

Confocal Fluorescence Microscopy

Rhodamine-phalloidin was used to stain F-actin distribution in neutrophils.

Neutrophils (200 μl of a suspension containing 2×10^6 cells) were exposed to 10^{-8} M PMA or the appropriate amount of DMSO (0.1%) for 5 min, and then fixed with an equal volume of 3% phosphate-buffered paraformaldehyde (final concentration, 1.5%) for 10 min. After fixation, the cells were allowed to settle on coverslips that were previously coated with 0.03% poly-L-lysine. After 20 min, the coverslips were gently washed with Kregs Ringer's phosphate dextrose buffer and then permeabilized by incubation with 0.1% buffered Triton X-100 for 5 min at room temperature followed by a wash with Kregs Ringer's phosphate dextrose. The cells were then stained by incubation with 1.65×10^{-7} M rhodamine-phalloidin for 10 min at 37°C. The coverslips were mounted on slides in a 1:1 solution of PBS:glycerol with p-phenylenediamine (0.1%) as a quenching agent. The slides were viewed using a Bio-Rad 600 laser scanning confocal microscope mounted on a Leitz microscope.

Statistical Analysis

Data are reported as mean ± SEM of the number of experiments indicated. All data were analyzed by analysis of variance for repeated measures, with correction for multiple comparisons (Sheffe).

Results and Discussion

Effects of Phorbol Esters and Synthetic DAG Analogs on Actin Assembly

We initially examined the effects of 4β-PMA on actin assembly in neutrophils. Fig. 1 a compares the effects of the phorbol ester with the receptor-mediated response elicited by fMLP. Exposure to 10^{-8} M 4β-PMA resulted in assembly of actin monomers into F-actin. The effect of 4β-PMA displayed a lag time of ~30 s, and peaked between 3 and 5 min. Stimulation by fMLP, in contrast, had reached a maximum by 30 s. The delay in response to 4β-PMA might be attributed to either (a) the time necessary for the phorbol ester to traverse the plasma membrane and interact with its putative receptor, ostensibly PKC (Nishizuka, 1984), and/or (b) the time required for the accumulation of active intermediates, likely phosphorylated proteins. The results in Fig. 2 a suggest that the former explanation accounts, at least in part, for the observed delay. In electropermeabilized cells, the initial lag was obviated and the peak response to 4β-PMA was reached more rapidly (within 2 min) than in intact cells. The differential behavior of intact and permeabilized cells was minimized when 1,2-dioctanoylglycerol (DiC$_8$), a short-chain membrane permeant DAG, was used as the stimulus (Fig. 2 b). Actin polymerization peaked between 1 and 2 min in both preparations, suggesting that DiC$_8$ enters intact cells more readily than 4β-PMA. It is noteworthy that, whereas the responses to both agonists in intact cells were sustained over the period analyzed, the polymerization induced in permeabilized cells was transient, decaying gradually over 10 min. This can be conceivably attributed to the progressive loss of an essential, unidentified component from the cytosol of the permeabilized cells. The observation that electroporated cells left at 37°C for 10 min and then stimulated with 4β-PMA responded less than those stimulated immediately after permeabilization is consistent with this hypothesis (Fig. 2 a, solid triangle). Figure 2 c illustrates that 4β-PMA–induced actin assembly was not sensitive to pretreatment with pertussis toxin in either intact or permeabilized cells. This suggested that the phorbol ester was acting through a similar (and pertussis-insensitive) pathway in both intact and permeabilized cells.

To ascertain the site of action of 4β-PMA, we next compared the effects of the phorbol ester and several chemically

a.

b.

c.
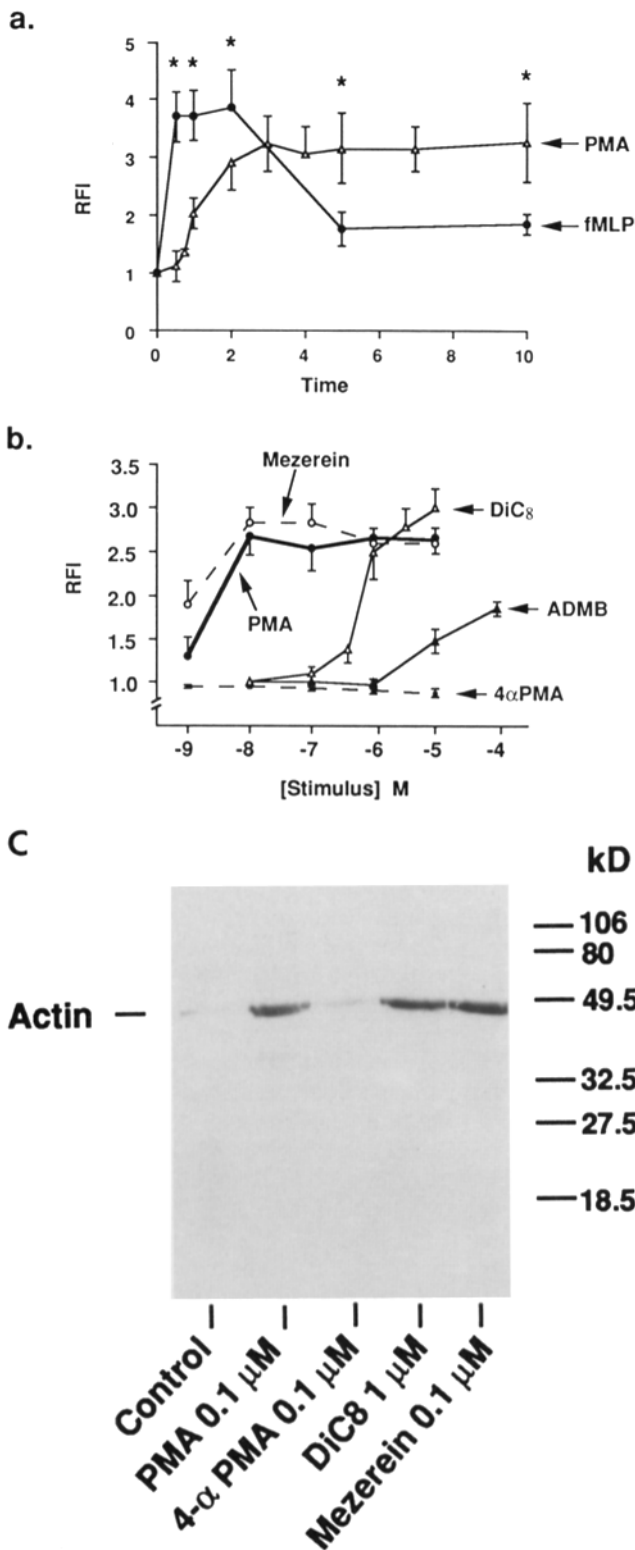
Figure 1. (*a*) Comparison of the time course of actin assembly at 37°C induced by PMA (10^{-7} M) or fMLP (10^{-8} M). F-actin content was measured by NBD-phallacidin staining of fixed and permeabilized cells at the specified time points after addition of the stimulus. Data are expressed as the RFI, relative to control neutrophils incubated in a comparable concentration of DMSO ($\leqslant 0.1\%$). Each value represents the mean ± SEM of four determinations. Asterisks indicate $P < 0.05$ with respect to the control, as determined by analysis of variance for repeated measures with correction for

unrelated PKC agonists. Fig. 1 *b* illustrates that the effects of 4β-PMA on actin assembly, measured by NBD-phallacidin staining, were detectable at 10^{-9} M and were maximal at $\sim 10^{-8}$ M, consistent with the reported affinity of PKC for the phorbol ester (Nishizuka, 1984). In addition, Fig. 1 *b* illustrates that exposure of neutrophils to DiC_8, mezerein, and the synthetic alcohol ADMB also resulted in dose-dependent actin assembly. Mezerein was slightly more potent than 4β-PMA, whereas DiC_8 and ADMB were two orders of magnitude less potent than the phorbol ester in their ability to induce actin polymerization. The relative potencies of these agents as promoters of actin assembly resemble their reported effects on PKC activity in vitro (Ebeling et al., 1985; Jaken et al., 1983; Miyake et al., 1984; Wender et al., 1986). The finding that a variety of chemically dissimilar activators of PKC can induce actin assembly supports the notion that the effects of 4β-PMA are mediated by the kinase or by another effector molecule with a very similar binding site. Accordingly, 4α-PMA, which is not an effective activator of PKC, failed to induce the formation of F-actin, even at concentrations as high as 10^{-5} M (Fig. 1 *b*).

Three additional and independent assays were employed to confirm that the increase in NBD-phallacidin binding induced by the PKC agonists was in fact due to an elevated F-actin content and was not artifactual. First, we compared the amount of F-actin incorporated into the Triton X-100–insoluble ("cytoskeletal") fraction of resting and stimulated cells by SDS-PAGE and immunoblotting using anti–actin antibodies. Fig. 1 *c* illustrates that exposure of neutrophils to 4β-PMA, DiC_8, or mezerein resulted in an increase in the actin content of the Triton X-100–insoluble fraction, indicative of the presence of F-actin. Consistent with the results of the NBD-phallacidin assay, 4α-PMA had no significant effect on actin polymerization when measured by detergent extraction and immunoblotting. We used scanning EM to determine if 4β-PMA resulted in membrane ruffling, which is generally accepted to be a consequence of polymerization of actin immediately subjacent to the plasma membrane (Sheterline et al., 1986; Roos et al., 1987). Fig. 3, *a* and *b* illustrates that exposure to 10^{-8} M 4β-PMA indeed induced the characteristic membrane ruffling associated with actin assembly. Lastly, using rhodamine-phalloidin to stain F-actin

multiple comparisons (Sheffe). (*b*) Effects of PKC activators on actin assembly in intact neutrophils at 37°C, as measured by NBD-phallacidin staining of fixed and permeabilized cells. Dose-response relationships are illustrated for 4βPMA, mezerein, DiC_8, ADMB, and 4αPMA. F-actin content was measured 5 min after addition of the specified stimulus. Data are expressed as the RFI relative to control neutrophils incubated in a comparable concentration of DMSO. Each value represents the mean ± SEM of four determinations. (*c*) Western blot of SDS polyacrylamide gel of the Triton X-100–insoluble fraction of neutrophils exposed to PKC stimulators for 5 min at 37°C: 4βPMA (10^{-7} M), mezerein (10^{-7} M), DiC_8 (10^{-6} M), and 4αPMA (10^{-7} M). The proteins on the SDS polyacrylamide gel were electrophoretically transferred to nitrocellulose and probed with a monoclonal anti–actin antibody followed by staining with alkaline phosphatase–conjugated goat anti–mouse IgG. Protein from identical numbers of neutrophils was loaded onto each lane. The positions of molecular weight standards are shown on the right side of the blot.

a. PMA

b. DiC8

c
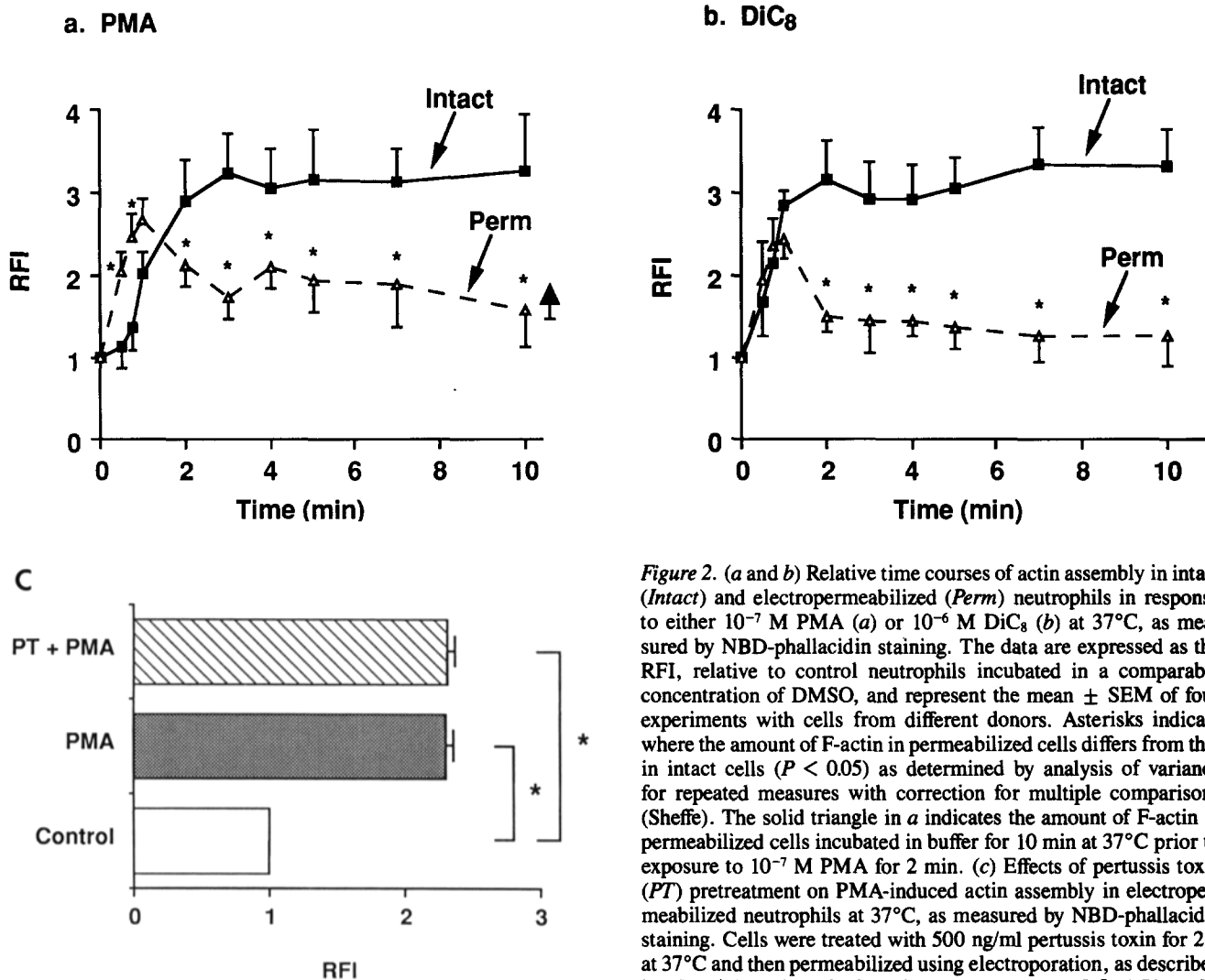
Figure 2. (a and b) Relative time courses of actin assembly in intact (*Intact*) and electropermeabilized (*Perm*) neutrophils in response to either 10^{-7} M PMA (a) or 10^{-6} M DiC8 (b) at 37°C, as measured by NBD-phallacidin staining. The data are expressed as the RFI, relative to control neutrophils incubated in a comparable concentration of DMSO, and represent the mean ± SEM of four experiments with cells from different donors. Asterisks indicate where the amount of F-actin in permeabilized cells differs from that in intact cells ($P < 0.05$) as determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe). The solid triangle in a indicates the amount of F-actin in permeabilized cells incubated in buffer for 10 min at 37°C prior to exposure to 10^{-7} M PMA for 2 min. (c) Effects of pertussis toxin (*PT*) pretreatment on PMA-induced actin assembly in electropermeabilized neutrophils at 37°C, as measured by NBD-phallacidin staining. Cells were treated with 500 ng/ml pertussis toxin for 2 h at 37°C and then permeabilized using electroporation, as described in Materials and Methods, prior to exposure to 10^{-7} M PMA for 2 min. The data are expressed as the RFI, relative to control neutrophils incubated in a comparable concentration of DMSO, and represent the mean ± SEM of three experiments with cells from different donors. Asterisks indicate differences from control cells ($P < 0.05$) as determined by analysis of variance for repeated measures with correction for multiple comparisons (Sheffe).

and scanning confocal fluorescence microscopy, we studied the alterations in the actin cytoskeleton induced by exposure to 4β-PMA. Fig. 4 illustrates that exposure to 10^{-8} M 4β-PMA induced dramatic reorganization of F-actin from a diffuse cytoplasmic location to focal aggregates in the area of the membrane ruffles. These three additional approaches validate the results obtained using NBD-phallacidin, and confirm that 4β-PMA and other PKC activators trigger assembly and spatial reorganization of the actin cytoskeleton.

Relationship Between Phorbol Ester–Induced Actin Polymerization and Protein Phosphorylation

The most likely mechanism whereby 4β-PMA exerts its effect on the microfilaments is the PKC-mediated phosphorylation of key intermediate proteins, with a resultant alteration in their conformation and/or activity. Phosphorylation by PKC occurs on serine and threonine residues of the substrate proteins, is antagonized by concomitant dephosphorylation, and is catalyzed by active phosphoserine and phosphothreonine phosphatases (Cohen, 1989). We therefore

reasoned that inhibition of phosphatase activity would be anticipated to enhance phorbol ester–induced phosphorylation and to potentiate actin assembly. This hypothesis was tested using okadaic acid, a potent and selective inhibitor of phosphatases 1 and 2A (Bialojan and Takai, 1988), which contribute importantly to the dephosphorylation of phosphorylated PKC substrates (reviewed by Cohen et al., 1990). Fig. 5 a illustrates the effects of okadaic acid on protein phosphorylation in the presence and absence of 4β-PMA. As reported, stimulation of the cells with 4β-PMA promoted phosphorylation of several polypeptides, including a 47-kD polypeptide (p47phox) known to be a component of the activated NADPH oxidase (Lomax et al., 1989). Similar results were obtained with 10^{-6} M DiC8 (data not shown). By itself, okadaic acid also induced the accumulation of phosphorylated proteins, indicating the presence of constitutively active kinases. Several of the polypeptides phosphorylated by 4β-PMA or DiC8 were also phosphorylated upon treatment with okadaic acid, raising the possibility that PKC or another kinase with similar substrate specificity is partially
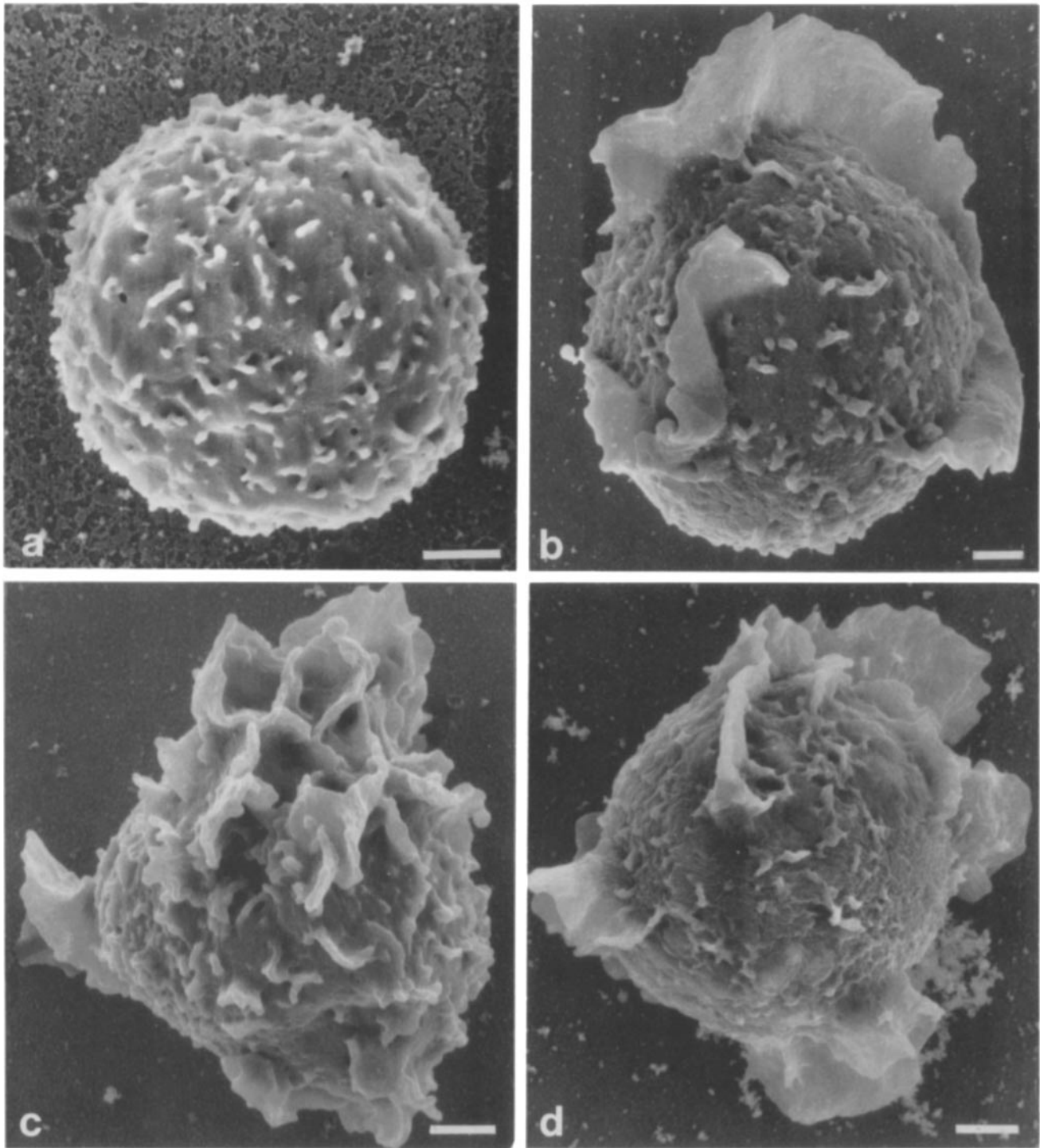
Figure 3. (a-d) Scanning electron micrographs of control (unactivated) neutrophil (*a*) and neutrophil exposed to 10^{-8} M PMA for 3 min in the absence (*b*) or presence of PKC inhibitors H7 (100 μM) (*c*) or staurosporine (50 nM) (*d*). Cells were preincubated with the specified inhibitor for 5 min at 37°C prior to exposure to the stimulus. Exposure to PMA results in the formation of membrane ruffling that is not prevented by pretreatment with either H7 or staurosporine. Each micrograph is representative of neutrophils from three separate experiments with cells isolated from different donors. Bars, 1 μm.

active in unstimulated cells. Simultaneous addition of 4β-PMA and okadaic acid produced a massive increase in phosphorylation (Fig. 5 *a*). A similar synergistic effect was observed when the phosphatase blocker was added together with DiC$_8$ (data not shown). Despite its marked effects on phosphorylation, however, incubation with okadaic acid for

5 min did not by itself promote actin assembly, nor did it potentiate the stimulatory response to 4β-PMA or DiC$_8$. In fact, Fig. 5 *b* illustrates that a slight inhibition was noted. Finally, even at submaximal doses of 4β-PMA (10^{-9} M) and DiC$_8$ (10^{-7} M), pretreatment with okadaic acid did not potentiate actin assembly (Fig. 5 *b*), but did potentiate
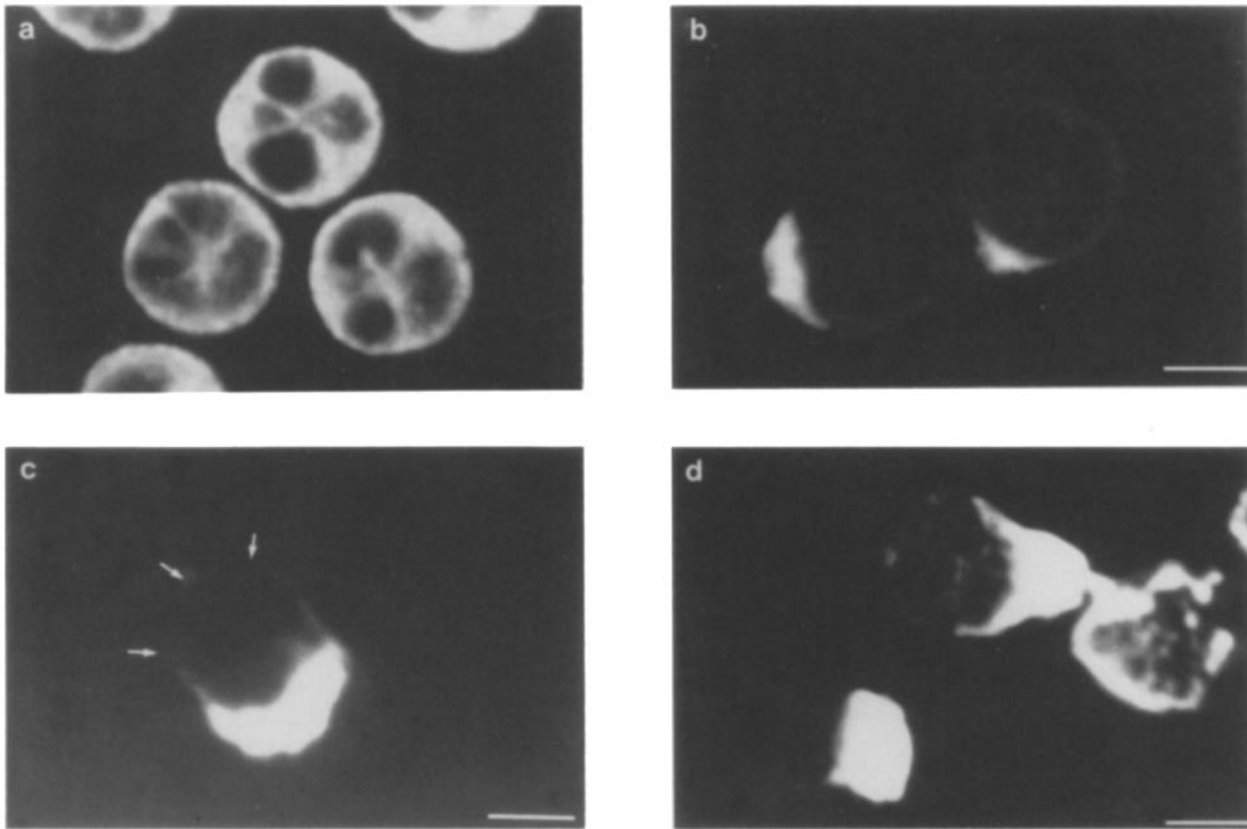
Figure 4. (*a–d*) Scanning confocal images of neutrophils stained with rhodamine-phalloidin to demonstrate the distribution of F-actin. Illustrated are images of control (unactivated) neutrophils (*a*) and neutrophils exposed to 10^{-8} M PMA for 1 min (*b*) or 5 min (*c*). *d* represents cells incubated in the presence of H7 (100 μM) for 5 min at 37°C prior to exposure to PMA 10^{-8} M for an additional 5 min. Exposure to PMA results in a time-dependent increase and redistribution of F-actin that is not prevented by pretreatment with either H7 (*d*) or staurosporine (not shown). In *c*, the small arrows indicate the location of the plasma membrane. In *d*, level of the optical section was chosen to demonstrate the F-actin content of the membrane ruffles for the two cells on the left side of the photomicrograph. The membrane ruffle of the cell on the right side of this photomicrograph was not in this optical plane. Each micrograph is representative of neutrophils from three separate experiments with cells isolated from different donors. The intensity of the confocal images was adjusted using image-processing software to illustrate the distribution of the F-actin, and, in these images, the apparent intensity of staining is *not* proportional to the intensity of the actual fluorescence images. Bars, 5 μm.
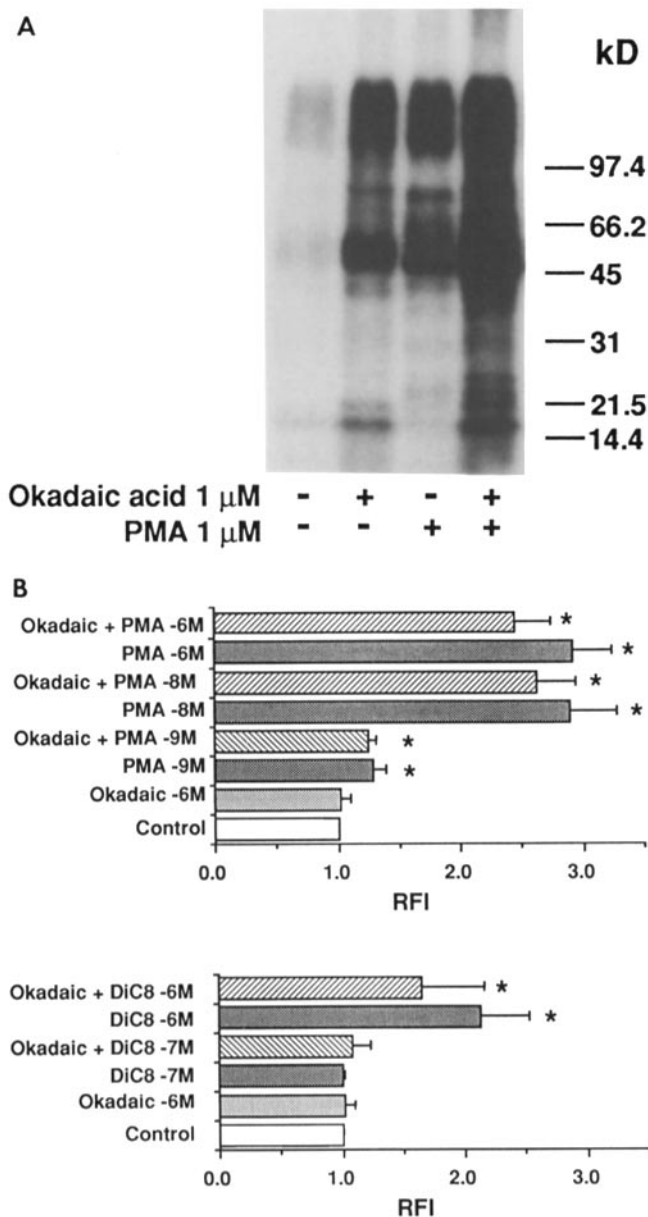
phosphorylation (data not shown). These results point to a dissociation between the activity of PKC and the polymerization of actin in cells stimulated by 4β-PMA or DiC$_8$.

Effects of PKC Inhibitors on Phorbol Ester–Induced Actin Assembly

To explore further the relationship between PKC activity and the cytoskeletal effects of phorbol esters, we studied the effects of several PKC inhibitors on phorbol ester–induced microfilament assembly. To circumvent problems with non-specific inhibition, we selected a variety of inhibitors known to act at different sites: (*a*) H7, thought to act competitively at the ATP binding site (Hidaka et al., 1984); (*b*) staurosporine, which has been reported to have both competitive and noncompetitive effects with respect to ATP (reviewed by Huang, 1989); (*c*) sphingosine, which competitively interacts with Ca^{2+}, phosphatidylserine and DAG (Hannun et al., 1986), thus preventing the formation of an active lipid-enzyme complex; and (*d*) calphostin C, which interacts with the regulatory domain of PKC (Kobayashi et al., 1989). At a concentration of 100 μM, H7 did not inhibit PMA-induced actin assembly (Fig. 6 *a*), as has been reported by Sha'afi et

al. (1988), nor did it prevent PMA-induced membrane ruffling (Fig. 3 *c*) or F-actin rearrangement (Fig. 4 *d*). Under comparable conditions, this concentration of H7 produced nearly complete inhibition of the 4β-PMA–induced superoxide production due to activation of the NADPH oxidase (Fig. 7 *a*). More importantly, the same concentration of H7 virtually abolished the effect of the phorbol ester on protein phosphorylation (Fig. 8 *a*). When the dose of H7 was increased to 200 μM, the 4β-PMA–induced actin assembly was inhibited only at low concentrations of 4β-PMA (10^{-9} and 10^{-8} M), but this inhibition was overcome by higher PMA concentrations (Fig. 6 *a*). Similar results were obtained using 50 nM staurosporine, a dose reported to block the activation of PKC (Tamaoki et al., 1986). In accordance with earlier reports (Dewald et al., 1989), 50 nM staurosporine inhibited 4β-PMA–induced superoxide production almost entirely (Fig. 7 *a*). Similarly, protein phosphorylation (Fig. 8 *a*) was eliminated by this dose of the blocker. In contrast, staurosporine inhibited only minimally the 4β-PMA–induced actin assembly (Fig. 6 *b*), and did not prevent 4β-PMA–induced membrane ruffling (Fig. 3 *d*) and F-actin redistribution (not shown). At a concentration of 100 nM, staurosporine sig-

A

	kD
	— 97.4
	— 66.2
	— 45
	— 31
	— 21.5
	— 14.4

Okadaic acid 1 μM   -   +   -   +
PMA 1 μM   -   -   +   +

B

Okadaic + PMA -6M *
PMA -6M *
Okadaic + PMA -8M *
PMA -8M *
Okadaic + PMA -9M *
PMA -9M *
Okadaic -6M
Control

0.0   1.0   2.0   3.0
RFI

Okadaic + DiC8 -6M *
DiC8 -6M *
Okadaic + DiC8 -7M
DiC8 -7M
Okadaic -6M
Control

0.0   1.0   2.0   3.0
RFI

Figure 5. (*a*) Effects of 1 μM okadaic acid on PMA (10^{-7} M) and DiC$_8$ (10^{-6} M)-induced protein phosphorylation in neutrophils. Cells were pretreated with diisopropylfluorophosphate, washed, incubated with 1 μM okadaic acid for 5 min at 37°C, permeabilized by electroporation, washed, an additional aliquot of okadaic acid added (final concentration, 1 μM) together with 10 μCi of [γ-^{32}P]-ATP, and then exposed to either 4βPMA, DiC$_8$, or DMSO, as specified, for an additional 5 min. The reaction was terminated by the addition of boiling sample buffer followed by PAGE and autoradiography, as described in Materials and Methods. This autoradiograph is representative of four separate experiments with cells isolated from different donors. (*b*) Effects of 1 μM okadaic acid on PMA- (*top panel*) and DiC$_8$-induced (*bottom panel*) actin assembly as measured by NBD-phallacidin staining in neutrophils. Cells were preincubated with 1 μM okadaic acid for 5 min at 37°C prior to the addition of the specified stimulus. Each value represents the mean ± SEM of four determinations. Asterisks indicate $P < 0.05$ with respect to the control, determined by analysis of variance with correction for multiple comparisons (Sheffe).

nificantly reduced the responses induced by low ($\leq 10^{-9}$ M) doses of 4β-PMA, as was found with H7. However, concentrations of 4β-PMA of $\geq 10^{-8}$ M largely (80%) restored the actin assembly response. Pretreatment with 1 μM sphingosine produced only a small, statistically insignificant reduction in actin polymerization (Fig. 6 *c*), and, even at a concentration of 10 μM, sphingosine inhibited less than half of the PMA-induced actin assembly. The latter concentration of sphingosine essentially eliminated the respiratory burst triggered by 10^{-8} M 4β-PMA (Fig. 7 *a*). Pretreatment with calphostin C, a highly specific inhibitor of PKC, in doses from 10^{-9}–10^{-6} M, also did not prevent actin assembly (Table I). Lastly, Fig. 6 *d* illustrates that the relative insensitivity of actin assembly to PKC inhibitors was not unique to phorbol ester–stimulated cells. A large component of the DiC$_8$-induced actin polymerization was similarly resistant to H7 and staurosporine at concentrations that greatly inhibited DiC$_8$-induced superoxide production (Fig. 7 *b*) and protein phosphorylation (data not shown).

The studies illustrating the inhibition of phorbol ester–induced protein phosphorylation by H7, staurosporine, and calphostin C (Fig. 8 *a*) were done with permeabilized cells, whereas the effects of the inhibitors on actin assembly were tested in intact cells. To ensure that the disparate effects on protein phosphorylation and actin assembly were not due to differences in accessibility of the inhibitors to their active sites, or to differences in the ATP concentration (which can competitively displace some of the inhibitors; see above), we examined the effects of H7, staurosporine, and calphostin C on phorbol ester–induced actin assembly in permeabilized cells. Fig. 8 *b* illustrates that, even under these conditions, where access of the inhibitors to the cytosol was optimized, PMA and DiC$_8$–induced actin assembly was largely resistant to inhibition. Also illustrated in Fig. 8 *b* is the observation that exposure of permeabilized cells to H7 and staurosporine resulted in a modest degree of actin assembly, an effect reported by others in intact cells (Keller et al., 1990; Niggli and Keller, 1991).

Taken together, these data suggest the existence of divergent pathways leading to actin polymerization and activation of NADPH oxidase in neutrophils stimulated with phorbol esters. Activation of the oxidase was susceptible to conventional inhibitors of PKC and correlated with protein phosphorylation, whereas actin polymerization appeared not to be directly proportional to protein phosphorylation and displayed a significant component (at least 50%) that, under the conditions used, was unaffected by the conventional PKC inhibitors.

Alternate Pathways Involved in Phorbol Ester–Induced Actin Assembly

The experiments outlined above suggested that the effects of PMA on microfilament assembly might occur independently of activation of, at least, conventional isoforms of PKC. Thus, we sought to define alternate pathways through which PMA might be acting. Recent reports have identified novel isoforms of protein kinase C (Ohno et al., 1988), which differ in both substrate specificity (Majumdar et al., 1991) and sensitivity to inhibitors (Gschwendt et al., 1989) from the conventional PKC isoforms (α, β1, β2, and γ) (see review by Huang, 1989). The predominant isoform present in hu-
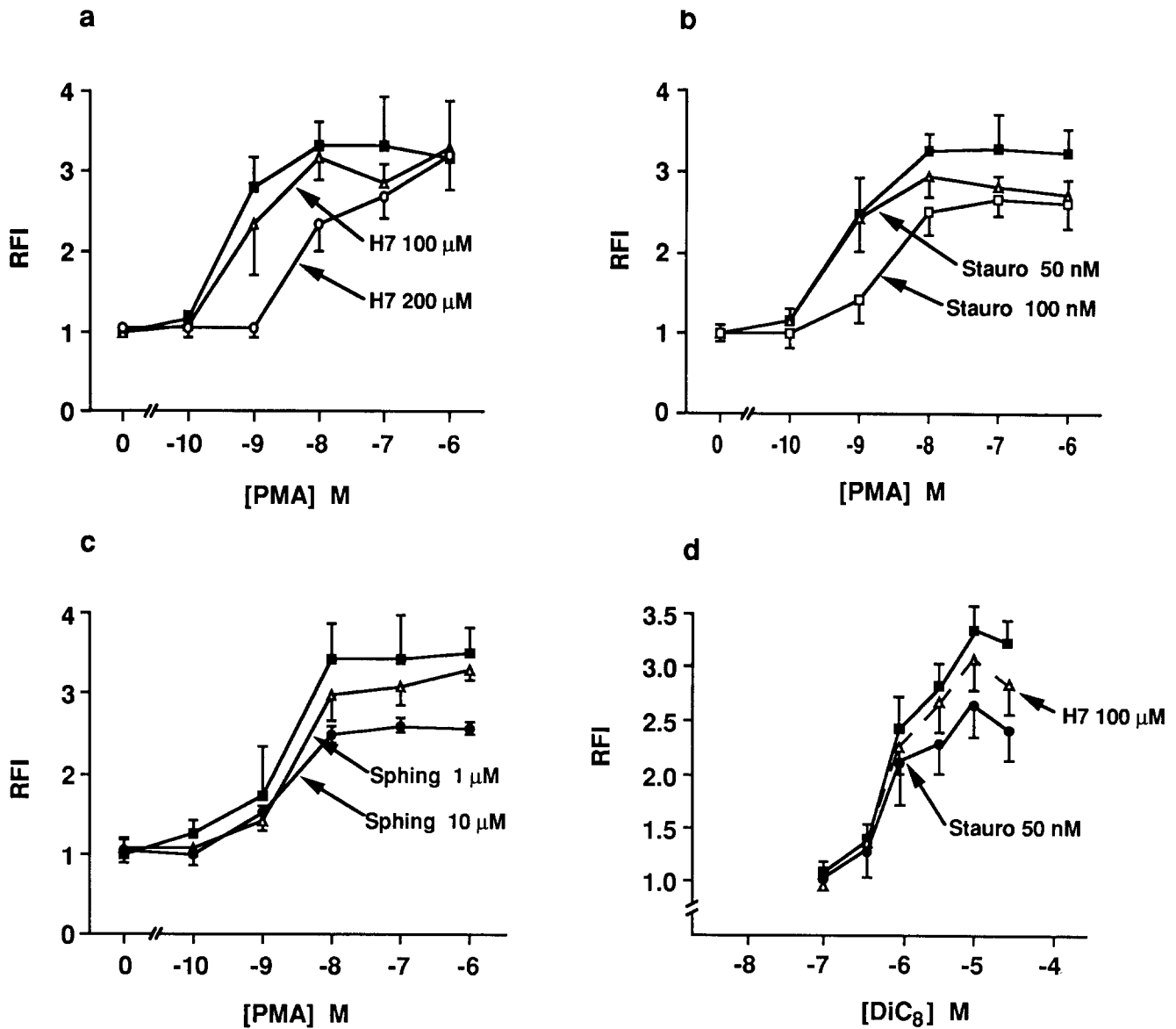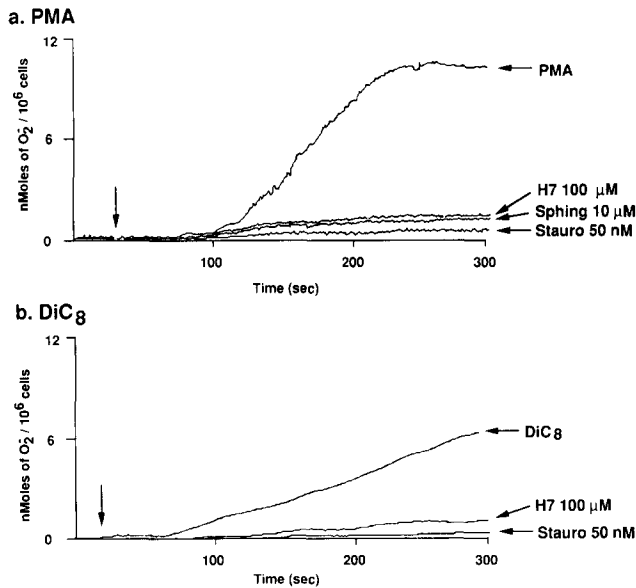
Figure 6. (a–d) Effects of PKC inhibitors on PMA- (a–c) and DiC₈-induced (d) actin assembly in intact neutrophils as measured by NBD-phallacidin staining. For these studies, the neutrophils were incubated with the specified inhibitor for 5 min at 37°C prior to exposure to the stimulus. The concentrations of the inhibitors are as follows: (a) H7; 100 and 200 μM; (b) staurosporine, (stauro) 50 and 100 nM; (c) sphingosine (Sphing), 1 and 10 μM; (d) H7, 100 μM, and staurosporine, 50 nM. Each value represents the mean ± SEM of four determinations.

man neutrophils is β-PKC (Majumdar et al., 1991), which is sensitive to conventional PKC inhibitors (see review by Huang, 1989), phosphorylates p47phox, and is involved in the activation of NADPH oxidase (Heyworth et al., 1989; Rotrosen and Leto, 1990). In addition, human neutrophils also contain nPKC, a calcium-independent isoform that has an entirely different phosphorylation profile, phosphorylating a 68-kD protein of unknown identity but not p47phox (Majumdar et al., 1991). Importantly, whereas the nPKC present in human neutrophils is susceptible to inhibition by long-chain fatty acyl-CoA esters such as oleoyl and palmitoyl CoA (Majumdar et al., 1991), it is resistant to conventional PKC inhibitors (Gschwendt et al., 1989). This raises the possibility that nPKC is selectively involved in cytoskeletal
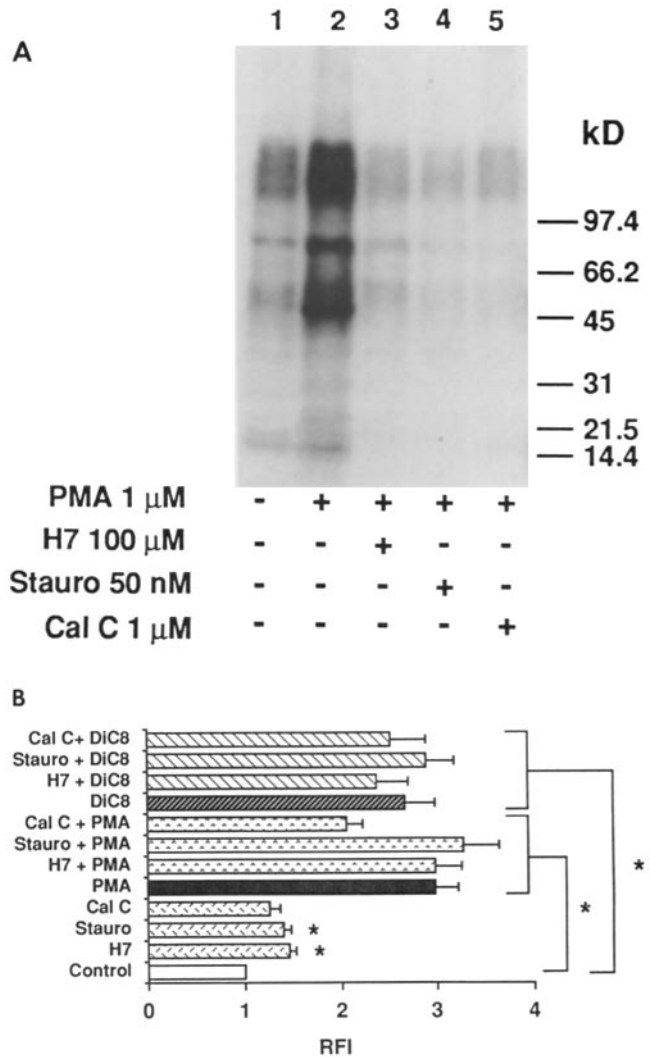
reorganization, accounting for the insensitivity of the PMA response to conventional PKC inhibitors such as H7 and staurosporine. To investigate this hypothesis, we tested the effects of long-chain fatty acyl-CoA esters on PMA-induced actin assembly. Fig. 9 illustrates that myristoyl, oleoyl, and palmitoyl CoA, but not their respective free fatty acids, completely inhibited PMA-induced actin assembly in electroper-meabilized neutrophils. No such inhibition was noted in intact cells (data not shown), suggesting that access to an intracellular location was necessary for inhibition. The long-chain fatty acyl-CoA esters inhibited the rearrangement of the actin cytoskeleton as determined by scanning confocal fluorescence microscopy (data not shown). Lastly, these concentrations of myristoyl, oleoyl, and palmitoyl CoA also

a. PMA

y-axis: nMoles of O_2 / 10^6 cells

Curves labeled: PMA, H7 100 μM, Sphing 10 μM, Stauro 50 nM

x-axis: Time (sec) — 100, 200, 300

b. DiC$_8$

y-axis: nMoles of O_2 / 10^6 cells

Curves labeled: DiC$_8$, H7 100 μM, Stauro 50 nM

x-axis: Time (sec) — 100, 200, 300

Figure 7. The effect of 10^{-8} M PMA (a) and 2×10^{-6} M DiC$_8$ (b) on superoxide production, measured as the superoxide dismutase–inhibitable reduction of ferricytochrome c in the absence or presence of 100 μM H7, 50 nM staurosporine (*Stauro*), or 10 μM sphingosine (*Sphing*), as indicated. The neutrophils were preincubated with the inhibitors for 5 min at 37°C prior to exposure to the stimuli. The concentration of 2×10^{-6} M DiC$_8$ was chosen because of the presence of cytochrome c, which binds DiC$_8$, thus lowering its free concentration. Each trace is representative of three experiments using cells from different donors. The arrow designates the time of addition of the stimulus.

inhibited the PMA-induced activation of the NADPH oxidase and protein phosphorylation (data not shown), consistent with their known abilities to inhibit both the conventional (Stasis et al., 1987) and *n*-isoforms (Majumdar et al., 1991) of PKC.

In summary, exposure of neutrophils to a variety of chemically dissimilar compounds known to stimulate PKC results in ruffling of the plasma membrane, assembly and spatial reorganization of the actin cytoskeleton, protein phosphorylation, and activation of the NADPH oxidase. However, whereas the effects of PMA on activation of the oxidase and protein phosphorylation were virtually abolished by conventional PKC inhibitors, membrane ruffling and actin polymerization were largely resistant to these inhibitors. Moreover, okadaic acid, which potentiated PMA-induced protein phosphorylation, did not potentiate the effects of the phorbol ester on actin assembly. Two types of explanations, which are not mutually exclusive, can be offered to account for these observations. Actin polymerization in response to PMA and related compounds may occur as a result of activation of specialized PKC isoforms, such as nPKC, that are not susceptible to classical PKC inhibitors. In view of the nearly complete inhibition of phosphorylation induced by staurosporine, H7, and calphostin C, this hypothesis implies that the putative substrates of the specialized PKC isozymes represent a very small fraction of the total phosphoprotein, therefore escaping detection in experiments such as those in Fig. 8 *a*. Alternatively, the effects of phorbol esters on the actin cytoskele-

A

Lanes: 1 2 3 4 5

kD markers: —97.4, —66.2, —45, —31, —21.5, —14.4

	1	2	3	4	5
PMA 1 μM	−	+	+	+	+
H7 100 μM	−	−	+	−	−
Stauro 50 nM	−	−	−	+	−
Cal C 1 μM	−	−	−	−	+

B

x-axis: RFI — 0, 1, 2, 3, 4

Bars (top to bottom):
Cal C+ DiC8
Stauro + DiC8
H7 + DiC8
DiC8
Cal C + PMA
Stauro + PMA
H7 + PMA
PMA
Cal C
Stauro *
H7 *
Control

Figure 8. (a) Effect of PKC inhibitors H7, staurosporine (*Stauro*), and calphostin C (*Cal C*) on protein phosphorylation induced by 10^{-6} PMA measured using [^{32}P]ATP in electropermeabilized cells. Cells pretreated with diisopropylfluorophosphate were permeabilized by electroporation followed by the addition of 10 μCi of [γ-^{32}P]ATP, inhibitor (H7, lane *3*; staurosporine, lane *4*; and calphostin C, lane *5*), and 10^{-6} PMA. Lane *1* represents control cells that were electropermeabilized, with [^{32}P]ATP added in the absence of inhibitors or stimuli. After 5 min at 37°C, the cells were washed, resuspended in boiling SDS sample buffer, and analyzed by SDS-PAGE and autoradiography, as described in Materials and Methods. (b) Effects of PKC inhibitors on PMA and DiC$_8$-induced actin assembly in permeabilized neutrophils as measured by NBD-phallacidin staining. For these studies, the neutrophils were electroporated, followed by the addition of 100 μM H7, 50 nM staurosporine, or 1 μM calphostin C, and either 10^{-6} M 4βPMA or 10^{-6} DiC$_8$ as indicated. Each value represents the mean ± SEM of at least four determinations. Asterisks indicate $P < 0.05$ with respect to the control, determined by analysis of variance with correction for multiple comparisons (Sheffe). There was no significant inhibition of PMA or DiC$_8$-induced actin assembly by either H7 or staurosporine, and calphostin C only partially inhibited the response to PMA but not to DiC$_8$.

*Table I. Effect of Calphostin C on PMA-Induced Actin Assembly*

Condition	RFI
Control	1.0
PMA 10^{-8} M	2.37 ± 0.13
PMA 10^{-6} M (PreRx calphostin C 10^{-7} M)	2.40 ± 0.22
PMA 10^{-6} M (PreRx calphostin C 10^{-6} M)	2.42 ± 0.25
PMA 10^{-6} M (PreRx calphostin C 10^{-5} M)	2.49 ± 0.04

Where indicated, intact neutrophils were pretreated (PreRx) with calphostin C at the specified concentration of 10^{-9} to 10^{-5} M for 5 min at 37°C prior to stimulation with PMA for an additional 5 min. F-actin content was determined using NBD-phallacidin as described in Materials and Methods. Data are presented as RFI $\pm$ SEM of $n = 3$ experiments.

ton may occur independently of activation of PKC, as has been reported for phospholipase D. Despite our uncertainty as to the extent of participation of PKC and the nature of the isozyme(s) involved, it is clear that different mechanisms underlie the activation of the oxidase and actin polymerization in neutrophils. Even if PKC activity is involved in both instances, very different levels of phosphorylated intermediates are required to elicit these responses, highlighting the divergent nature of the pathways leading to the two responses (Tortora et al., 1990; Trudel et al., 1990).
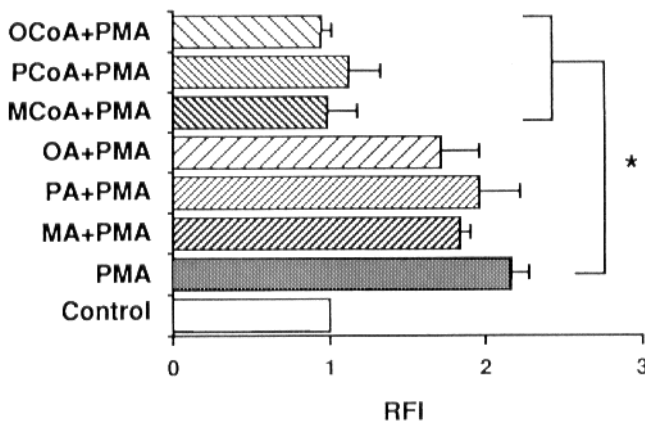
Figure 9. Effect of pretreatment with long-chain fatty acyl CoA esters on PMA-induced actin assembly in neutrophils as measured by NBD-phallacidin staining. For these studies, electropermeabilized neutrophils were incubated in the presence or absence of 10 μM of the free fatty acids myristic acid (*MA*), oleic acid (*OA*), or palmitic acid (*PA*), or the corresponding fatty acyl CoA (myristoyl CoA [*MCoA*], palmitoyl CoA [*PCoA*], or Oleoyl CoA [*OCoA*]), as indicated, for 1 min at 37°C prior to the addition of 10^{-6} M 4βPMA and then incubated for an additional 5 min. Each value represents the mean $\pm$ SEM of four determinations. Asterisks indicate that pretreatment with either myristoyl CoA, palmitoyl CoA, or oleoyl CoA inhibited significantly the response to PMA ($P < 0.05$), as determined by analysis of variance with correction for multiple comparisons (Sheffe).

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