

Antagonists of Calcium Fluxes and Calmodulin Block Activation of the p21-Activated Protein Kinases in Neutrophils¹

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Neutrophils stimulated with fMLP or a variety of other chemoattractants that bind to serpentine receptors coupled to heterotrimeric G proteins exhibit rapid activation of two p21-activated protein kinases (Paks) with molecular masses of ~63 and 69 kDa (γ - and α -Pak). Previous studies have shown that products of phosphatidylinositol 3-kinase and tyrosine kinases are required for the activation of Paks. We now report that a variety of structurally distinct compounds which interrupt different stages in calcium/calmodulin (CaM) signaling block activation of the 63- and 69-kDa Paks in fMLP-stimulated neutrophils. These antagonists included selective inhibitors of phospholipase C (1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione), the intracellular Ca²⁺ channel (8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate), CaM (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; N-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide; trifluoperazine), and CaM-activated protein kinases (N-[2-(N-(chlorocinnamyl)-N-methylaminomethyl)phenyl]-N-[2-hydroxyethyl]-4-methoxybenzenesulfonamide). This inhibition was dose-dependent with IC₅₀ values very similar to those that interrupt CaM-dependent reactions in vitro. In contrast, less active analogues of these compounds (1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione; N-(6-aminohexyl)-1-naphthalenesulfonamide; N-(4-aminobutyl)-1-naphthalenesulfonamide; promethazine; 2-[N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzyl-amine]) did not affect activation of Paks in these cells. CaM antagonists (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; trifluoperazine), but not their less-active analogues (N-(6-aminohexyl)-1-naphthalenesulfonamide; promethazine), were also found to block activation of the small GTPases Ras and Rac in stimulated neutrophils along with the extracellular signal-regulated kinases. These data strongly suggest that the Ca²⁺/CaM complex plays a major role in the activation of a number of enzyme systems in neutrophils that are regulated by small GTPases. *The Journal of Immunology*, 2001, 166: 2643–2650.

Neutrophils stimulated with fMLP or a variety of other chemoattractants that couple to heterotrimeric G proteins exhibit rapid activation of a large number of protein kinases that participate in the functional responses of these cells. These protein kinases include two p21-activated protein kinases (Paks)³ with molecular masses of 63 and 69 kDa (γ - and

α -Pak; Refs. 1–5) and certain mitogen-activated protein kinase (MAPK) cascades (e.g., extracellular signal-regulated kinases (ERK-1/2), p38-MAPK; Refs. 6–10). Paks are Ser/Thr protein kinases that undergo autophosphorylation/activation upon interacting with the active (GTP-bound) forms of the small GTPases (p21) Rac or Cdc42 (11). Activation of the Paks in neutrophils can be blocked by inhibitors of heterotrimeric G proteins (pertussis toxin) (2, 5), phosphatidylinositol 3-kinase (PI 3-K; i.e., wortmannin, LY 294002) (12), and tyrosine kinases (13, 14). Paks or Pak-like kinases contain binding sites for the $\beta\gamma$ -subunits of complex G proteins (15), guanine nucleotide exchange factors (GEF; i.e., Pak-interacting exchange factors) (16), and adaptor proteins (Nck) (17, 18). Paks may directly interact with the cytoskeleton through p95 paxillin-kinase linker, which binds directly to both Pak-interacting exchange factors and the focal adhesion adaptor protein paxillin (19). Thus, Paks may be capable of integrating messengers from a number of signal transduction pathways.

Paks can participate in a broad range of cellular events that include rapid cytoskeletal responses, activation/potentialiation of several distinct MAPK cascades and apoptosis (for review see Ref.

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³ Abbreviations used in this paper: Pak, p21-activated protein kinase; CRIB, Cdc42/Rac interactive binding domain; PI 3-K, phosphatidylinositol 3-kinase; CaM, calmodulin; PI-PLC, phosphatidylinositol-specific phospholipase C; CaM-PK, CaM-activated protein kinases; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; p47-phox, the 47-kDa protein component of the phagocyte oxidase; O₂⁻, superoxide; W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; W-5, N-(6-aminohexyl)-1-naphthalenesulfonamide;

TFP, trifluoperazine; PMZ, promethazine; PKC, protein kinase C; GEF, guanine nucleotide exchange factor; W-13, N-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide; W-12, N-(4-aminobutyl)-1-naphthalenesulfonamide; U-73122, 1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione; U-73343, 1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione; TMB-8, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate; KN-93, N-[2-(N-(chlorocinnamyl)-N-methylaminomethyl)phenyl]-N-[2-hydroxyethyl]-4-methoxybenzenesulfonamide; KN-92, 2-[N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine].

20 and 21). Recent studies have implicated Pak in the activation of NF- κ B in macrophages (22), tumor growth (23) and the pathogenesis of HIV (24, 25). Paks can also catalyze the phosphorylation *in vitro* of both the 47- and 67-kDa subunits of the superoxide (O_2^-)-generating system of phagocytic leukocytes (NADPH-oxidase) (5, 26). However, it is not known whether Paks participate in the phosphorylation of these oxidase subunits *in vivo*.

Recent studies have shown that heterotrimeric G proteins can activate Ras, Src family tyrosine kinases, and the extracellular signal regulated kinases (ERKs-1/2) through a variety of signal transduction pathways (27, 28). One such pathway contains phosphatidylinositol-specific phospholipase C (PI-PLC) and the Ca^{2+} /calmodulin (CaM) complex as major components in the activation of Src and Ras (28). We have recently demonstrated that D-erythro-sphingosine blocks activation of the 63- and 69-kDa Paks in neutrophils if added to the cells either before or after stimulation with fMLP (29). Interestingly, D-erythro-sphingosine can inhibit a number of enzymes activated by the Ca^{2+} /CaM complex (30).

In this paper, we describe the effects of selective antagonists of PLC, the intracellular Ca^{2+} channel, the Ca^{2+} /CaM complex, and CaM-activated protein kinases (CaM-PK) on the activation of Paks in neutrophils. The data indicate that the Ca^{2+} /CaM complex has a major role in regulating the Paks in these cells along with other enzyme systems activated by small GTPases.

Materials and Methods

Materials

N-(6-aminoethyl)-5-chloro-1-naphthalenesulfonamide (W-7); *N*-(6-aminoethyl)-1-naphthalenesulfonamide (W-5); *N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide (W-13); *N*-(4-aminobutyl)-1-naphthalenesulfonamide (W-12); 1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione (U-73122); 1-[6-((17 β -3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-2,5-pyrrolidinedione (U-73343); 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate (TMB-8); *N*-[2-(*N*-(chlorocinnamyl)-*N*-methylaminomethyl)phenyl]-*N*-[2-hydroxyethyl]-4-methoxybenzenesulfonamide (KN-93); and 2-[*N*-(4-methoxybenzenesulfonyl)amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzyl-amine] (KN-92) were purchased from Calbiochem (La Jolla, CA). Purified mouse mAbs to human Ras was obtained from PharMingen/Transduction Laboratories (San Diego, CA). A mouse mAb to full-length human Rac was purchased from Upstate Biotechnology (Lake Placid, NY). Affinity-purified, rabbit polyclonal Abs that recognized only the active (doubly phosphorylated) forms of MAP/ERK kinase (MEK)1 and MEK2 (phospho-MEK-1/2 (Ser²¹⁷/Ser²²¹) Ab) were obtained from New England Biolabs (Beverly, MA). Affinity-purified, rabbit polyclonal Abs that recognized only the active (doubly phosphorylated) forms of ERK (p44/ERK1 and p42/ERK2) were obtained from Promega (Madison, WI). Affinity-purified rabbit polyclonal Abs that recognized both the phosphorylated and nonphosphorylated forms of ERK (p44/42 (ERK-1/2) MAPK Abs) were also purchased from New England Biolabs. Goat anti-rabbit IgG labeled with HRP, goat anti-mouse IgG labeled with HRP, a Super Signal substrate Western blotting kit for luminol-enhanced chemiluminescence, and an ImmunoPure binding/elution buffer system for stripping and reblotting Western blots were purchased from Pierce (Rockford, IL). Sources of all other materials are described elsewhere (1–3).

Preparation of neutrophils

Guinea pig peritoneal neutrophils were prepared as described previously (31). These preparations contained >90% neutrophils with viabilities always >90%.

Detection of renaturable protein kinases (Paks) in polyacrylamide gels

Paks and certain other protein kinases were detected directly in gels by their ability to undergo renaturation and catalyze the phosphorylation of a peptide substrate fixed within a gel that corresponds to amino acid residues 297–331 of the 47-kDa protein component of the phagocyte oxidase (p47-phox). This technique was performed as described elsewhere (2) except the amount of cells was reduced to 3×10^6 /ml.

Detection of activated Rac and Ras

Activated forms of Rac or Ras were measured in neutrophil lysates by their ability to bind specifically to GST-fusion proteins containing the Cde42/Rac interactive binding (CRIB) domain of Pak1b (crib domain, amino acid residues 56–227) (GST-Pak-CRIB) or the Ras-binding domain of Raf (GST-Raf-RBD) coupled to glutathione agarose beads (32, 33). The beads were subsequently isolated, washed, and subjected to SDS-PAGE as described previously (32, 33). Specific binding of Rac to the GST-Pak crib fusion protein was determined by Western blotting with a specific Ab to this small GTPase. Conditions for Western blotting are described below. Fusion proteins were prepared as described previously (32, 34).

Immunoblotting/detection of activated ERKs and MEK in stimulated neutrophils

Neutrophils (3×10^6 /ml) were stimulated and lysed as described (1). Aliquots of these samples were separated by SDS-PAGE (35 μ g/lane) on 9.0% (v/v) polyacrylamide slab gels and transferred electrophoretically to Immobilon-P membranes as described (1). Activated ERK and MEK were assayed by Western blotting with Abs that recognized only the activated (doubly phosphorylated) forms of these kinases (35). Activated kinases and the small GTPases were visualized with a luminol-enhanced chemiluminescence detection system (Pierce), which monitored the activity of HRP bound to the secondary Ab (35). Membranes were incubated with the primary Ab against 1.0 μ g/ml Rac or Ras (1:500 dilution) for 1 h at room temperature. All other Ab dilutions and conditions for Western blotting are detailed by Huang et al. (14).

In certain experiments (Fig. 6), products of the chemiluminescence detection system were removed by washing the membranes two times (10 min/wash) with TBST (20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 0.01% (v/v) Tween 20). These blots were then reprobbed with a different Ab as described above so that both Ags could be visualized simultaneously (Ref. 35; Fig. 6). At the end of these experiments, both the immunodetection system and the bound Abs were removed from the blot by incubating the membranes with ImmunoPure elution buffer (Pierce) for 30–60 min at room temperature followed by two washes with TBST. The blots were then stained with an Ab that recognized both the phosphorylated and nonphosphorylated forms of ERK to confirm that equal amounts of protein were present in each lane of the gel.

Miscellaneous procedures

O_2^- release from neutrophils was measured as described previously (2).

Analysis of data

Unless otherwise noted, all of the autoradiographic observations were confirmed in at least three separate experiments performed on different cell preparations. The numbers of observations (*n*) are also based on different cell preparations.

Results

Effects of Ca^{2+} /CaM antagonists on activation of the Paks in neutrophils

Neutrophils stimulated with the chemoattractant fMLP exhibit rapid activation of two Paks with molecular masses of ~63 and 69 kDa along with two unidentified renaturable protein kinases with masses of ~49 and 40 kDa (Fig. 1; Refs. 2 and 4). These kinases can be detected directly in gels by their ability to undergo renaturation and catalyze the phosphorylation of a peptide substrate fixed within a gel. Positions of the protein kinases are visualized by autoradiography after exposure of the gel to [γ -³²P]ATP (2). The peptide used corresponds to amino acid residues 297–331 of p47-phox and contains several of the phosphorylation sites of this protein.

Certain naphthalenesulfonamides (W-7, W-13) have been designed as selective antagonists of CaM (36). The order of potency of these compounds *in vitro* is W-7 > W-13 with the unchlorinated analogues of these molecules (W-5, W-12) being considerably less active (36). Thus, W-5 and W-12 serve as excellent controls for evaluating nonspecific cellular effects of W-7 and W-13, respectively. Treatment of neutrophils with 50 μ M W-7 for 5.0 min

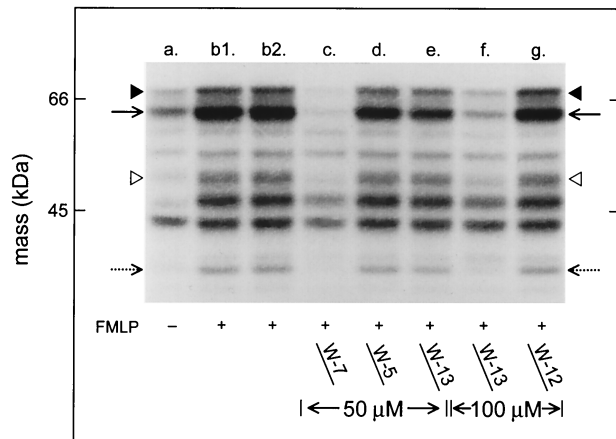


FIGURE 1. Effects of $\text{Ca}^{2+}/\text{CaM}$ antagonists on activation of the Paks in neutrophils. Paks were assayed in neutrophil lysates by their ability to undergo renaturation and catalyze phosphorylation of the p47-*phox* peptide fixed within a gel as described in *Materials and Methods*. Cells were treated with the inhibitors at the concentrations specified for 5.0 min at 37°C and then stimulated with $1.0\ \mu\text{M}$ fMLP for 15 s. Lane *a* is for unstimulated cells not treated with an inhibitor. Lanes *b1* and *b2* are for two different preparations of stimulated cells not treated with an inhibitor. The positions of the 69- and 63-kDa Paks are designated by a filled arrowhead and a solid arrow, respectively. The renaturable 49- and 40-kDa kinases are marked by an open arrowhead and a dashed arrow, respectively.

before stimulation with $1.0\ \mu\text{M}$ fMLP completely blocked activation of the 63- and 69-kDa Paks along with the 49- and 40-kDa kinases (Fig. 1*c*). In contrast, $50\ \mu\text{M}$ W-13 was only partially effective in blocking activation of these kinases and $50\ \mu\text{M}$ W-5 lacked activity (Fig. 1, *d* and *e*). Increasing the concentration of W-13 to $100\ \mu\text{M}$ markedly reduced activation of the 63- and 69-kDa Paks, whereas the unchlorinated derivative W-12 was inactive even at this concentration (Fig. 1, *f* and *g*).

Fig. 2 presents dose-response data for blockade of Pak activation by W-7 and several other structurally diverse, selective inhibitors that block different aspects of Ca^{2+} signaling. The antagonists employed consisted of inhibitors of phospholipase C (PLC) (U-73122) (37), the intracellular Ca^{2+} channel (TMB-8) (38), the $\text{Ca}^{2+}/\text{CaM}$ complex (trifluoperazine (TFP)) (39), and $\text{Ca}^{2+}/\text{CaM}$ -activated protein kinases (KN-93) (40). These compounds were chosen, in part, because of the availability of less active analogues to monitor possible nonspecific effects of the antagonists on cells. The less-active analogues of TFP, U-73122, and KN-93 are promethazine (PMZ), U-73343, and KN-92, respectively (37, 39, 40). All of the antagonists listed above blocked activation of the 63- and 69-kDa Paks in fMLP-stimulated neutrophils in a dose-dependent manner at their effective pharmacological doses (Refs. 37–42; see below).

The decreases in Pak activity with these compounds were estimated by densitometry performed on x-ray films by comparing the peak heights of the bands in lane *b* with those in lanes *c* or *d* in Figs. 1 and 2. Treatment of neutrophils with $50\ \mu\text{M}$ W-7, $15\ \mu\text{M}$ TFP, $1.0\ \mu\text{M}$ U-73122, $200\ \mu\text{M}$ TMB-8, or $50\ \mu\text{M}$ KN-93 before stimulation with $1.0\ \mu\text{M}$ fMLP for 15 s reduced the content of ^{32}P in the 63- and 69-kDa bands by $93 \pm 3\%$ and $92 \pm 3\%$, $87 \pm 14\%$ and $82 \pm 17\%$, $83 \pm 12\%$ and $84 \pm 10\%$, $78 \pm 14\%$ and $73 \pm 25\%$, and $72 \pm 5\%$ and $73 \pm 9\%$ (SD, $n = 3-5$), respectively. In contrast, similar treatment of the cells with $50\ \mu\text{M}$ W-5, $15\ \mu\text{M}$ PMZ, $1.0\ \mu\text{M}$ U-73343, or $50\ \mu\text{M}$ KN-92 did not significantly reduce the content of ^{32}P in the 63- or 69-kDa bands (data not shown; $n = 3-5$).

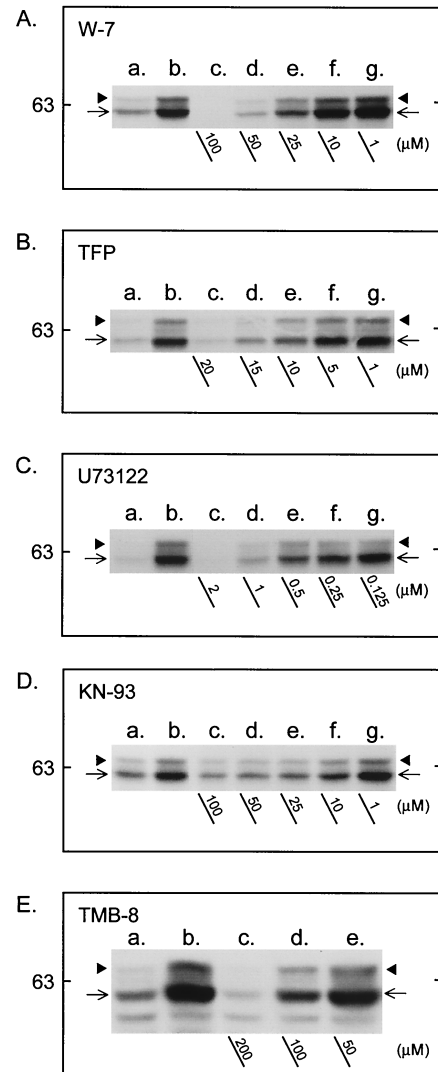


FIGURE 2. Effects of different concentrations of antagonists on activation of the Paks in neutrophils. Neutrophils were treated with various amounts of W-7 (A), TFP (B), U-73122 (C) and TMB-8 (E) for 5.0 min or KN-93 (D) for 30 min and then stimulated with $1.0\ \mu\text{M}$ fMLP for 15 s. Paks were monitored by their ability to undergo renaturation and catalyze phosphorylation of the p47-*phox* peptide fixed within a gel as described in *Materials and Methods*. In all cases, lane *a* is for cells treated with 0.25% (v/v) DMSO for 15 s (unstimulated neutrophils) and lane *b* is for cells stimulated with $1.0\ \mu\text{M}$ fMLP for 15 s. The positions of the 69- and 63-kDa Paks are designated by an arrowhead and arrow, respectively.

Addition of $50\ \mu\text{M}$ W-7, $100\ \mu\text{M}$ W-13, $15\ \mu\text{M}$ TFP, $1.0\ \mu\text{M}$ U-73122, $200\ \mu\text{M}$ TMB-8, or $100\ \mu\text{M}$ KN-93 to the phosphorylation step of the “in gel” renaturation assay with the p47-*phox* peptide substrate did not affect this reaction ($n = 2$; data not shown). These data strongly indicate that these antagonists did not interact with the 63- and 69-kDa Paks themselves, but on upstream components involved in the activation of the kinases (see *Discussion*). The concentrations of inhibitors and conditions used did not affect cell viability, as measured by the exclusion of trypan blue or by the release of lactate dehydrogenase from the cells (data not shown; Ref. 37, 38, and 41).

Neutrophils stimulated with $1.0\ \mu\text{M}$ fMLP release large quantities of O_2^- (i.e., $46 \pm 9\ \text{nmol}\ \text{O}_2^-/\text{min}/10^7$ cells) as a result of the activation of the NADPH-oxidase system (2). Compounds W-7, TFP, U-73122, and TMB-8 are known to inhibit O_2^- release from stimulated neutrophils (37, 38, 41). The concentrations of

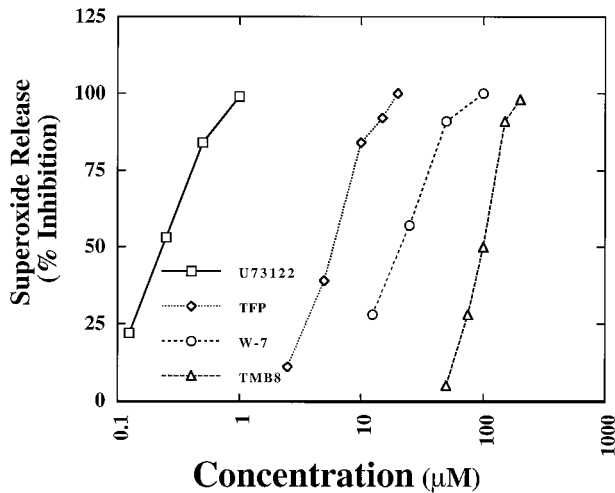


FIGURE 3. Effects of different concentrations of antagonists on O_2^- release from neutrophils. Neutrophils were treated with various amounts of U-73122 (\square), TFP (\diamond), W-7 (\circ) and TMB-8 (\triangle) for 5.0 min at 37°C and then stimulated with $1.0 \mu\text{M}$ fMLP. O_2^- was assayed as referenced in *Materials and Methods*. Data points represent mean values of two to three different experiments.

these drugs that blocked O_2^- release (Fig. 3) were very similar to those that inhibited activation of the 63- and 69-kDa Paks (Fig. 2). In contrast, treatment of neutrophils with (50–100 μM) KN-93 for 15–30 min at 37°C before stimulation with $1.0 \mu\text{M}$ fMLP had no effect on O_2^- release ($n = 4$; data not shown) even though this antagonist was effective against Pak (Fig. 2D).

Effects of Ca^{2+} /CaM antagonists on the activation of Rac in stimulated neutrophils

The fact that a variety of antagonists of Ca^{2+} signaling events were equally effective in blocking activation of the Paks and NADPH-oxidase complex in neutrophils suggested that these inhibitors might be effecting a common component of these systems. As noted above, activated Rac (GTP-bound) can trigger autophosphorylation/activation of Pak (11). Rac-GTP is also an obligatory subunit of the NADPH-oxidase system (43). The effects of CaM antagonists on the activation of Rac in fMLP-stimulated neutrophils were examined with a fusion protein containing the p21-binding domain of Pak that binds only the GTP-bound form of Rac (33, 44, 45). Rac exhibited maximal activation within 15 s of cell stimulation, followed by significant inactivation at 3.0 min (Fig. 4A). This pattern of activation was virtually identical with that exhibited by the 63- and 69-kDa Paks in fMLP-stimulated neutrophils (Fig. 4B; Refs. 2 and 4). Incubation of neutrophils with 50 μM W-7 or 15 μM TFP for 5.0 min at 37°C before stimulation with $1.0 \mu\text{M}$ fMLP for 15 s blocked activation of Rac, whereas 50 μM W-5 or 15 μM PMZ did not effect this process (Fig. 4C). This treatment of neutrophils with W-7, W-5, TFP, and PMZ reduced activation of Rac by $91 \pm 5\%$, $9 \pm 10\%$, $97 \pm 5\%$, and $36 \pm 8\%$ (SD, $n = 3$), respectively. Treatment of neutrophils with 200 nM wortmannin for 10 min at 37°C before stimulation with $1.0 \mu\text{M}$ fMLP for 15 s reduced the activation of Rac by $68 \pm 9\%$ (range, $n = 2$) (Fig. 2D) as reported in previous studies (33, 44). In contrast, treatment of neutrophils with 50 μM KN-93 for 30 min at 37°C before stimulation with $1.0 \mu\text{M}$ fMLP for 15 s did not effect the activation of Rac ($n = 2$; Fig. 2D).

Effects of Ca^{2+} /CaM antagonists on the activation of Ras and the extracellular regulated kinases (ERKs) in neutrophils

Previous studies have shown that CaM antagonists can either block (27, 28) or prolong (46) the activation of ERKs in various cell types. Because ERKs are effector proteins for the small GTPase Ras, we examined the effects of CaM antagonists on the activation of Ras, MEK, and ERK in neutrophils (Figs. 5 and 6). Previous studies have shown that neutrophils stimulated with fMLP exhibit rapid activation of Ras (8) and ERK-1/2 (6–10, 35). We confirmed the activation of Ras in guinea pig neutrophils stimulated with fMLP using a fusion protein that contained the Ras binding domain of Raf coupled to glutathione agarose beads. This fusion protein binds only the activated, GTP-bound form of Ras (32). Ras exhibited maximal activation within 15–30 s followed by significant inactivation at 1.0–3.0 min (Fig. 5A). This activation of Ras was insensitive to 200 nM wortmannin ($n = 3$; data not shown). Treatment of neutrophils with 50 μM W-7 or 15 μM TFP for 10 min before stimulation with $1.0 \mu\text{M}$ fMLP for 30 s markedly reduced the activation of Ras, whereas compounds W-5 (50 μM) and PMZ (15 μM) had little or no effect on this reaction (Fig. 5B). This treatment with 50 μM W-7, 50 μM W-5, 15 μM TFP, or 15 μM PMZ reduced the activation of Ras by $80 \pm 18\%$, $10 \pm 9\%$, $97 \pm 5\%$, and $16 \pm 27\%$ (SD, $n = 3$), respectively.

Activation of ERK-1/2 and MEK in neutrophils was monitored with Abs that recognized only the activated (doubly phosphorylated) forms of these kinases. p42-ERK, a small amount of p44-ERK, and MEK undergo a pronounced activation in neutrophils at ~ 1.0 –3.0 min after stimulation of these cells with fMLP (35). Previous studies have shown that MEK-2 is the predominant isoform of this kinase in human neutrophils and undergoes activation in fMLP-stimulated cells (47). Data presented in Fig. 6 demonstrate the effects of CaM antagonists on the activation of ERK-1/2 and MEK in neutrophils. The cells were incubated with the antagonists for 10 min at 37°C and then stimulated with $1.0 \mu\text{M}$ fMLP for 3.0 min. A single blot was first stained for activated ERKs (Fig. 6A) and then stained again for activated MEK (Fig. 6B). As was the case with Ras, 50 μM W-7 and 15 μM TFP markedly reduced the activation of ERKs and MEK in these cells whereas 50 μM W-5 and 15 μM PMZ had little or no effect. Visualization of MEK by the chemiluminescence detection system required a longer reaction time than that needed for p42-ERK (Fig. 6A), which resulted in the p42-ERK band being over-exposed in Fig. 6B. Treatment of the cells with 50 μM W-7, 50 μM W-5, 15 μM TFP, and 15 μM PMZ reduced the activation of p42-ERK by $96 \pm 4\%$, $21 \pm 10\%$, $100 \pm 0\%$, and $19 \pm 26\%$ ($n = 2$ –3), respectively. These data were calculated by densitometry from blots stained for p42-ERK alone as shown in Fig. 6A. Thus, the Ca^{2+} /CaM complex plays a major role in activating the small GTPases Ras and Rac in neutrophils along with their downstream effectors (e.g., Paks, NADPH-oxidase, ERKs).

Discussion

The overall pathway that triggers activation of Rac and Pak in neutrophils is largely unknown. Subunits of complex G proteins coupled to the fMLP receptor are involved in triggering activation of PI-PLC and PI 3-K in neutrophils (48–51). Inositol(1,4,5)-trisphosphate, a product of PI-PLC, promotes a transient increase in intracellular Ca^{2+} in neutrophils by triggering the release of this cation from intracellular storage depots (48). In this paper, we report that a variety of structurally diverse, selective antagonists of PLC, the intracellular Ca^{2+} channel, CaM and CaM-PK block activation of the 63- and 69-kDa Paks. Antagonists of CaM were also found to block activation of Rac and Ras in neutrophils along with

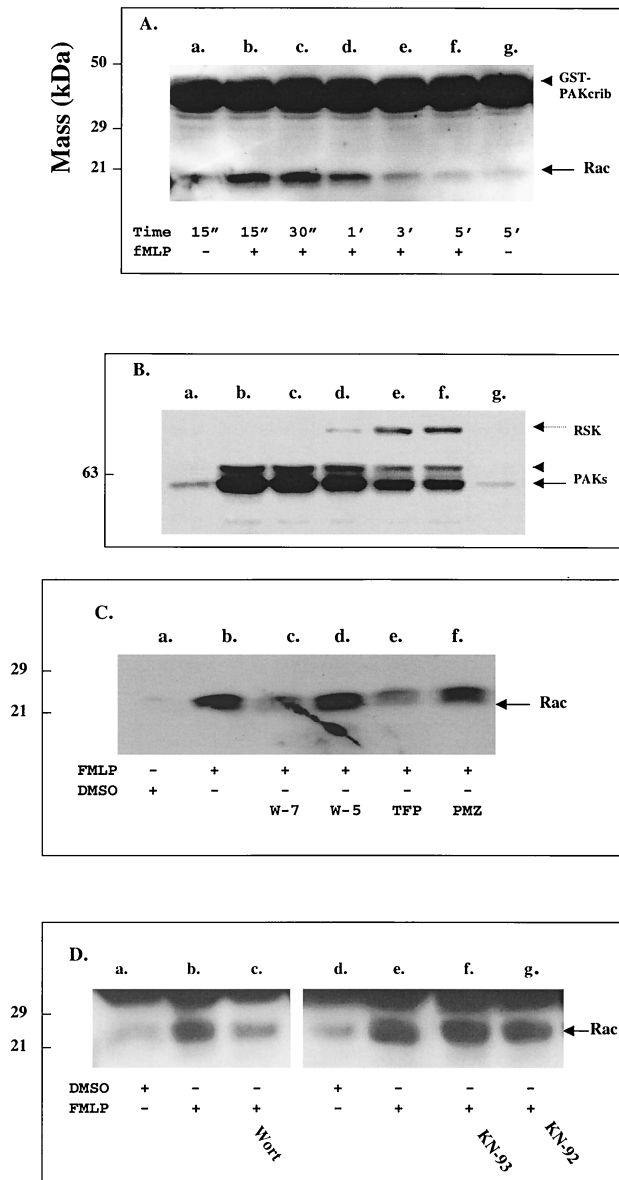


FIGURE 4. Activation of Rac (A) and Pak (B) in stimulated neutrophils. Effects of $\text{Ca}^{2+}/\text{CaM}$ antagonists (C) and other inhibitors (D) on the activation of Rac. A, Time-course for the activation of Rac in neutrophils stimulated with $1.0 \mu\text{M}$ fMLP. Activation of Rac was measured in a pull-down assay with a GST-Pak-CRIB fusion protein as described in *Materials and Methods*. Cells were incubated for 5.0 min at 37°C in the standard assay medium and then stimulated with fMLP ($1.0 \mu\text{M}$) for the times indicated. The position of Rac is designated by an arrow. The location of the GST-Pak-CRIB fusion protein is designated by an arrowhead. B, Time course for the activation of the 63- and 69-kDa Paks in neutrophils stimulated with $1.0 \mu\text{M}$ fMLP. Paks were monitored by their ability to undergo renaturation and catalyze phosphorylation of the p47-phox peptide fixed within a gel as described in *Materials and Methods*. Lanes a–g are for the same assay conditions as in A. The positions of the 69- and 63-kDa Paks are designated by a filled arrowhead and an arrow, respectively. The protein kinase that undergoes activation at time points ≥ 1.0 min (dotted arrow) is p90^{RSK2} . C, Effects of various antagonists on the activation of Rac. Cells were treated with the inhibitors for 5.0 min at 37°C and then stimulated with $1.0 \mu\text{M}$ fMLP for 15 s. Lane a is for unstimulated neutrophils. Lane b is for stimulated cells. Lanes c–f are for stimulated neutrophils treated with the following: $50 \mu\text{M}$ W-7 (c), $50 \mu\text{M}$ W-5 (d), $15 \mu\text{M}$ TFP (e), and $15 \mu\text{M}$ PMZ (f). D, Effects of various antagonists on the activation of Rac. Cells were incubated at 37°C with or without inhibitors for 10 min (lanes a–c) or 30 min (lanes d–g) and then stimulated with $1.0 \mu\text{M}$ fMLP for 15 s. Lanes a and d are for unstimulated cells. Lanes b and

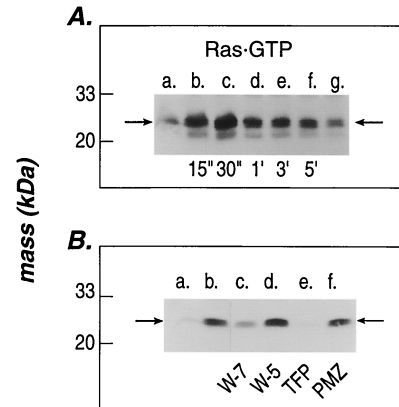


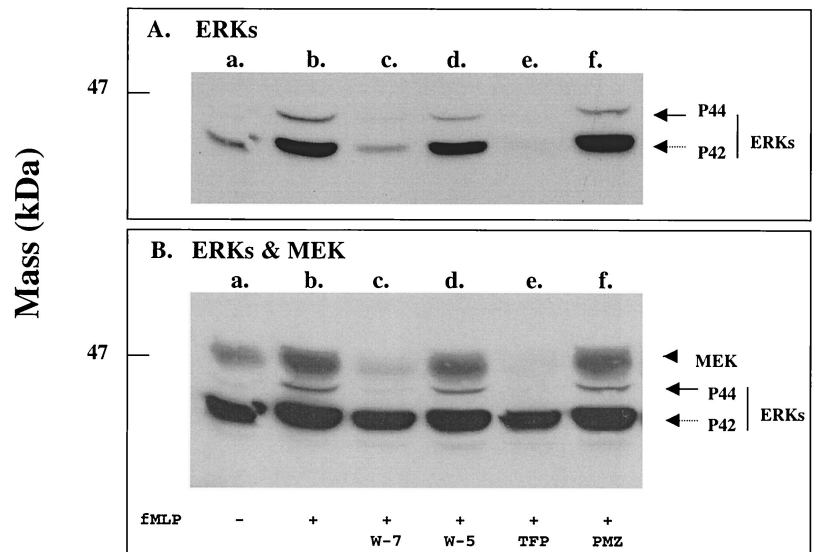
FIGURE 5. Effects of $\text{Ca}^{2+}/\text{CaM}$ antagonists on activation of Ras in neutrophils. Activation of Ras was measured in a pull-down assay with a GST-Raf-RBD fusion protein as described in *Materials and Methods*. A, Time-course for the activation of Ras in neutrophils stimulated with $1.0 \mu\text{M}$ fMLP. Cells were incubated for 5.0 min at 37°C in the standard assay medium and then stimulated with fMLP for the times indicated. B, Effects of various antagonists on the activation of Ras. Cells were treated with the inhibitors for 5.0 min at 37°C and then stimulated with $1.0 \mu\text{M}$ fMLP for 30 s. Lane a is for unstimulated neutrophils. Lane b is for stimulated cells. Lanes c–f are for stimulated cells treated with the following: $50 \mu\text{M}$ W-7 (c), $50 \mu\text{M}$ W-5 (d), $15 \mu\text{M}$ TFP (e), and $15 \mu\text{M}$ PMZ (f). The position of activated Ras is designated by the arrow.

other downstream targets of these small GTPases. The significance of these and other novel observations are discussed below.

It is not possible to employ the techniques of molecular biology to investigate the involvement of the $\text{Ca}^{2+}/\text{CaM}$ complex in Pak activation in primary neutrophils because these cells are short-lived. Therefore, particular care was taken to employ structurally distinct antagonists, which blocked different stages in triggering the increase in cytosolic Ca^{2+} and the subsequent target/effector proteins. The choice was also restricted to those compounds for which less active analogues were available. The order of effectiveness of the CaM antagonists in blocking activation of Paks (i.e., $\text{TFP} > \text{W-7} > \text{W-13} > \text{PMZ}$, W-5, and W-12) (Figs. 1 and 2) was identical with that reported for CaM -dependent enzymes *in vitro* (39). Although TFP and W-7 can also inhibit protein kinase C (PKC) at high concentrations (52), PKC is not involved in activating the Paks in neutrophils (1, 4, 5). Moreover, the concentrations of TFP and W-7 required for blockade of Pak activation (i.e., ~ 10 and $25 \mu\text{M}$, respectively) (Fig. 2) were very similar to that observed for CaM -dependent processes (39) and severalfold less than that required to inhibit PKC (52). The IC_{50} values for TFP, W-7, W-5, and PMZ blocking the CaM mediated activation of cAMP phosphodiesterase *in vitro* are 2–10, 28, 240, and 200–340 μM , respectively (39). The concentrations of TFP, W-7, U-73122, KN-93, and TMB-8 routinely used to block CaM , CaM , PLC, CaM -PK, and the intracellular Ca^{2+} channel *in vivo* are ~ 10 , 35, 1.0, 20, and 100–300 μM , respectively (37–39, 42). Each of these antagonists were effective at blocking activation of the Paks in neutrophils at these doses (Fig. 2). In contrast, the antagonists W-7 ($50 \mu\text{M}$) and TFP ($15 \mu\text{M}$) did not effect the increases in

e are for stimulated cells. Lanes c, f, and g are for stimulated cells treated with the following: 200 nM wortmannin (c), $50 \mu\text{M}$ KN-93 (f), and $50 \mu\text{M}$ KN-92 (g).

FIGURE 6. Effects of Ca^{2+} /CaM antagonists on activation of the ERKs (A) and MEK (B) in stimulated neutrophils. Activation of ERKs (A, arrows) and MEK (B, filled arrowhead) was monitored by Western blotting with Abs that only recognized the activated (doubly phosphorylated) forms of these kinases. Stimulation of the cells and Western blotting were performed as described in *Materials and Methods*. The membrane was first blotted with an Ab to activated ERKs (A) and then reblotted with an Ab to activated MEK (B). Cells were treated with the inhibitors for 5.0 min at 37°C and then stimulated with $1.0 \mu\text{M}$ fMLP for 3.0 min. Lane a is for unstimulated neutrophils. Lane b is for stimulated cells. Lanes c–f are for stimulated cells treated with the following: $50 \mu\text{M}$ W-7 (c), $50 \mu\text{M}$ W-5 (d), $15 \mu\text{M}$ TFP (e), and $15 \mu\text{M}$ PMZ (f).



Ca^{2+} permeability or cytosolic Ca^{2+} that occur in fMLP-stimulated neutrophils (Refs. 38 and 53; data not shown), which indicates that these inhibitors are selective for certain pathways and do not have a general disruptive effect on the fMLP-receptor.

The GEF that promotes activation of Rac (and hence Pak) in fMLP-stimulated neutrophils is blocked by antagonists of PI 3-K and tyrosine kinases (33, 44). Only the γ -isoform of PI 3-K is activated when neutrophils are stimulated with fMLP (49–51). Unlike other isoforms of PI 3-K, the γ -isoform is directly activated by $\beta\gamma$ -subunits of complex G proteins and is not activated by binding to tyrosine phosphorylated proteins (49–51). Thus, the tyrosine kinase involved in the activation of Rac is not likely to be “upstream” of PI 3-K. One possible explanation for these data is that the relevant GEF requires both products of PI 3-K and tyrosine phosphorylation for activation (Fig. 7). Interestingly, Vav, a GEF for Rac, undergoes enhanced phosphorylation/activation by a Src-related kinase when bound to PIP_3 (54). Location of Vav or a similar GEF upstream of Rac would account for the sensitivity of the Pak stimulatory pathway to pertussis toxin, wortmannin, and herbimycin.

How does CaM fit into the scenario described above? Although the answer to this question is not yet known, a modest amount of speculation may be appropriate here. Certain receptors coupled to pertussis-toxin-sensitive G proteins can trigger activation of Src-related tyrosine kinases, Ras and ERK through a stimulatory pathway that contains PI-PLC and CaM (27, 28). Fig. 7 presents a modified portion of that model (28). The ability of CaM antagonists to block activation of Ras and ERK in neutrophils (Figs. 5 and 6) is consistent with a similar stimulatory pathway being present in these cells. Addition of a GEF to this pathway, which is synergistically activated by PI 3-K and tyrosine phosphorylation as described above, will account for all of the data presented herein and in earlier publications (2–5, 12–14). This modified model predicts that the Ca^{2+} /CaM complex is required for activation of a tyrosine kinase that lies upstream of the GEF for Rac. Thus, most (see below) Ca^{2+} /CaM antagonists block activation of Pak (Figs. 1 and 2) by preventing activation of Rac (Fig. 4). Activation of the Src-related kinase Lyn by a mechanism dependent upon Ca^{2+} /CaM has been described in macrophages (55). This scheme can also explain why agonists that only increase intracellular Ca^{2+} in neutrophils (thapsigargin, ionophore A23187) do not trigger activation of Pak (1, 2, 4) because $\beta\gamma$ -subunits of complex G proteins are

also required to effectively activate PI 3-K and thus Rac in these cells. Activation of Tiam1, a GEF for Rac, is catalyzed by CaM-PK and blocked by KN-93 in fibroblasts (42). The inability of KN-93 to block activation of Rac in fMLP-stimulated neutrophils (Fig. 4D) suggests that Tiam1 is either not involved in this reaction in neutrophils or its activation under these circumstances does not require CaM-PK.

In contrast to studies reported in this paper and elsewhere (33, 44), a previous paper has reported that activation of Rac in human neutrophils is insensitive to an antagonist of PI 3-K ($10 \mu\text{M}$ LY294002) and genistein (45). The “pull-down” assay used to monitor activated Rac only measures the fraction of this GTPase that is accessible to the fusion protein and may not detect membrane associated Rac or Rac that forms high affinity complexes

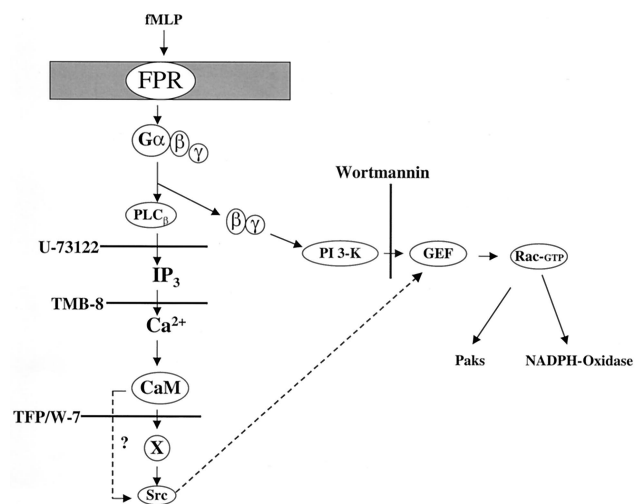


FIGURE 7. A pathway for the activation of Paks during stimulation of neutrophils with fMLP. Occupation of the fMLP receptor (FPR) results in disassociation of the pertussis toxin sensitive heterotrimeric G protein coupled to this receptor to yield the α -GTP-bound and $\beta\gamma$ -subunits. Activation of PI-PLC β by these subunits results in an increase in cytosolic Ca^{2+} , activation of CaM and Src-family tyrosine kinases (Src). The exact link between the Ca^{2+} /CaM complex and Src is not known. Src and PI 3-K γ are thought to be required for activation of the GEF for Rac. Unproven pathways are indicated by dashed lines. FPR, Formyl peptide receptor; IP_3 , inositol(1,4,5)-trisphosphate.

with other effector proteins (44). Differences in assay conditions (e.g., detergents, incubation times) can alter the amount of "protected Rac" and could account for some of the discrepancies in the literature. A corollary of this situation is that the pull-down assay for Rac and the renaturation assay for Pak may monitor different populations of Rac.

It is noteworthy that KN-93, but not KN-92, blocked activation of the Paks in neutrophils (Fig. 2D) but did not effect the activation of Rac (Fig. 4D) or O_2^- release (see *Results*). Thus, KN-93 blocks activation of Pak by a mechanism different from that of W-7 and TFP. KN-93 does not bind to CaM but competes for the CaM binding site on CaM-PK (40). The concentrations of KN-93 that blocked activation of Paks (Fig. 2) were similar to those that inhibit CaM-PK in other cell types (40, 42). The question as to whether a CaM-PK may be involved in the activation of Pak (e.g., by direct phosphorylation?) is currently under investigation.

In summary, we provide evidence that the Ca^{2+} /CaM complex plays a major role in activation of the Paks and ERKs in neutrophils through stimulation of their upstream effectors Rac and Ras. Identifying the direct target(s) of CaM in these pathways may markedly increase our knowledge of the GEFs in neutrophils that control a variety of cellular responses.

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