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Wortmannin and 1-butanol block activation of a novel family of protein kinases in neutrophils

Jiabing Ding[b], John A. Badwey[a,*]

[a]*Boston Biomedical Research Institute, 20 Staniford St., Boston, MA 02114, USA*
[b]*Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA*

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Abstract

Neutrophils contain four uncharacterized protein kinases with molecular masses of ca. 69, 63, 49 and 40 kDa that are rapidly activated upon stimulation of these cells with the chemoattractant fMet-Leu-Phe [Ding, J. and Badwey, J.A. (1993) J. Biol. Chem. 268, 17326–17333]. We now report that wortmannin and 1-butanol block activation of all four of these kinases. These reagents are known to inhibit superoxide generation in neutrophils stimulated with this agonist. Wortmannin inhibits phosphatidylinositol 3-kinase and blocks activation of phospholipase D, whereas 1-butanol can reduce the generation of phosphatidate in cells by serving as a substrate for phospholipase D. These data suggest that phosphatidylinositol 3-kinase and phospholipase D may be involved in the activation of several novel protein kinases in neutrophils and that one or more of these kinases is/are involved in superoxide release.

Key words: Neutrophil: Cell signalling; Protein kinase; Superoxide

1. Introduction

Neutrophils stimulated with the chemoattractant fMLP display a variety of phenomena that include shape changes, chemotaxis, degranulation and the production of large quantities of O_2^- (for review, see [1]). Cells treated with this agonist also exhibit rapid activation of several uncharacterized protein kinases with molecular masses of ca. 69, 63, 49 and 40 kDa [2–5]. These kinases can be detected by a procedure based on their ability to undergo renaturation and autophosphorylation in a gel, or to catalyze the phosphorylation of a peptide or protein substrate fixed in the gel [3–5]. The novel 69, 63, 49 and 40 kDa kinases are not activated when neutrophils are stimulated with calcium ionophores, activators of protein kinase C (e.g. PMA), or the combination of these agents (e.g, [3,4,6]). Activation of these kinases was blocked by treating the cells with *pertussis* toxin, which indicates a role for a heterotrimeric G-protein in this response [4–6].

Previous studies have shown that the microbial metabolite wortmannin blocks a signal transduction sequence in fMLP-stimulated neutrophils that is necessary for degranulation and O_2^- production [7,8]. This signal is dependent upon a heterotrimeric G-protein but independent of Ca^{2+} [8]. Wortmannin has recently attracted considerable interest since it directly inhibits PI 3-kinase [9–11] and blocks activation of phospholipase D in neutrophils [12–14]. Primary alcohols also block degranulation and O_2^- release in cells stimulated with fMLP but not PMA [15–17]. In this communication, we describe the effects of wortmannin and 1-butanol on the activation of the 69, 63, 49 and 40 kDa protein kinases.

2. Materials and methods

2.1. Materials

Wortmannin was purchased from Kamiya Biomedical Company, Thousand Oaks, CA. Isomers of butanol were obtained from Fischer Scientific, PA. Sources of all other materials are provided elsewhere [3,4].

2.2. Preparation of neutrophils

Guinea pig peritoneal neutrophils were prepared as described previously [18].

2.3. Superoxide release and preparation of solubilized neutrophils for assaying the renaturable protein kinases

These procedures are described in detail in [19] and [3], respectively.

2.4. Detection of renaturable protein kinases in polyacrylamide gels

In this procedure, protein kinases are detected directly in gels by their ability to undergo renaturation and catalyze the phosphorylation of a peptide substrate that corresponds to amino acid residues 297 to 331 of p47-phox 'fixed' in the gel. This technique is presented in [4].

2.5. Analysis of data

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

3. Results

3.1. Effects of wortmannin on guinea pig neutrophils

Neutrophils stimulated with 1.0 μM fMLP release O_2^- at a rate of ca. 45 nmol O_2^-/min/10^7 cells (e.g. [4]). This

* Corresponding author. Fax: (1) (617) 523 6649.

Abbreviations: fMLP, fMet-Leu-Phe; O_2^-, superoxide; PMA, 4β-phorbol 12-myristate 13-acetate, p47-phox, the 47 kDa protein component of the phagocyte oxidase; PI 3-kinase, phosphatidylinositol 3-kinase.

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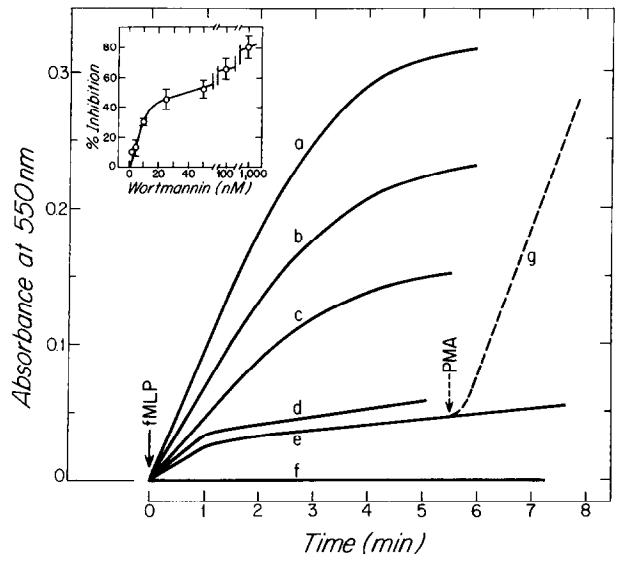

Fig. 1. Effects of wortmannin on superoxide release by neutrophils. Reaction progress curves demonstrate the effects of wortmannin on the release of O_2^- from neutrophils stimulated with 1.0 μM fMLP. Cells were incubated with wortmannin for 5 min prior to the addition of fMLP, which was added last to initiate the reactions (solid arrow). The concentrations of wortmannin were: (a) 0 nM (control); (b) 10 nM; (c) 50 nM; (d) 100 nM; and (e) 1.0 μM. Addition of 50 nM PMA to neutrophils previously treated with 1.0 μM wortmannin and fMLP (broken arrow) resulted in the restimulation of O_2^- release (curve g, dotted line). Curve f is for unstimulated cells. The inset shows the dose–response curve for wortmannin in inhibiting O_2^- release. These data represent the mean% inhibition ± S.D. from four separate experiments performed on different cell preparations.

response was immediate and transient with the majority of the event occurring in the first 3 min (Fig. 1, curve a). Treatment of the cells with wortmannin for 5 min prior to stimulation with 1.0 μM fMLP resulted in a dose-dependent inhibition of O_2^- release. Half-maximal inhibition occured at 46 ± 18 nM (S.D., $n = 4$) wortmannin (Fig. 1, inset). In contrast, 1.0 μM wortmannin did not block O_2^- release from cells stimulated with PMA (e.g. Fig. 1, curve g). Wortmannin (1.0 μM) inhibited O_2^- release from neutrophils stimulated with 1.0 μM fMLP or 50 nM PMA by 81 ± 8% and 8 ± 8% (S.D., $n = 6$–7), respectively. Similar observations have been published previously for human neutrophils [7,8].

Neutrophils stimulated with fMLP exhibit a rapid activation of four renaturable protein kinases with molecular masses of ca. 69, 63, 49 and 40 kDa. These kinases can catalyze the phosphorylation of a peptide substrate that corresponds to amino acid residues 297 to 331 of p47-phox [4] (Fig. 2, part A). This peptide contains several of the potential phosphorylation sites of the complete p47-phox protein (e.g. serines 303, 304, 315, 320 and 325) [20]. All four of these kinases exhibited maximal activity within 15 s of the addition of the stimulus followed by significant inactivation at 3 min. The kinetics of the activation of these kinases was similar to the kinetics of phosphorylation of p47-phox and O_2^- release in intact neutrophils stimulated with fMLP [4] (Fig. 1, curve a). A 96 kDa protein kinase was also activated at time periods ≥ 1 min. Wortmannin (100 nM) blocked activation of the 69, 63, 49 and 40 kDa protein kinases (Fig. 2, part B). The time-course of inhibition was very similar for each of these kinases. Partial inhibition occurred at 50 nM and 25 nM wortmannin, whereas 10 nM was largely without effect. For example, the percent inhibition of the 63 kDa protein kinase in cells treated with 1.0 μM, 100 nM and 10 nM wortmannin for 5 min and then stimulated with fMLP for 1 min was estimated by densitometry at 81 ± 9% (S.D., $n = 5$), 76 ± 12% (S.D., $n = 3$) and 17 ± 22% (S.D., $n = 3$), respectively. Wortmannin did not block the activation of the 96 kDa kinase nor did it effect the renaturable protein kinases in unstimulated cells (Fig. 2, part B).

3.2. Effects of butanol on neutrophils

The effects of different isomers of butanol on the release O_2^- from stimulated neutrophils are presented in Fig.

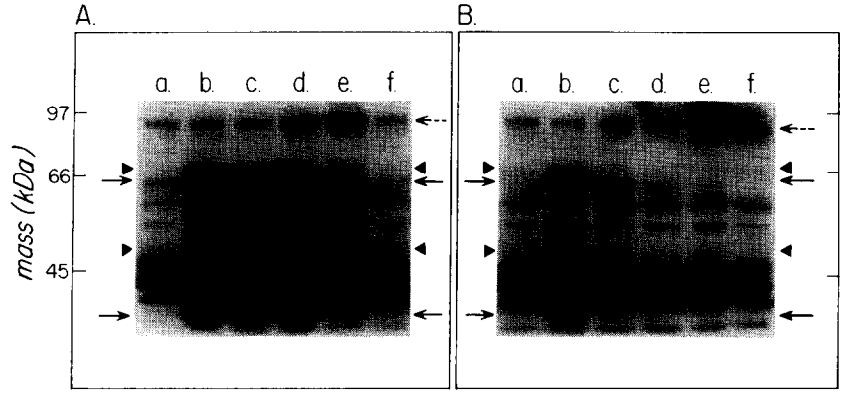

Fig. 2. Effects of wortmannin on the activation of the renaturable protein kinases that catalyze phosphorylation of the p47-phox peptide. Neutrophils were incubated in the absence (A) and presence of 0.10 μM wortmannin (B) for 5 min prior to stimulation with 1.0 μM fMLP. The cells were treated with: (a) 0.25% (v/v) dimethyl sulfoxide for 15 s (unstimulated cells); (b) fMLP for 15 s; (c) fMLP for 30 s; (d) fMLP for 1 min; (e) fMLP for 3 min; and (f) 0.25% (v/v) dimethyl sulfoxide for 3 min. The positions of the 63 and 40 kDa kinases are designated by unbroken arrows, whereas the 69 and 49 kDa kinases are marked by arrowheads. Molecular mass markers are provided on the left.

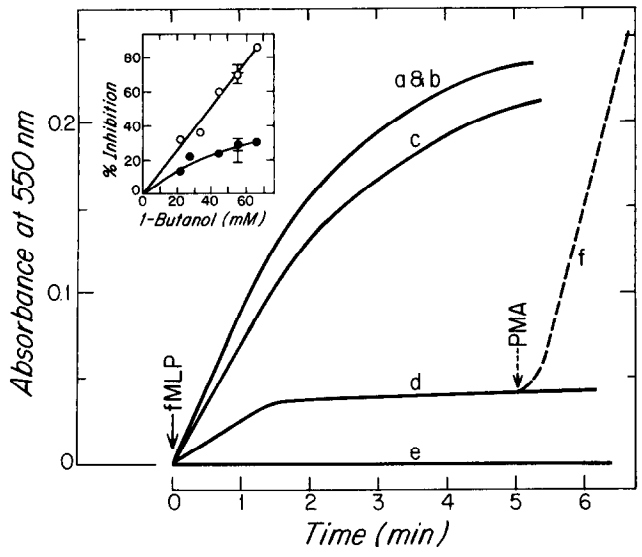

Fig. 3. Effects of different isomers of butanol on superoxide release by neutrophils. Reaction progress curves demonstrate the effects of the isomers of butanol on superoxide release by neutrophils. Cells were incubated with the alcohols (55 mM) for 5 min prior to the addition of 1.0 μM fMLP, which was added last to initiate the reactions (solid arrow). The curves shown were from stimulated cells: (a) not treated with an isomer of butanol; (b) treated with tert-butanol; (c) treated with 2-butanol; and (d) treated with 1-butanol. Curve e is for unstimulated cells. Addition of 50 nM PMA (broken arrow) to neutrophils previously treated with 55 mM 1-butanol and fMLP resulted in the restimulation of O_2^- release (curve f, dotted line). The inset shows the dose–response curves for 1-butanol in inhibiting O_2^- release from cells stimulated with 1.0 μM fMLP (○) or 50 nM PMA (●). The error bars at 55 mM 1-butanol represent the mean% inhibition ± S.D. from 7 separate experiments.

3. 1-Butanol, 2-butanol and tert-butanol at concentrations of 55 mM inhibited O_2^- release from cells stimulated with 1.0 μM fMLP by 74 ± 2%, 20 ± 5% and 7 ± 3% (S.D., $n = 4$), respectively. In contrast, 1-butanol (55 mM) inhibited O_2^- release from cells stimulated with 50 nM PMA by 27 ± 7% (S.D., $n = 8$). 1-Butanol also blocked the activation of the 69, 63, 49 and 40 kDa protein kinases with similar kinetics (Fig. 4, Part B). As was the case with wortmannin, this inhibition was most pronounced at time periods ≥ 30 s. A previous study has provided evidence for early and late phases of phosphatidate production in fMLP-stimulated neutrophils, with only the late pool generated by phospholipase D being functionally linked to O_2^- generation [14]. Unlike wortmannin, 1-butanol also partially blocked the activation of the 96 kDa kinase (Fig. 4, part B, lanes e and f). In contrast, 2-butanol and tert-butanol were not as effective as 1-butanol in blocking the activation of these enzymes. For example, the percent inhibition of the 63 kDa protein kinase in cells treated with 1-, 2- and tert-butanol (55 mM) for 5 min and then stimulated with fMLP for 1 min was estimated by densitometry at 77 ± 8%, 39 ± 4% and 24 ± 1% (S.D., $n = 3$), respectively.

Addition of 1.0 μM wortmannin or 55 mM 1-butanol to the assay mixture employed to detect the renaturable protein kinases in the gel did not affect the activity of these enzymes. This suggests that these reagents did not interact with the 69, 63, 49 and 40 kDa kinases themselves but on upstream components that are involved in the activation of these enzymes. Finally, wortmannin (1.0 μM) and 1-butanol did not affect the viability of neutrophils (as measured by exclusion of trypan blue) nor did these reagents scavange O_2^- in a cell-free system (i.e. xanthine oxidase plus purine).

4. Discussion

In this communication, we report that wortmannin and 1-butanol block activation of a novel family of protein kinases in neutrophils. As noted above, wortmannin directly inhibits PI 3-kinase [9–11] and blocks activation of phospholipase D in these cells [12–14]. In contrast, 1-butanol can directly inhibit protein kinase C [21] and

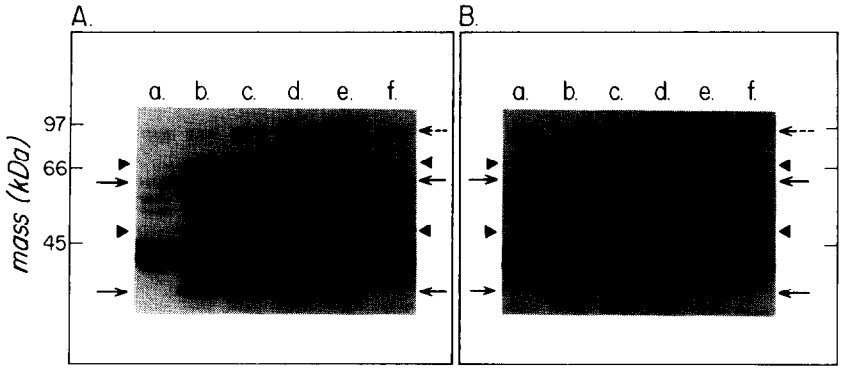

Fig. 4. Effects of 1-butanol on the activation of the renaturable protein kinases that catalyze phosphorylation of the p47-phox peptide. Neutrophils were incubated in the absence (A) and presence of 55 mM 1- butanol (B) for 5 min prior to stimulation with 1.0 μM fMLP. The cells were treated with: (a) 0.25% (v/v) dimethyl sulfoxide for 15 s (unstimulated cells); (b) fMLP for 15 s; (c) fMLP for 30 s; (d) fMLP for 1 min; (e) fMLP for 3 min; and (f) 0.25% (v/v) dimethyl sulfoxide for 3 min. The positions of the 63 and 40 kDa kinases are designated by unbroken arrows, whereas the 69 and 49 kDa kinases are marked by arrowheads. Molecular mass markers are provided on the left.

may inhibit phosphatidate production (along with diglyceride) by serving as a substrate in the phospholipase D reaction (e.g. [17]). PI 3-kinase and phospholipase D are involved in the production of increased amounts of phosphatidylinositol 3,4,5-trisphosphate and diglyceride in fMLP-stimulated neutrophils (e.g. [14,22,23]). These compounds selectively activate the β and ζ-isozymes of protein kinase C, respectively (e.g. [24,25]). Both of these species of protein kinase C are present in neutrophils (e.g. [26,27]). The concentrations of wortmannin that inhibit PI 3-kinase in neutrophils (IC_{50} = 50 nM) [10] are very similar to those that block both O_2^- release (IC_{50} = 46 ± 18 nM; Fig. 1) and the activation of the 69, 63, 49 and 40 kDa protein kinases (Fig. 2). Wortmannin inhibits PI 3-kinase from myeloid cells activated in vitro by either the $\beta\gamma$ subunits of heterotrimeric G-proteins or by a tyrosine phosphorylated peptide at saturating ATP (2.0 mM) with IC_{50} values of 43 nM and 17 nM, respectively [11]. It is interesting to note in this context that the ζ-isozyme of protein kinase C is insensitive to phorbol esters (e.g. [24]) and that neutrophils treated with PMA do not exhibit activation of the 69, 63, 49 and 40 kDa protein kinases [3–5]. In fact, neutrophils treated with PMA exhibit a decrease in the basal activities of these enzymes [3,4]. Thus, PI 3-kinase, phospholipase D and/or an isozyme of protein kinase C that is insensitive to PMA may be involved in the stimulatory pathway which triggers activation of these novel protein kinases.

As noted earlier, wortmannin and 1-butanol inhibit O_2^- generation and degranulation in neutrophils stimulated with fMLP but not PMA [8,15–17]. These reagents should therefore be useful not only in sorting out the biochemical events involved in the activation of these kinases, but also in defining their physiological substrates and functions.

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