Human neutrophil phospholipase D activation by *N*-formylmethionyl-leucylphenylalanine reveals a two-step process for the control of phosphatidylcholine breakdown and oxidative burst

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A comparative study of real-time kinetics of respiratory burst, monitored by H_2O_2 -dependent chemiluminescence, and phospholipase D (PLD)-mediated phosphatidylcholine breakdown has been undertaken on human neutrophils stimulated by *N*-formylmethionyl-leucylphenylalanine in the absence of cytochalasin B. The fungal metabolite 17-hydroxywortmannin (HWT), an inhibitor of NADPH oxidase activation, decreases phosphatidic acid (PA) production by 30 % at a concentration of 1 nM. Higher concentrations (10 nM–1 μM) inhibit PA formation maximally by 50 % as compared with control. In all cases, the inhibition is delayed by 20–30 s after addition of the agonist. Thus the full PA generation is actually the result of an early (HWT-insensitive) and a late (HWT-sensitive) phosphatidylcholine breakdown. However, under all conditions, alkylacylglycerol remains at the basal level. PLD activity is dependent on Ca^{2+} influx, but is fully inhibited in cells depleted of Ca^{2+} with EGTA and Quin 2. The effect of HWT on the respiratory burst was investigated by measuring the kinetics of H_2O_2 -induced chemiluminescence. This method allows to distinguish various phases of superoxide ion production: a lag, an increase in H_2O_2 formation (early phase), the duration of H_2O_2 production (late phase) and the termination of the oxidative burst. The lag remains constant for all HWT concentrations. A concentration of 10 nM-HWT, which fully inhibits the HWT-sensitive part of PA production, decreases superoxide ion production with a delay of about 20 s after addition of the agonist. Higher HWT concentrations, which have no additional effect on PLD inhibition, equally affect an early and a late phase of the burst. Thus high doses of HWT have a site of action which decreases the whole burst but does not affect the PLD any more. Therefore HWT and Ca^{2+} provide evidence for a two-step process for PLD activation. Only the delayed PA generation is functionally linked to a late phase of the oxidative burst.

INTRODUCTION

Human neutrophils release reactive oxygen products upon activation, which are mediators of inflammation. The NADPH oxidase system is responsible for the respiratory burst. The system converts oxygen into superoxide, which in turn dismutates to H_2O_2 . The reactions necessary for signal transduction from the *N*-formylmethionyl-leucylphenylalanine (fMLP) receptor to the oxidase are known to be very complex. Different agonists, e.g. fMLP, leukotriene B_4 and platelet-activating factor (PAF), elicit different response patterns [1,2]. The simple linear model for the activation of the burst, in which phospholipase C (PLC) produces InsP_3 and DAG, and in turn induces the release of Ca^{2+} and activation of protein kinase C (PKC), is only one of the signal-transduction pathways [3]. It is evident that other pathways are as important to control the burst, but very few components involved have been identified [4,5].

17-Hydroxywortmannin (HWT), a fungal metabolite, is known as a potent inhibitor of the respiratory burst [5]. HWT was shown to inhibit a signalling pathway that functions in addition to PLC activation in agonist-stimulated neutrophils [6].

An alternative path, triggered by fMLP, activates phospholipase D (PLD) [7,8], which breaks down phosphatidylcholine (PC) and generates phosphatidic acid (PA) and alkylacylglycerol (DG) [9,10]. The mechanism(s) upstream from the PLD are inhibited by HWT [11]. Decreasing PA formation by adding ethanol results in a decrease in the burst signal and indicates the

importance of this phospholipid [12]. PA can be converted, by a PA phosphohydrolase, into DG, which in turn could activate PKC [13]. The inhibition of DG formation from PA by propranolol indicates that PA also controls the oxidase activity [14]. However, the relative roles of PA and DG in burst control may depend on experimental conditions, since DG production has been shown to increase in the presence of cytochalasin B [15,16]. Real-time studies demonstrate that the fMLP-induced burst also occurs in the absence of cytochalasin B [1].

In the present work we measure the time course of PLD-mediated PC-breakdown products and respiratory burst upon stimulation with fMLP in the absence of cytochalasin B. We provided evidence that the full PLD activation involves two transduction pathways, one of which affects the late phase of the respiratory burst. The results are discussed with respect to signal transduction.

EXPERIMENTAL

Materials

1-*O*-[^3H]Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, mixture of C_{18} and C_{16} alkyl ethers (^3H]PAF; 80 Ci/mmol), was purchased from Amersham International (Amersham, Bucks., U.K.). fMLP, quin2/AM, PA and BSA were supplied by Sigma (St. Quentin Fallavier, France). Peroxidase was purchased from Boehringer (Mannheim, Germany). Plasmagel was obtained from Laboratoires Roger Bellon (Neuilly, France), and Percoll

Abbreviations used: PLC, phospholipase C; PLD, phospholipase D; fMLP, *N*-formylmethionyl-leucylphenylalanine; HWT, 17-hydroxywortmannin; PAF, platelet-activating factor; PA, phosphatidic acid; PC, phosphatidylcholine; DAG, diacylglycerol; DG, alkylacylglycerol; PKC, protein kinase C.

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was from Pharmacia. HWT and 11-desacetoxywortmannin were supplied by Dr. T. G. Payne, Preclinical Research, Sandoz, Basel, Switzerland. Silica-gel plates were from Merck (Darmstadt, Germany).

Methods

Buffers. Three salt solutions have been used for experiments: Buffer A to label and wash the cells (20 mM-Hepes, 137 mM-NaCl, 2.6 mM-KCl and 20 mM-glucose, pH 7.4); Buffer B (20 mM-Hepes, 137 mM-NaCl, 2.6 mM-KCl, 20 mM-glucose, 1.3 mM-CaCl₂ and 1.0 mM-MgCl₂), for investigating PLD activation; alternatively, the respiratory-burst test buffer [6] (130 mM-NaCl, 4.6 mM-KCl, 1.1 mM-KH₂PO₄, 20 mM-glucose, 1.0 mM-CaCl₂ and 20 mM-Hepes) was also used.

Cell preparation. The methods used for neutrophil preparation have been previously reported by Dewald *et al.* [6] and Record *et al.* [17].

PLD activity measurement. PC was labelled by metabolism of either [³H]lysoPAF or [³H]PAF (1 µCi/10⁷ cells, for 15 min, 37 °C). [³H]monoalkylglycerols originating from [³H]lysoPAF degradation ran close to PA in our t.l.c. system, at variance with labelling performed with PAF. Therefore results reported herein have been obtained on cells labelled with [³H]PAF, whose concentration never exceeds 6 nM, as previously reported [7]. Excess PAF was removed by three washings with 2.5 % BSA in buffer A. Neutrophils were suspended in buffer B at 10⁷/ml. For HWT treatment, cells were incubated with it for 5 min before addition of the agonist. Cells were stimulated by addition of 1 µM-fMLP. All the experiments performed in this paper were done without cytochalasin B. The reaction was stopped by addition of chloroform/methanol in accordance with Bligh & Dyer [18] in the presence of 0.25 M-HCl, 2.3 mM-EDTA and PA (10 µg/sample) [7]. The labelled products were separated by t.l.c. with the solvent system of Cohen *et al.* [19]. on silica-gel plates sprayed with 0.25 M-oxalic acid and dried at 40 °C. The plates were scanned for radioactivity with a t.l.c. linear analyser (Berthold LB 2848). Fractions, based both on the location of a lipid standard and the t.l.c. linear analyser, were scraped from t.l.c. plates and assayed by liquid-scintillation counting. Further details of this procedure have been published [7].

Results are reported as the level of ³H in a particular fraction as a percentage of total label recovered. The average of total radioactivity in a fraction after stimulation by fMLP for 60 s was as follows: PA, 5400 d.p.m.; phosphatidylethanol, 1850 d.p.m.; DG, 1239 d.p.m.; PC, 364400 d.p.m.

Ca²⁺ depletion. Total Ca²⁺ depletion was obtained by incubating 10⁸ neutrophils for 20 min at 37 °C, pH 7.4, in the presence of 25 µM-quin2/AM in buffer A supplemented with 1 mM-EGTA to prevent Ca²⁺ influx. After washing, the cells were suspended in the same buffer (10⁷ cells/ml) and used for PLD measurement.

Respiratory burst assay. Luminol-dependent chemiluminescence, a sensitive assay for H₂O₂, was measured as described by Wymann *et al.* [1]. Briefly, to 10⁶ neutrophils/ml of test buffer 9 µM-luminol, 90 µM-NaN₃ and 9 units of horseradish peroxidase were added. The horseradish peroxidase was used instead of myeloperoxidase released upon stimulation of neutrophils to oxidize luminol; NaN₃ inhibits the myeloperoxidase. Samples were warmed for 10 min at 37 °C before the experiment. Chemiluminescence was measured with a purpose-built luminometer at a rate of 200 ms per channel [20]. The measurements were recorded and analysed with a personal computer (AT 286). Stimuli were injected with a micro-syringe (1–10 µl) into the stirred sample and mixing occurred within the first channel measured. Each measurement was calibrated by injecting a known amount of H₂O₂ before stimulation. The chemilumi-

nescence signal of stimulated neutrophils was divided by the integrated chemiluminescence of the calibration pulse and multiplied by the amount of H₂O₂ used for the pulse. The burst activity is expressed as pmol of H₂O₂/s per 10⁶ cells.

We have checked the effect of cell labelling on the respiratory burst, and no difference was observed from control neutrophils (results not shown). This is consistent with the absence of a priming effect of PAF when cytochalasin B is omitted [21].

Data presentation. Measurements have been performed on 3–6 separate neutrophil preparations. For PLD experiments, results are means ± s.e.m. and are expressed as percentages of total label recovered. Vertical bars in the Figures represent the s.e.m. (not indicated when smaller than the symbols).

RESULTS

PC breakdown in absence of cytochalasin B and effect of 11-desacetoxywortmannin

In the present work, we never used cytochalasin B in the pre-incubation medium. Under these conditions, DG remains at the basal level (Fig. 1a) upon stimulation with fMLP. We observed the single formation of [³H]alkyl-PA in neutrophils directly stimulated by fMLP (Figs. 1a and 1b), as shown in our previous work [7], with a time course and an extent similar to those reported by Bauldry *et al.* [22]. Since a direct correlation has been established between PA and superoxide formation [14,22], the effect of wortmannins on the production of these two products has been investigated under experimental conditions allowing the formation only of PA.

The 11-desacetoxywortmannin, ineffective for inactivation of the NADPH oxidase [5], is also ineffective in inhibiting the PLD hydrolytic and transphosphatidylolation activities (Fig. 1b).

Dose- and time-dependent inhibition by HWT or ethanol of PLD-mediated PC breakdown

HWT, initially known as an inhibitor of NADPH oxidase activation [5], is also a potent inhibitor of PLD, as 1 nM decreases PA production by 30 % (Fig. 2a) whereas concentrations from 10 nM to 1 µM inhibit the PLD activity similarly (Figs. 2b, 2c and 2d). Inhibition reaches a plateau at 50 % of the control after 30 s. This inhibition is always delayed for about 20 s after addition of the agonist, although HWT had been present for 5 min before cell activation. The drug, at a concentration of 1 µM, also inhibits by approx. 50 % the production of phosphatidylethanol by PLD (result not shown).

In order to define better the delay in HWT-mediated PA inhibition, more time points were checked during the first 60 s of cell stimulation (Fig. 3a). The difference between the two upper curves represents the portion of PA produced by the path that can be inhibited by HWT (Fig. 3a, curve c). The HWT-inhibited PA formation starts to deviate from the control only 20 s after stimulation, and the curve levels off already after 30 s (Fig. 3a, curve b). This residual activity represents the early PA generation that is insensitive to HWT. Therefore the overall PLD activity results from the activation of two transduction pathways leading to the formation of an 'early' (HWT-insensitive) and a 'late' (HWT-sensitive) pool of PA. Curve a of Fig. 3(a) represents the sum of curves b and c and fully describes the observed PA production in the absence of HWT. From an entire involvement of the HWT-insensitive pathway during the first 20–30 s, the HWT-sensitive pathway becomes progressively more important, accounting for 50 % of PA produced at 60 s after addition of the agonist. Therefore both pathways account for the same final amount of product formed.

In presence of 0.5 % ethanol, PA decreased and the production was not delayed (Fig. 3b, curve d) as compared with the kinetics

Fig. 1. PC breakdown upon stimulation with 1 μ M-fMLP, in the absence of cytochalasin B

(a) PC-related DG formation in non-treated cells (●) and in cells treated with 1 μ M-HWT (▼) or 0.5% ethanol (▲). (b) PC breakdown products, PA (○) and phosphatidylethanol (PEt) (▼), formed in the presence (●, ▼) or absence of (○, ▽) of 1 μ M-11-desacetoxy-wortmannin.

of PA produced in presence of 1 μ M-HWT alone. This result demonstrates that ethanol affects the pathway that is insensitive to the drug. The amount of PA recovered in presence of 0.5% ethanol reaches a plateau at about 25% of the total amount of PA obtained in the absence of ethanol, and represented about half the amount obtained in the presence of HWT (Fig. 3b). This observation indicates that ethanol also affects the HWT-sensitive part of the total PA production. From Fig. 3(b) we conclude that the two PA pools discriminated by HWT originated from a PLD-type of activity.

Role of Ca^{2+} pools in activation of PLD

Neutrophils stimulation with fMLP triggers, to the same extent, both Ca^{2+} influx and mobilization of Ca^{2+} -storage pools [23,24]. We demonstrate herein that total Ca^{2+} depletion obtained by a combination of EGTA and quin 2 fully inhibits PLD activity (Fig. 4, curve d). When Ca^{2+} influx only was quenched by EGTA (Fig. 4, curve b), a rapid inhibition of PA is noticed at 10 s. Under these experimental conditions production of PA levels off at 30 s after addition of the agonist.

The residual amounts of PA in presence of EGTA could actually originate from both the HWT-insensitive and -sensitive pathways. To investigate the role of Ca^{2+} influx in regulating these two pathways, cells have been incubated with a combination of EGTA and HWT. The first 30 s of curve c in Fig. 4 monitor the HWT-insensitive PA pool (see Fig. 3a): PA production is again highly inhibited at 10 s; however, we observe an additional clear-cut inhibition at 30 s, i.e. when the HWT-sensitive activation pathway is triggered (Fig. 3a). When neutrophils are stimulated by fMLP, the major cytosolic Ca^{2+} increase occurs during the first 10 s [25]. This observation might account for the strong inhibition (75%) of PA production observed at 10 s in Fig. 4, curve b (EGTA alone), and also found in conditions of Fig. 4, curve c (EGTA + HWT).

Fig. 2. Time-dependence of PLD-mediated PA production upon stimulation with 1 μ M-fMLP

Cells were preincubated (see under 'Methods') with HWT at 1 nM (▼, a), 10 nM (●, b), 0.1 μ M (■, c) and 1 μ M (▲, d) as compared with untreated cells (○, a). In (b), (c) and (d) the PA production in absence of HWT (----) is reported for clarity. Results are means $\pm$ S.E.M. for three separate determinations.

When the two PLD pathways are operating (i.e. after 30 s; see Fig. 3a) incubation of cells with HWT + EGTA further decreased by about one-half (Fig. 4, curve c) the PA production obtained in the presence of EGTA alone (Fig. 4, curve b). These data indicate that Ca^{2+} originating from intracellular stores triggers, to the same extent, some PA formation arising from both the HWT-sensitive (Fig. 4, curves b minus c) and -insensitive (Fig. 4, curves c minus d) pathways.

The late PA production controls the duration of the respiratory burst

The inhibitory effect of HWT on superoxide ion formation has been established by measurements with cytochrome *c* [6]. This technique does not allow one to distinguish between various phases of the oxidative burst. In the present work we use an H_2O_2 -dependent chemiluminescence recording of the burst, which monitors the successive activation steps of NADPH oxidase [1].

These phases are defined by a short lag (Fig. 5; see also Fig. 6 inset), an increase in H_2O_2 production (early phase), followed by

Fig. 3. HWT discriminates between two pools of PA in cells stimulated with 1 μM -fMLP

(a) In comparison with non-treated cells (a) (○), the effect of HWT was measured at 1 μM (b) (▲). Curve c monitors the difference between curves a and b (▽). (b) PA production in presence of 0.5% ethanol (d) (▼) compared with the residual PA in the presence of 1 μM HWT (b) (----) reported in (a).

Fig. 4. Role of Ca^{2+} in regulating PLD activity upon stimulation with 1 μM -fMLP

In comparison with non-treated cells (○, curve a), neutrophils incubated with EGTA (▲, curve b), or EGTA + HWT (1 μM) (▼, curve c), or EGTA + quin 2 (■, curve d) are shown.

Fig. 5. Activity of NADPH oxidase induced by 1 μM -fMLP

The top curve is the control without HWT or ethanol; second curve, in the presence of 0.5% ethanol; third curve, in the presence of 1 nM-HWT.

Fig. 6. Activity of NADPH oxidase induced by 1 μM -fMLP in the presence of various concentrations of HWT

Dose-dependent inhibition by HWT of the H_2O_2 -dependent chemiluminescence. From top to bottom the HWT concentrations are: 10 pM, not treated, 0.1 nM, 1 nM, 10 nM, 0.1 μM , 1 μM . The insert displays the tracing of the first 18 s at higher resolution.

a period of decreasing H_2O_2 formation (late phase) monitoring the duration of the respiratory burst. Termination of the burst occurs beyond 120 s (Fig. 5). The amount of H_2O_2 monitors the activity of NADPH oxidase at each time.

The superoxide dismutase-inhibitable cytochrome *c* reduction technique monitors an accumulation of superoxide ions, thereby impairing distinction between the various phases of NADPH oxidase activation.

Stimulation with 1 μM -fMLP of neutrophils which were preincubated for 5 min with HWT (1 nM) or stimulation of neutrophils in the presence of ethanol yields identical progress curves (Fig. 5). Both compounds have no effect on the increase in H_2O_2 production (early phase), but instead affect the late phase. Fig. 5 demonstrates that the late phase can be inhibited independently of the early phase. Thus inhibition of the activation of PLD by HWT or depletion of the PLD product (PA) by ethanol have similar effects on the oxidative burst, and demonstrate a functional link between PLD and the NADPH oxidase activity. These observations demonstrate that PLD activity is responsible for the burst activity during a late phase of the process, i.e. the NADPH oxidase activity is maintained by the presence of PC-derived PA.

The HWT dose-dependence of respiratory burst was measured in cells which were preincubated for 5 min with 10 pM-1 μM -HWT before stimulation with 1 μM -fMLP (Fig. 6). The traces obtained with HWT-treated cells show oscillations of the burst. These oscillations have been attributed to the shape change of cells [26]. Preincubation with HWT has no effect on the lag (Fig. 6, inset). HWT doses smaller than 10 nM do not inhibit the initial rate of oxidase activation. However, the late phase of the burst is progressively inhibited by HWT concentrations ranging from 0.1 to 10 nM (Fig. 6). The HWT concentration which fully inhibits the late phase of PA production (10 nM) (Fig. 2) completely abolishes the H_2O_2 -dependent chemiluminescence after 40 s. Higher HWT concentrations (which have no additional effect on PLD) further decrease the amplitude of the burst but leave the shape of the progress curve unaffected. Thus the kinetics of signal transduction do not change with HWT concentrations greater than 10 nM; however, the amount of activatable overall oxidase activity is decreased. This observation unravels a new component of transduction sequences triggered by fMLP which is distinct from PLD.

Therefore our results discriminate between two transduction sequences, triggered time-dependently, for control of the oxidative burst. One is sensitive to high concentrations of HWT (> 10 nM) and does not involve PLD. The second pathway controls the duration of the oxidative burst, is sensitive to low concentrations of HWT (< 10 nM), and requires PLD-mediated PA formation.

DISCUSSION

The PLC signal-transduction path which generates InsP_3 and DAG, which are responsible for Ca^{2+} release and PKC activation, was thought for years to be the only mechanism leading to activation of the respiratory burst. However, a Ca^{2+} -independent path which works in concert with the PLC path was detected by using Ca^{2+} -depleted cells. This or yet another Ca^{2+} -independent path which can be inhibited by HWT plays a significant role in activating the oxidase [6]. The situation is now more complex, and, in addition to the PLC signalling pathway, there are other efficient routes leading to oxidase activation involving other phospholipases [7,8].

A recent report demonstrated a direct correlation between PA and superoxide ion formation in human neutrophils [22]. Using HWT, which inhibits both PLD and NADPH oxidase activation [11,5] we have addressed the question of whether various pools of PA could control different steps of NADPH oxidase activation. So far, no kinetic analysis of HWT inhibition has been performed on PLD activity, and its effect on superoxide ion production has been studied only by using cytochrome *c* reduction [6]. This latter technique cannot allow one to distinguish between different steps of NADPH oxidase activation, at variance with chemiluminescence [1,20].

Our results unravel the presence of two different PA pools produced at different times upon cell activation by fMLP (Fig. 2). Both PA pools originate from a PLD activity, as shown by the decrease in PA in presence of ethanol (Fig. 3b) and the formation of phosphatidylethanol (results not shown). Moreover, PA originating through PC-related phospholipase C and DG kinase is unlikely to occur in human neutrophils [8].

Under our experimental conditions, PA is the unique second messenger produced by PC hydrolysis, occurring in neutrophils directly stimulated by fMLP. This is consistent with the results of Bauldry *et al.* [22]. Although PA has been demonstrated to activate the NADPH oxidase [27] and superoxide generation [12,14], our work provides the first evidence for two distinct pools of this phospholipid. Only the second pool, which is generated by the late PLD activation, is functionally linked to NADPH oxidase activation (Figs. 5 and 6). This functional link is established by the decreases in both PA and H_2O_2 production by HWT and ethanol (Figs. 3 and 5). In addition, other reports have shown a role of PA in the oxidative burst [22], but without discriminating between various phases of the process. It is proposed that PA could also act on the burst through PKC, since PA is almost as potent an activator as is phosphatidylserine towards the enzyme [28]. Direct interaction between PA and the 47 kDa protein [29,30], which is highly enriched in basic amino acid residues [31], is a further possible route of activation. In turn, the PKC might be not activated by PC-derived diacylglycerols [32].

Decreasing the first PA pool by ethanol (Fig. 3) had no effect on the early phase of the burst (Fig. 5). A possible explanation could be that this pool is located in a different plasma-membrane domain, not accessible to those reactions leading to oxidase activation. We have demonstrated that the overall PA formation occurs in the plasma membrane ([7]; G. Ribbes, P. Gélais, M. Record & H. Chap, unpublished work). The role of the early

generation of PA (during the first 30 s) remains to be defined, since the PtdInsP_2 -PLC/PKC pathway controls only the lag of the burst [1,6].

Our kinetic measurements of PLD in the presence of Ca^{2+} chelators and HWT bring also new insights in the time course of PLD activation. The present work demonstrates a complete inhibition of PLD (Fig. 3) when the two chelators (EGTA and quin 2) are used together. This indicates that the PLD path is Ca^{2+} -dependent and therefore not identical with the Ca^{2+} -independent HWT-inhibitable paths described previously [6]. Our results differ from a previous investigation [11], which reports a total Ca^{2+} depletion in neutrophils induced by EGTA only. Actually both EGTA and quin 2 are required to quench Ca^{2+} fully in human neutrophils, especially when challenged with fMLP, which induces a similar extent of Ca^{2+} release from intracellular stores and Ca^{2+} influx [23,24].

Previous reports indicate that the Ca^{2+} -independent path which does not activate PKC is inhibitable by HWT [6,11], this path being responsible for a full inhibition of fMLP-induced respiratory burst at a concentration of 50 nM-HWT [11]. Using a rapid and sensitive method, which records the very first seconds of the burst, we demonstrated that complete inhibition of the burst requires higher doses of HWT (1 μM , Fig. 6).

Chemiluminescence monitoring of the respiratory burst allows us to distinguish various periods in the NADPH oxidase activity not detected in measurements performed by cytochrome *c* reduction [6,12]. Up to now, only the transduction sequence involved in the control of the lag time has been clearly established [1,2,6]. By using the combination of receptor and non-receptor agonists, i.e. phorbol 12-myristate 13-acetate and fMLP, NADPH oxidase activation has been shown to require two transduction sequences [6]. The lag of the burst can be attributed to the delayed activation of PKC (1). The lag remains unchanged with HWT (Fig. 6, insert), which confirms the independence of the PtdInsP_2 -PLC path to HWT [6].

The receptor agonist fMLP thus triggers, for the early phase, a route which apparently does not require PC-derived PA, but involves a component displaying a low affinity towards HWT. Effectively concentrations of HWT 100 times higher than for full PLD inhibition are required to abolish the burst completely since the early phase. Myosin light-chain kinase might be involved in the HWT low-affinity pathway, since a recent report demonstrates that wortmannin in the range of 1–10 μM fully inhibits that kinase [33]. In this respect, the cytoskeleton has been shown to play an essential role in NADPH oxidase activation [26,34].

Instead, the route leading to the control of the duration of burst involves PLD (Figs. 3 and 5), whose activation mechanism exhibits a high affinity towards HWT (Fig. 2).

It is noteworthy that inhibiting PLD leads to a subsequent inhibition of the late phase of the burst. One might have assumed that this phase of the burst would only result from the arrest of the superoxide ions produced during the early phase. Instead we demonstrate that the decreasing part of the burst is dependent on the presence of PA, i.e. the duration of the late phase is stimulated by this second messenger. Altogether, our data suggest that each step of the burst would be controlled by a specific transduction pathway.

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REFERENCES

1. Wymann, M. P., Von Tscharner, V., Deranleau, D. A. & Baggiolini, M. (1987) *J. Biol. Chem.* **262**, 12048–12053
2. Reibman, J., Korchak, H. M., Vossball, L. B., Haines, K. A., Rich, A. M. & Weissmann, G. (1988) *J. Biol. Chem.* **263**, 6322–6328

3. Farago, A. & Nishizuka, Y. (1990) *FEBS Lett.* **268**, 350–354
4. Della Bianca, V., Grzeskowiak, M., Cassatella, M. A., Zeni, L. & Rossi, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 556–565
5. Baggiolini, M., Dewald, B., Schnyder, J., Ruch, W., Cooper, P. & Payne, T. G. (1987) *Exp. Cell Res.* **169**, 408–418
6. Dewald, B., Thelen, M. & Baggiolini, M. (1988) *J. Biol. Chem.* **263**, 16179–16184
7. Glas, P., Ribbes, G., Record, M., Terc, F. & Chap, H. (1989) *FEBS Lett.* **251**, 213–218
8. Billah, M. M., Eckel, S., Mullmann, T. J., Egan, R. W. & Siegel, M. I. (1989) *J. Biol. Chem.* **264**, 17069–17077
9. Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1–4
10. Billah, M. M. & Anthes, J. C. (1990) *Biochem. J.* **269**, 281–291
11. Reinhold, S. L., Prescott, S. M., Zimmerman, G. A. & McIntyre, T. M. (1990) *FASEB J.* **4**, 208–214
12. Bonser, R. W., Thompson, N. T., Randall, R. W. & Garland, L. G. (1989) *Biochem. J.* **264**, 617–620
13. Billah, M. M., Pai, J. K., Mullmann, T. J., Egan, R. W. & Siegel, M. I. (1989) *J. Biol. Chem.* **264**, 9069–9076
14. Rossi, F., Grzeskowiak, M., Della Bianca, V., Calzetti, F. & Gandini, G. (1990) *Biochem. Biophys. Res. Commun.* **168**, 320–327
15. Honeycutt, P. J. & Niedel, J. E. (1986) *J. Biol. Chem.* **261**, 15900–15905
16. Tyagi, S. R., Burnham, D. N. & Lambeth, J. D. (1989) *J. Biol. Chem.* **264**, 12977–12982
17. Record, M., Laharrague, P., Fillola, G., Thomas, J., Ribbes, G., Fontan, P., Chap, H., Corberand, J. & Douste-Blazy, L. (1985) *Biochim. Biophys. Acta* **819**, 1–9
18. Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–918
19. Cohen, P., Broekman, M. J., Verkley, R., Lisman, J. W. W. & Derksen, A. (1971) *J. Clin. Invest.* **50**, 762–772
20. Wymann, M. P., Von Tscharnner, V., Deranleau, D. A. & Baggiolini, M. (1987) *Anal. Biochem.* **165**, 371–378
21. Bass, D. A., McPhail, L. C., Schmitt, J. D., Morris-Natschke, S., McCall, C. E. & Wykle, R. L. (1989) *J. Biol. Chem.* **264**, 19610–19617
22. Bauldry, S. A., Bass, D. A., Cousart, S. L. & McCall, C. E. (1991) *J. Biol. Chem.* **266**, 4173–4179
23. Von Tscharnner, V., Deranleau, D. A. & Baggiolini, M. (1986) *J. Biol. Chem.* **261**, 10163–10168
24. Truett, A. P., III, Verghese, M. W., Dillon, S. B. & Snyderman, R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1549–1553
25. Korchack, H. M., Voshall, L. B., Haines, K. A., Wilkenfeld, C., Lundquist, K. F. & Weissman, G. (1988) *J. Biol. Chem.* **263**, 11098–11105
26. Wymann, M. P., Kernen, P., Deranleau, D. A. & Baggiolini, M. (1989) *J. Biol. Chem.* **264**, 15829–15834
27. Bellavite, P., Corso, F., Dusi, S., Grzeskowiak, M., Della-Bianca, V. & Rossi, F. (1988) *J. Biol. Chem.* **263**, 8210–8214
28. Lee, M. H. & Bell, R. M. (1989) *J. Biol. Chem.* **264**, 14797–14805
29. Parkinson, J. F. & Gabig, T. G. (1988) *J. Bioenerg. Biomembr.* **20**, 653–677
30. Clark, R. A. (1990) *J. Infect. Dis.* **161**, 1140–1147
31. Volpp, B. D., Nauseef, W. M., Donelson, J. E., Moser, D. R. & Clark, R. A. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7195–7199
32. Leach, K. L., Ruff, V. A., Wright, T. M., Pessin, M. S. & Raben, D. M. (1991) *J. Biol. Chem.* **266**, 3215–3221
33. Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H. & Matsuda, Y. (1992) *J. Biol. Chem.* **267**, 2157–2163
34. Wymann, M. P., Kernen, P., Bengtsson, T., Andersson, T., Baggiolini, M. & Deranleau, D. A. (1990) *J. Biol. Chem.* **265**, 619–622

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