Calcium-sensing soluble adenylyl cyclase mediates TNF signal transduction in human neutrophils

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Through chemical screening, we identified a pyrazolone that reversibly blocked the activation of phagocyte oxidase (phox) in human neutrophils in response to tumor necrosis factor (TNF) or formylated peptide. The pyrazolone spared activation of phox by phorbol ester or bacteria, bacterial killing, TNF–induced granule exocytosis and phox assembly, and endothelial transmigration. We traced the pyrazolone's mechanism of action to inhibition of TNF-induced intracellular Ca\(^{2+}\) elevations, and identified a nontransmembrane ("soluble") adenylyl cyclase (sAC) in neutrophils as a Ca\(^{2+}\)-sensing source of cAMP. A sAC inhibitor mimicked the pyrazolone's effect on phox. Both compounds blocked TNF–induced activation of Rap1A, a phox–associated guanosine triphosphatase that is regulated by cAMP. Thus, TNF turns on phox through a Ca\(^{2+}\)-triggered, sAC-dependent process that may involve activation of Rap1A. This pathway may offer opportunities to suppress oxidative damage during inflammation without blocking antimicrobial function.

Neutrophils, the most abundant leukocytes in blood, provide a critical element of host defense. Defects in neutrophil numbers, migration across endothelium or bactericidal mechanisms lead to life-threatening infections. At the same time, neutrophil activation is a major contributor to inflammatory tissue damage (1). Thus, it is a goal of antiinflammatory therapy to target neutrophils, but it is a challenge to do so without impairing host defense.

Two families of neutrophil products play key roles in host defense and tissue damage. Activated neutrophils degranulate to release antibiotic proteins, including proteases that degrade connective tissue. Activated neutrophils also secrete reactive oxygen intermediates (ROIs), products of phagocyte oxidase (phox), a multicomponent enzyme whose catalytic flavocytochrome is a transmembrane protein of the granules. ROIs contribute directly to bacterial killing (2) but also promote proteolytic tissue damage by: triggering K\(^{+}\) influx into the phagolysosome, desorbing proteases from their proteoglycan bed (3), activating neutrophil proteases (4) and matrix metalloproteinases (5), inactivating antiproteases (1), and activating NF-κB and AP1, transcription factors for numerous genes with proinflammatory products, including chemokines that attract neutrophils. Thus, inhibition of neutrophil ROI production would be expected to reduce inflammatory proteolysis through multiple mechanisms.

Maximal neutrophil activation can be induced artificially with PMA or physiologically by inflammatory peptides and proteins, such as TNF (6). The soluble, physiologic agonists are far more effective if the neutrophils are adherent to extracellular matrix proteins by way of integrins (7). Adherence (8) and TNF (9) combine to trigger oscillatory elevations of intracellular Ca\(^{2+}\), which take the form of spatially restricted, moving waves in neutrophils responding to N-formylpeptide (10). However, it remains unclear how TNF activates neutrophils. To help answer this question, we
Figure 1. Identification of compounds that inhibit the neutrophil respiratory burst in response to TNF. (A) Chemical structures and concentration-response curves. Compounds were added at 37°C for 30 min before stimulation with TNF (red squares) or PMA (black triangles). H_2O_2 release at 90 min is displayed as percentage of H_2O_2 release seen with TNF or PMA in the presence of the vehicle, DMSO. (B) Reversibility. Neutrophils incubated with DMSO (D), 2, or 5 were left unmanipulated (black bars) or washed (open bars) with buffer before both sets were

Data from: Han et al., JEM, 2005.
RESULTS AND DISCUSSION

We screened 15,000 compounds for those that blocked H$_2$O$_2$ release by TNF-treated, adherent neutrophils, but spared H$_2$O$_2$ release when the same cells were triggered by PMA. The PMA countercheck allowed us to exclude compounds that were toxic, inhibited phox or protein kinase C, or interfered with the assay. Four compounds were both selectively inhibitory and consistently effective with neutrophils from different donors. We chose compound 2 (2-[3-chloro-phenyl]-5-phenyl-2,4-dihydro-pyrazol-3-one) for analysis here. The others (1, 3, and 4) work in different ways, as will be reported elsewhere (Han et al., unpublished data). 5 ((3-morpholino-4-yl-proplyl)-(5,6,7,8-tetrahydro-benzo[4,5]thieno[2,3-d][pyrimidin-4-y1]-amine) shares its core structure with 1 and 3, but had no effect on H$_2$O$_2$ release and was used as a control. Concentration-response curves of 2, using neutrophils from three donors, yielded a mean 50% inhibitory concentration (IC$_{50}$) ± SEM of 1.6 ± 0.4 μM for H$_2$O$_2$ release (Fig. 1 A). A new screen of 1,100 congeners of 2 identified 2’ (5-methyl-4-[2-methylquinolin-8-ylamino]-methyl)-2-phenyl-2,4-dihydro-pyrazol-3-one), with an IC$_{50}$ of 24 ± 6 nM (Fig. 1 A). Because 2’ was identified after most of these studies were completed, we focus here on 2. At 5 μM, 2 yielded >95% inhibition of TNF-triggered H$_2$O$_2$ release with cells from all donors tested. This concentration was used for all further experiments.

When neutrophils were treated with 2 and then washed, they regained responsiveness to TNF (Fig. 1 B). The cells remained susceptible to inhibition when 2 was added back (Fig. 1 B). Thus, inhibition by 2 was fully reversible. When we added 2 to neutrophils that already were releasing H$_2$O$_2$ in response to TNF, the respiratory burst slowed quickly (Fig. 1 C). Thus, the process inhibited by 2 was needed to launch the TNF-triggered respiratory burst and to sustain it.

Next, we tested the effects of 2 on neutrophils’ responses to bacteria and to the major neutrophil stimulant released by *Escherichia coli*, formylated methionyl-leucyl-phenylalanine (fMLF). H$_2$O$_2$ release triggered by fMLF was blocked by 2. However, 2 did not inhibit H$_2$O$_2$ release triggered by *Salmonella enterica* var. Typhimurium, and only reduced slightly the respiratory burst that was elicited by *Listeria monocytogenes* (Fig. 1 D). 2 also did interfere with the killing of these pathogens by neutrophils (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20050778/DC1).

Degranulation delivers the phox flavocytochrome to phagosomal and plasma membranes (11) where the cytosolic components of phox are recruited. Therefore, we considered that 2 might block activation of phox by blocking degranulation. However, 2 did not block TNF-induced exocytosis of the specific granule marker, lactoferrin, and only inhibited slightly the release of the azurophil granule marker, myeloperoxidase (Fig. 1 E).

The large-scale respiratory burst triggered by TNF or fMLF requires that neutrophils spread on extracellular matrix (6, 7). Pretreatment with 2 allowed neutrophils to spread normally on serum-coated glass in response to PMA. With TNF as a stimulus in 2-pretreated cells, spreading was initiated, which suggested that 2 did not interfere with binding of TNF or transmission of some signals to the cytoskeleton. However, 2 caused TNF-triggered spreading to halt prematurely, which suggested inhibition of a later stage in TNF signaling (Fig. 2 A).

Interference with spreading of neutrophils in response to TNF raised the question of whether 2 might block migration across endothelium—the process by which neutrophils enter tissues. However, pretreatment of neutrophils with 2 blocked neither the adhesion of the cells to, nor their migration across, monolayers of TNF-activated human umbilical vein endothelial cells (HUVECs; Fig. 2, available at http://www.jem.org/cgi/content/full/jem.20050778/DC1). Moreover, pretreatment of HUVECs with 2 did not influence the integrity of the monolayer nor the cells’ ability to respond to TNF by supporting adhesion and transmigration of untreated neutrophils (unpublished data). Similarly, a 2-d incubation with 2 did not affect the morphology or adherence of primary mouse peritoneal exudate macrophages, nor the ability of TNF to synergize with IFN-γ in inducing them to release nitric oxide (unpublished data). These experiments demonstrated that 2 inhibited only a selective aspect(s) of TNF signal transduction.

Neutrophil responses to TNF require protein tyrosine phosphorylation involving Syk (12, 13); Src family kinases, Hck and Fgr (14); and Pyk2 (15, 16). However, treatment of adherent neutrophils with 2 had little impact on overall protein tyrosine phosphorylation induced by TNF (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20050778/DC1). Nor did 2 inhibit recombinant Src (Fig. S3 B), Syk (Fig. S3 C), or TNF-induced (auto)phosphorylation of Pyk2 on tyrosine 402 (Fig. S3 D), which is critical for Pyk2’s kinase activity (17).

Cytosolic components of phox—p40$^{phox}$, p47$^{phox}$, and p67$^{phox}$—form a M$_c$ ~240,000 complex (18) in resting neu-
trophils, with p47phox serving as an essential adaptor. The complex translocates to the membrane upon activation. 2 had no effect on TNF-induced translocation of p47phox to the membrane (Fig. 3 A). Because 2 inhibited neither the assembly of phox (Fig. 3 A) nor its catalytic activity when triggered by PMA (Fig. 1 A), we hypothesized that 2 might interfere with a later stage in phox activation, namely, TNF-dependent activation of one of the guanosine triphosphatases bound to phox, Rac2 (19) or Rap1A (20). Rac2-deficient neutrophils have severe migration defects (21), whereas 2-treated neutrophils migrated normally (Fig. S2). Thus, Rac2 was unlikely to be the locus of regulation by 2, and we focused on Rap1A.

Rap1A is bound stoichiometrically to the phox flavocytochrome in a guanosine triphosphate (GTP)- and phosphorylation-dependent manner (22). Treatment of neutrophils with fMLF or GM-CSF activated Rap1A (i.e., converted it to the GTP-bound state; reference 23). We found that TNF treatment of adherent neutrophils also activated Rap1A, and 2 blocked TNF-induced activation of Rap1A (Fig. 3 B).

Rap1A can be activated by a guanine nucleotide exchange protein, exchange protein directly activated by cAMP (Epac; references 24, 25). The cAMP that activates Epac can arise from the long-studied G protein–regulated, transmembrane adenylyl cyclases (tmACs) or from a recently discovered nontransmembrane (“soluble”) adenylyl cyclase, sAC, which is regulated by intracellular Ca2+ and bicarbonate (26, 27). We considered tmACs unlikely candidates for neutrophil activation because their pharmacologic stimulation inhibits most neutrophil functions, including TNF-induced activation of phox (28). Thus, we asked if neutrophils contain sAC.

Immunoblot revealed sAC in highly purified neutrophil preparations (Fig. 4 A). Neutrophils were the source of the immunoreactivity because they were stained uniformly and specifically by three mAbs, each directed against a different epitope of sAC, as shown for one mAb in Fig. 4 B. The granular pattern of staining raised the possibility that sAC may reside near phox, but definitive analysis of sAC’s subcellular localization awaits immuno-electron microscopy.

Next, we selected pharmacologic reagents to distinguish between possible sources of cAMP in a mammalian cell. A chemical screen identified a specific inhibitor of recombinant human sAC, 2-(1H-benzoimidazole-2-ylsulfanyl)-propionic acid (5-bromo-2-hydroxy-benzylidene)-hydrazide (KH7) that spares tmACs (29). Reciprocally, we determined a concentration range in which 2,5′-dideoxyadenosine (ddAdo) acts as a selective inhibitor of tmACs, sparing sAC. Like 2, KH7 inhibited TNF-triggered activation of Rap1A (Fig. 3 B), and suppressed TNF-triggered H2O2 release with an IC50 ± SEM of 4.7 ± 0.3 μM while sparing PMA-triggered H2O2 release (Fig. 4 C). In contrast, the selective inhibitor of tmACs, ddAdo, had no effect on the activation of Rap1 (Fig. 3 B) or on the TNF-induced respiratory burst (Fig. 4 C). KH7 seemed to elicit a small degree of Rap1A activation by itself, the basis of which is not known. These findings suggest that TNF-triggered activation of Rap1 is mediated by sAC, not by tmACs.
Unlike KH7, 2 did not inhibit recombinant sAC (data not shown). Thus, we hypothesized that 2 might block a step upstream of sAC, such as TNF-induced elevation of intracellular Ca\(^{2+}\). We first demonstrated that neutrophil sAC could respond to elevations of intracellular Ca\(^{2+}\). Ionomycin, a Ca\(^{2+}\) ionophore, induced production of cAMP in neutrophils, and the ionomycin-induced cAMP production was blocked by the sAC inhibitor, KH7 (Fig. 4 D); this indicated that sAC was responsible for the increase in cAMP.

**Figure 4.** sAC as a critical element of TNF signaling in neutrophils. (A) Western blot. Neutrophils were lysed and subjected to SDS-PAGE/Western blot with anti-sAC mAb R21. Lane 1 (control): lysate of Chinese hamster ovary cells transfected with sperm isoform of sAC. Lanes 2, 3: neutrophil lysates treated without (lane 2) or with (lane 3) protease inhibitor diisopropylfluorophosphate. (B) Immunofluorescence microscopy. Neutrophils were stained with or without biotinylated anti-sAC mAb R41 followed by streptavidin-Alexafluor 594. (C) Effects of sAC inhibitor KH7 and tmAC inhibitor ddAdo on the respiratory burst. Compounds were added to adherent neutrophils at 37°C for 30 min before stimulation with TNF (red squares) or PMA (black triangles). (D) sAC activity in neutrophils (5 × 10\(^6\)/well) preincubated 30 min with the phosphodiesterase inhibitor, isobutylmethylxanthine (100 μM) and DMSO (D), 2, or KH7, then stimulated with ionomycin (1 μM). cAMP accumulation was measured at 0 min and 2 min after stimulation. (E) Impact on TNF-triggered Ca\(^{2+}\) elevation in Fluo-3/AM-loaded neutrophils. Left panels are pseudocolored images of adherent neutrophils pretreated with DMSO (D) or 2 for 30–60 min and then treated with TNF or no stimulus (NS). Average relative fluorescence intensity (RFI) for all cells in similar microscopic fields is plotted as a function of time on the right. Arrow marks addition of TNF. Some cells were pretreated with KH7 (25 μM) as indicated. (F) Speculative model.
cAMP, which confirmed that 2 does not inhibit endogenous sAC. To test whether 2 blocked TNF-induced Ca2+ elevation, we loaded adherent neutrophils with the Ca2+-sensitive fluorochrome, Fluo3/AM, and studied their fluorescence by way of videomicroscopy. TNF triggered an almost instantaneous elevation of intracellular Ca2+ that persisted throughout the observation period (~2 min; Fig. 4 E). The elevation seemed to be nearly constant when expressed as average fluorescence intensity per microscopic field in fields that contained hundreds of cells. However, inspection of individual cells revealed asynchronous, irregular oscillations, as reported (30). Pretreatment of the cells with KH7 did not suppress the TNF-induced increase in intracellular Ca2+ (Fig. 4 E), although 2 had no effect on Ca2+ elevation that was induced artificially by ionomycin (not depicted). In contrast, pretreatment with KH7 did not suppress the TNF-induced increase in intracellular Ca2+ (Fig. 4 E). We propose that 2 be named “neucalcin-1” for neutrophil calcium inhibitor, and 2’ be named “neucalcin-2.”

These findings outline a pathway for TNF signal transduction that is distinct from the known routes that lead to transcriptional regulation and apoptosis. Fig. 4 F outlines a speculative model: exposure of adherent neutrophils to TNF leads to elevation of intracellular Ca2+ (9, 30), which stimulates sAC (26, 27) to generate cAMP. The elevated cAMP causes activation of Rap1A, perhaps by way of Epac. Activated Rap1A may turn on phox, whose components have been assembled in response to TNF, fMLF, and other soluble, physiologic agonists. This proposed pathway is independent of the previously described TNF signaling cascade involving phosphatidylinositol 3-kinase and tyrosine kinases that mediate degradation and the degradation–dependent assembly of phox (13, 16). The sAC-dependent and sAC-independent pathways seem to converge on phox. The two chemically distinct inhibitors used in this study, neucalcin and KH7, seem to act at two different points in the sAC-dependent pathway (Fig. 4 F). However, further work is needed to identify the molecular target of neucalcins, the means by which TNF triggers the elevation of intracellular Ca2+, the role (if any) of Epac, and the participation and mechanism of Rap1A in the activation of phox.

It may seem paradoxical that in previous studies, direct measurement of cAMP levels in TNF-treated neutrophils did not reveal an elevation; on the contrary, TNF prolonged a decline in cAMP that was induced when neutrophils adhered to biologic surfaces (28). Those observations can be reconciled with our new observations by proposing that the elevations of cAMP that are required to sustain the activation of phox by TNF are spatially confined and quantitatively insufficient to nullify the decrease in cAMP that proceeds at the same time in a larger cellular compartment.

Opposing effects of cAMP have been reported on protein kinase B, depending on whether the cAMP effector is protein kinase A or Epac (31). A similar situation may pertain to Rap1A. Pharmacologically induced, global, sustained elevations in cAMP that suppress neutrophil functions are likely to activate protein kinase A, which can phosphorylate Rap1A and block its association with phox (22). In contrast, because TNF-induced Ca2+ elevations in neutrophils are oscillatory (9, 30) and probably localized (10), sAC-mediated production of cAMP and the ensuing activation of Rap1A also may be oscillatory and localized. It has been argued that Rap1A must undergo cycles of association with, and dissociation from, phox to sustain phox activity (32, 33). The kinetic studies in Fig. 1 C are consistent with the concept that sustained H2O2 release in response to TNF may require repetitive Rap1A activation.

Thus, we have identified a new enzyme in neutrophils, sAC; a new pathway for TNF signal transduction, centered on sAC; the ability of endogenous sAC to sense Ca2+ elevations; a possible functional role for the Ca2+ elevations in adherent, TNF-stimulated neutrophils; evidence that cAMP can not only inhibit but also activate neutrophils; and chemical agents, neucalcins, that may serve as tools to discover how TNF leads to Ca2+ elevation. These findings help to generate a list of targets whose inhibition might allow selective intervention in neutrophil functions.

Blocking nonbacterial activation of phox might be a more effective way to reduce neutrophil–dependent proteolysis of tissue than attempting to inhibit individual proteases. The latter approach suffers from the redundancy of proteases released by neutrophils, and those produced by other cells but activated, in part, by neutrophils. In contrast, selective interference with the inflammatory activation of phox may retard the release of neutrophil proteases from their proteoglycan matrix in the lysosomes (3); prevent the oxidative activation of neutrophil proteases (4); blunt the oxidative activation of matrix metalloproteinases (5); diminish the oxidative inactivation of the major antiproteases secretory leukocyte protease inhibitor, α1-antiprotease and α2 macro- globulin (1); and diminish the oxidative contribution to NF-κB– and AP1-dependent expression of chemokines and other inflammatory mediators.

In sum, this work shows that it is feasible to block the activation of phox by inflammatory factors without interfering with neutrophil migration or phox–dependent killing of ingested bacteria.

**MATERIALS AND METHODS**

**Cells.** All cells were used under Institutional Review Board–approved protocols. Neutrophils were isolated from heparinized (10 U/ml) blood of healthy, consenting adult donors to >95% purity using Polymorphprep (Axis-Shield PoC AS) according to the manufacturer’s instructions. Contaminating erythrocytes were lysed by hypotonic shock for 45 s with 0.2% saline. Neutrophils were resuspended in Krebs Ringer phosphate with glucose (KRPG) formulated as described (34). HUVECs were isolated from umbilical cords and cultured as described (35). Percutaneous macrophages were isolated from C57BL/6 mice 4 d after injection of 2 ml of thioglycollate broth (4%) and cultivated as described (34).

**H2O2 release and degradation.** The neutrophil respiratory burst was measured as described (34). In brief, 96-well flat-bottomed plates (Primaria, Falcon) were coated with 50 μl/well of FBS in 5% CO2 in air at 37°C for at least 1 h and washed three times with 0.9% saline. 1.5 × 104 neutrophils
were added to triplicate wells containing 100 μl of reaction mixture (2.4 nmol scopoletin, 0.5 μg horseradish peroxidase, and 1 mM NaO₂) and stimulated with either buffer control, TNF (Preprotech) or PMA, each at 100 ng/ml. The reduction of scopoletin by H₂O₂ was recorded every 15 min on a plate-reading fluorometer until H₂O₂ release reached plateau. The amount of H₂O₂ released was calculated as described (34). The supernate from the H₂O₂ release assay with 1.5 × 10⁶ neutrophils was used to measure degranulation using lactoferrin or myeloperoxidase ELISA kit (Oxis International, Inc.). Spontaneous cell death from the same supernate was measured using Cytotoxicity Detection Kit (Roche Molecular Biochemicals), an assay for lactate dehydrogenase. The total cell content of lactoferrin, myeloperoxidase, and lactate dehydrogenase was determined after lysing cells with 1% Triton X-100.

Automated assay screening. A collection of 15,000 compounds with drug-like characteristics from ChemDiv Labs was screened robotically in the High Throughput Screening Facility at The Rockefeller University to identify specific inhibitors of TNF-triggered H₂O₂ release in human neutrophils. KH7 was identified in the same library during an independent screen for inhibition of sAC. H₂O₂ release was measured as above with the following modifications. Black instead of clear, 96-well tissue culture plates (Falcon, catalog number 353945) were used to reduce background fluorescence as measured in a Perkin-Elmer Fusion microplate reader. A Titerate MultiDrop 96/384 dispenser was used for bulk reagent dispensing, Bi-Tek Eks 405 Select system was used for plate washing, and a PerkinElmer MiniTrakV liquid handling dispenser was used for the delivery of compound aliquots to each well. Percent inhibition by each compound was calculated using the formula: (T₀−T₉₀)/(T₀−T₉₀), where T₀ and T₉₀ are fluorescence readings at each well at 0 and 90 min, respectively, and Tc₀ and Tc₉₀ are the mean fluorescence readings in the compound-free control wells at 0 and 90 min, respectively. Compounds that showed >90% inhibition of the TNF-triggered respiratory burst were retested with TNF and PMA as stimuli to eliminate nonspecific, toxic chemicals and protein kinase C inhibitors.

Test of reversibility of inhibition. Neutrophils were incubated with each compound at 37°C for 30 min in FBS-coated tubes (Falcon), washed twice with cold KRPG (5 min each at 4°C), and plated in 96-well plates before immediate stimulation with TNF or an equivalent volume of KRPG buffer as a control. On a separate plate, compounds were added back to the neutrophils from which compounds had been washed off, and cells were stimulated to evaluate the neutrophils’ capacity to respond. Neutrophils incubated with each compound without washing also were stimulated for comparison.

Bacteria-triggered respiratory burst. Neutrophils were incubated with DMSO or each compound for 30 min at 37°C and exposed to 10% autologous serum-opsinized S. enterica var. Typhimurium (14028S; American Type Culture Collection) or L. monocytogenes (104035; American Type Culture Collection) at a multiplicity of infection of 0.5 bacteria per neutrophil. H₂O₂ release was measured as described before.

Bacterial killing. Neutrophils were preincubated with or without 2 for 30 min at 37°C and exposed to 10% autologous serum-opsinized S. enterica var. Typhimurium (14028S; American Type Culture Collection) or L. monocytogenes (15323; American Type Culture Collection) at a multiplicity of infection of 0.5 bacteria per neutrophil. Neutrophils were lysed at the indicated times with 1% sodium deoxycholate for assays with Salmonella or 1% Triton X-100 for assays with Listeria. Bacteria recovered from each condition were grown in Luria Bertani agar plates at 37°C overnight, and the colonies were counted.

Morphology. Acid-washed glass cover slips were placed in a 12-well tissue culture plate, coated with FBS in 5% CO₂ at 37°C for at least 1 h, and washed three times with 0.9% saline. Neutrophils (2 × 10⁶) were added to each well in 1 ml of reaction mixture, and incubated or not with each compound at 37°C for 30 min before stimulation with TNF (100 ng/ml), PMA (100 ng/ml), or an equal volume of KRPG. Cells were fixed with 2% paraformaldehyde and 3.7% formaldehyde buffer, and photographed through a phase-contrast microscope. Results are typical of those based on inspecting living cells at 15-min intervals over 2 h.

Transendothelial migration assay. HUVECs were isolated and cultured in M199 medium (GIBCO BRL) supplemented with 20% normal human serum, penicillin, and streptomycin as described (35). Experiments used cells at passage two that were cultured on hydrated type I collagen gels in 96-well plates. Where shown, HUVECs were stimulated with TNF (50 pg/ml) in 5% CO₂ at 37°C for the final 12–18 h. Neutrophils were isolated from the peripheral blood of healthy adult volunteers by density gradient sedimentation in a discontinuous gradient of Ficoll (GE Healthcare) and Histopaque (Sigma-Aldrich), washed in Hanks’ balanced salt solution with 0.1% human serum albumin, resuspended to 0.5 × 10⁶ cells/ml, and added to HUVEC monolayers. Test compounds were incubated with the neutrophils at room temperature for 30 min before transendothelial migration, and allowed to remain during the duration of the assay. After neutrophils were allowed to transmigrate for 30 min at 37°C, nonadherent neutrophils were washed off. Cells were fixed with 2% PFA, and the remaining adherent and transmigrated cells were fixed with the endothelial monolayer by incubation overnight in 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1 sodium cacodylate buffer. HUVECs and neutrophils were stained differentially with Wright-Giemsa stain, and adherent and transmigrated cells were counted in multiple fields from the six replicates of each condition tested. Total adhesion was calculated as the total number of adherent and transmigrated cells per high-powered field. The percentage of adherent cells that transmigrated below the endothelial layer was called percentage of transendothelial migration.

Immunoprecipitation and Western blot. Tissue culture plates (Primaria, Falcon) were coated with 3 ml of FBS in 5% CO₂ at 37°C for at least 1 h and washed twice with 0.9% saline. Neutrophils (15 × 10⁶) were added to each plate containing 4 ml of reaction mixture, incubated with each compound at 37°C for 30 min, and stimulated with buffer control or TNF (100 ng/ml). When cells were spread fully (20–40 min), they were treated with 5 mM diisopropylfluorophosphate to inhibit serine proteases, and lysed with 125 μl of SDS lysis buffer (10 mM Tris-Cl, pH 7.6; 150 mM NaCl; 1% SDS; 1 mM PMSF; 1 mM Na pyrophosphate; 1 mM NaF; 1 mM vanadate; 5 μg/ml each of aprotinin, leupeptin, chymostatin, pepstatin A) for Western blot or with 200 μl of nondenaturing modified radioimmunoprecipitation assay buffer (10 mM Tris-Cl, pH 7.6; 150 mM NaCl; 1% Triton X-100; 1 mM PMSF; 1 mM Na pyrophosphate; 1 mM NaF; 1 mM vanadate; 5 μg/ml each of aprotinin, leupeptin, chymostatin, pepstatin A) for immunoprecipitation. The cell lysate was passed through a 26-gauge needle six times to shear DNA, and centrifuged at 20,000 × g for 15 min to remove cell debris and DNA. The protein concentration was determined using a Bio-Rad kit. Cell lysates were separated by SDS-PAGE and transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Inc.). The membranes were incubated with 5% milk in TBST (100 mM Tris-Cl, pH 7.5; 9% NaCl; 0.1% Tween-20) for 1 h at 37°C, and then overnight at 4°C with antiphosphotyrosine antibody (Santa Cruz Biotechnology, Inc.), anti-Syk antibody (Transduction Laboratories), antiphospho-specific Pyk2 antibodies (Biosource International), or anti-Pyk2 antibody (Upstate Biotechnology). αC protein was immunoblotted with monoclonal antibody R21 and immunostained with monoclonal antibodies R21, R41, and R32 produced in the laboratories of two of us (L. Levin and J. Buck). Membranes were washed with TBST and incubated with secondary antibody conjugated with horseradish peroxidase in 5% milk in TBST for 1 h at 37°C. After further washing with TBST, bound antibody was detected by enhanced chemiluminescence (Pierce Chemical Co.).

In vitro kinase assay for Src and Syk. Kinase assays were performed according to the instructions provided by UBI, from which recombinant Src and Syk were purchased. In brief, after incubation of recombinant Src (40 U) with each compound at room temperature for 30 min, Src kinase re-
action buffer (100 mM Tris-HCl, pH 7.2; 125 mM MgCl2; 25 mM MnCl2; 2 mM EGTA; 0.25 mM Na3VO4; 2 mM DTT), Src kinase substrate peptide (375 μM) and γ[32P]ATP were added to the reaction mixture. After incubation at 30°C for 10 min, the reaction was stopped by addition of 40% TCA. After transferring each sample to a 96-well plate, unincorporated radioactivity was washed off with 0.75% phosphoric acid and acetone, and the assay square was read in a scintillation counter. For Syk kinase assay, reaction buffer contained 50 mM Tris-HCl, pH 7.5; 0.1 mM EGTA; 0.1 mM Na3VO4; 0.15 M 2-mercaptoethanol and substrate was poly (Glu8-Tyr); 4:1 ( Santa Cruz Biotechnology, Inc.). Each condition was done in duplicate.

Translocation of p47phox. Neutrophil lysates were prepared as above with 2.5% Triton X-100 lysis buffer (final concentration of 1% Triton X-100 after dilution in plate at residual KRPG). The cell lysate was passed through a 25-gauge needle six times to shear DNA and centrifuged at 20,000 g for 15 min to remove cell debris and DNA. The supernatant was centrifuged again at 100,000 g for 1 h to pellet membrane fraction. The pellet was washed with PBS and resuspended in 2% Triton X-100 lysis buffer. Membrane fraction from each condition was separated by SDS-PAGE and Western blot with anti-p47phox Ab (gift from T. Lesto, National Institutes of Health, Bethesda, MD; reference 36).

cAMP accumulation assay. Neutrophils (5 × 106/well) were plated in FBS-coated 24-well tissue culture plates (Coming) in 500 μl of KRPG. The cells were pretreated with 3-isobutyl-1-methylxanthine (100 μM), and 2 (5 μM) or KH7 (25 μM) for 30 min. Ionomycin (1 μM, Sigma-Aldrich) was added to each well to stimulate calcium release, and cAMP was allowed to accumulate for 0 or 2 min at 37°C. The assay was stopped, cells were lysed by adding 500 μl of 0.2 N HCl, and cAMP concentration was determined using the Correlate-EIA Direct cAMP Kit (Assay Designs, Inc.).

Rap1 activation assay. Lysates of neutrophils that had been pretreated for 30 min with DMSO, 2, KH7 (25 μM), or dAdo (25 μM; Calbiochem; reference 37) and then stimulated for 30 min with TNF or buffer alone were incubated with agarose beads coupled to recombinant Rap1GDS-Rap1 binding domain to affinity purify GTP-bound Rap1. Beads were boiled in SDS sample buffer and the supernate was subjected to SDS-PAGE and Western blot with anti-Rap1 antibody (Santa Cruz Biotechnology, Inc.). Western blot of total cell lysates served as a control for equal input into the Rap binding domain affinity purification.

Immunofluorescence staining of sAC in human neutrophils. Neutrophils were fixed and permeabilized with 3.3% paraformaldehyde, 0.05% glutaraldehyde, and 0.25 mg/ml saponin in PBS for 5 min at room temperature. The reactions were stopped with the same volume of 20 mM glycine buffer. The cells were washed three times with PBS. After permeabilization, cells were blocked in 2% BSA for at least 1 h. Cells were stained with biotin-conjugated anti-sAC (R21, R41, or R52) monoclonal antibody (1:250) overnight in 2% BSA; stained for 45 min with streptavidin Alexa Fluor 594 (Molecular Probes) in 2% BSA, 0.01% Triton X-100; and mounted with gelvatol/diaminobicyclooctane (Sigma-Aldrich). Fluorescent images were recorded by a digital camera (Hamamatsu) connected to an inverted epifluorescent microscope (Nikon).

Determination of neutrophil intracellular free Ca2+ ([Ca2+]i). The Ca2+-sensitive fluorescent probe Fluor-3/AM (Molecular Probes) was used for determination of changes in [Ca2+]i. Neutrophils were incubated in the dark for 40 min at room temperature with Fluor-3/AM (10 μM) in M199 (GIBCO BRL) containing 0.025% pluronics acid, 2.5 mM probenecid, 0.5% human serum albumin, and 20 mM Hepes. Cells were washed twice and incubated with M199 containing 2.5 mM probenecid, 0.5% human serum albumin, and 20 mM Hepes in the dark for 20 min at room temperature to allow hydrolysis of the dye ester. 100 PMN with DMSO, 2, or KH7 were plated in 35-mm glass-bottomed coverslip dishes (Mat Tek Corporation) precoated with FBS, and allowed to settle for 10 min. Changes in neutrophil [Ca2+]i, in response to TNF (100 ng/ml) or ionomycin (1 μM) were measured using a Zeiss Axiovert 200M wide-field microscope. Digitized images were captured every 5 s before and after TNF stimulation through a charge-coupled device camera controlled by MetaMorph software (Universal Imaging). Quantitative analysis of images was performed with MetaMorph software. In brief, after background subtraction, the total averaged intensity per field was measured per time point. Relative fluorescence intensities per field for each time point were plotted.

Online supplemental material. Figs. S1–S3 illustrate the impact of 2 on bacterial killing by neutrophils, the interaction of neutrophils with TNF-activated HUVEC monolayers, and the impact of inhibitors on biochemical events involved in TNF-induced activation of adherent neutrophils. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20050778/DC1.

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