Free Radical Research, Vol. 35, pp. 723–733
Reprints available directly from the publisher
Photocopying permitted by license only

The Photoprotein Pholasin As a Luminescence Substrate for Detection of Superoxide Anion Radicals and Myeloperoxidase Activity in Stimulated Neutrophils

S. REICHL, A. VOCKS, M. PETKOVIĆ, J. SCHILLER and J. ARNHOLD*

Medical Department, Institute of Medical Physics and Biophysics, University of Leipzig, Liebigstr. 27, 04103 Leipzig, Germany

Accepted by Dr M. Davies

(Received 21 February 2001; In revised form 4 April 2001)

Pholasin, the photoprotein of the common piddock *Pholas dactylus*, emits an intense luminescence upon oxidation. The contribution of superoxide anion radicals and myeloperoxidase (MPO) to Pholasin luminescence in stimulated neutrophils was investigated. Data on Pholasin luminescence were compared with results of superoxide anion radical generation detected by the cytochrome c test as well as with the release of elastase and MPO. In *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulated neutrophils, most of the luminescence is caused by superoxide anion radicals, whereas MPO shows only a small effect as shown by coincubation with superoxide dismutase (SOD) as well as potassium cyanide (KCN), an inhibitor of MPO. However, both, $O_2^{\cdot-}$ and MPO contribute to light emission in fMLP/cytochalasin B and phorbol myristoyl acetate (PMA) stimulated cells. Thus, the kinetics of $O_2^{\cdot-}$ generation and MPO release can be very well detected by Pholasin luminescence in stimulated neutrophils.

Degranulation of azurophilic granules was assessed using an ELISA test kit for released MPO or detection of elastase activity with MeO–Suc–Ala–Ala–Pro–Val–*p*-nitroanilide in the supernatant of stimulated cells. Both approaches revealed concurrently similar results concerning the amount and kinetics of enzyme release with data of Pholasin luminescence. Both, cytochrome c measurements and Pholasin luminescence indicate that fMLP/cytochalasin B and PMA stimulated neutrophils produce more $O_2^{\cdot-}$ than fMLP stimulated cells. Thus, Pholasin luminescence can be used to detect, sensitively and specifically, $O_2^{\cdot-}$ production and MPO release from stimulated neutrophils.

Keywords: Pholasin luminescence; Neutrophils; Superoxide anion radicals; Degranulation; Myeloperoxidase; Elastase

Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks balanced salt solution; MPO, myeloperoxidase; PMA, phorbol myristoyl acetate; RLU, relative light units; SOD, superoxide dismutase

INTRODUCTION

Pholasin, the photoprotein of the common piddock *Pholas dactylus* with a molecular weight

*Corresponding author. Tel.: +49-341-9715705. Fax: +49-341-9715709. E-mail: arnj@medizin.uni-leipzig.de

of 34 kDa,[1] emits an intense luminescence upon oxidation.[2] This protein has been used to detect very sensitively the oxidative activity in suspensions of stimulated neutrophils.[3] These phagocytosing cells produce a variety of reactive oxygen species (ROS) including superoxide anion radicals, hydrogen peroxide, hypochlorous acid, hydroxyl radicals and singlet oxygen as well as release enzymes like elastase or myeloperoxidase (MPO) from their granules.[4]

MPO (donor: hydrogen peroxide oxidoreductase, EC 1.11.1.7) is involved in the production of ROS and substrate oxidation. It is activated via hydrogen peroxide and converted into the oxidatively active intermediates, compounds I and II.[5] Compound I produces the powerful oxidising and chlorinating species hypochlorous acid.[6] Compounds I and II oxidise a variety of substrates including aromatic amino acids, drugs and others.[5] Native MPO also reacts with superoxide anion radicals to give compound III.[7] Such activated compounds of MPO exist in the extracellular space after MPO release from neutrophils.

Previous investigations on model systems have shown which ROS generated from cells can be involved in Pholasin luminescence.[2,8] Furthermore, the role of different compounds of MPO on Pholasin luminescence has been investigated.[8,9] Superoxide anion radicals generated by the xanthine/xanthine oxidase system induce a strong luminescence of Pholasin.[2] This light emission is completely suppressed by superoxide dismutase (SOD). Native MPO as well as compound III of MPO are unable to oxidise Pholasin, whereas Pholasin acts as a substrate for compound I and II of MPO.[8,9] While the addition of HOCl causes a luminescence of Pholasin,[8,10] hypochlorous acid produced by the MPO–H_2O_2–Cl^- system or stimulated neutrophils is not involved in light emission of Pholasin as evidenced by experiments with the HOCl scavengers, methionine and taurine.[8] Hydrogen peroxide in micromolar concentrations is also unable to emit light from Pholasin.[10]

On the basis of these data, approaches using Pholasin have been developed to determine in suspensions of phagocytosing cells the production of superoxide anion radicals by NADPH oxidase as well as to assess the release of enzymes from azurophilic granules. Only 25,000 or less cells are necessary to obtain valuable results using Pholasin. The chemotactic tripeptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), a combination of fMLP and cytochalasin B and the phorbol ester phorbol myristoyl acetate (PMA) were used for stimulation of neutrophils. These stimulators act in a different way in the signal transduction cascade of stimulated neutrophils leading to the activation of NADPH oxidase. The chemotactic tripeptide fMLP, causes an activation of phospholipase C (PLC). This enzyme releases diacylglycerols (DAG) from phosphatidylinositol bisphosphate.[11] DAGs activate protein kinase C (PKC) which is responsible for activation of NADPH oxidase. Cytochalasin B influences cytoskeleton dependent processes and enhances the response of soluble stimulators concerning oxidative activity and enzyme degranulation.[12] PMA activates PKC directly.[13]

Pholasin luminescence was used to investigate the production of ROS generated from neutrophils. The aim of this study was to clarify which ROS influence the Pholasin luminescence generated from stimulated neutrophils and the contribution of secreted MPO to this luminescence. Data on Pholasin luminescence of stimulated neutrophils were compared with results of superoxide anion radical generation detected by the cytochrome c test as well as with the activity of elastase measured by release of *p*-nitroaniline from MeO–Suc–Ala–Ala–Pro–Val–*p*-nitroanilide and the amount of MPO determined with antibodies against MPO in the supernatant of stimulated cells.

MATERIALS AND METHODS

Chemicals

Pholasin was obtained from Knight Scientific (Plymouth, UK) (Pholasin® is the registered trade mark of Knight Scientific). An aqueous stock solution of Pholasin 3.6×10^{-7} mol l^{-1} was stored at −80°C. Human neutrophil MPO was a product of Planta GmbH (Vienna, Austria). Ficoll histopaque, xanthine, MeO–Suc–Ala–Ala–Pro–Val-*p*-nitroanilide, Hanks balanced salt solutions (HBSS) with and without $Ca^{2+}$and Mg^{2+} and without phenol red as well as PMA were purchased from Sigma (Deisenhofen, Germany). Cytochalasin B was a product of Calbiochem Novabiochem (Bad Soden, Germany). SOD from bovine erythrocytes (activity: 5185 U mg^{-1}), xanthine oxidase from butter milk (activity: 0.39 U mg^{-1}), cytochrome c and all other chemicals were obtained from Fluka (Neu-Ulm, Germany). A MPO ELISA test kit from Calbiochem/Novabiochem (Bad Soden, Germany) was used for the determination of MPO released from neutrophils.

Isolation and Purification of Neutrophils

Neutrophils were isolated from freshly drawn heparinized blood (10 U ml^{-1} heparin) from healthy volunteers by dextran-enhanced sedimentation of erythrocytes, Ficoll histopaque density centrifugation, lysis of remaining red blood cells with distilled water and washing of cells with HBSS in the absence of any divalent cations. Neutrophils were stored in Hank's medium at a concentration of 1×10^6 cells ml^{-1} at 4°C. The isolated cells were used within 2 h after preparation.

Measuring of Cytochrome C reduction

10^6 cells were incubated with a cytochrome c solution (5×10^{-5} mol l^{-1}, final concentration) and in some cases with cytochalasin B (10^{-5} mol l^{-1}, final concentration) in HBSS in a cuvette for UV/VIS spectroscopy. Cells were stimulated by addition of 100 µl fMLP (10^{-6} mol l^{-1}) or PMA (10^{-7} mol l^{-1}). The total measuring volume was 1000 µl. All incubations were performed at 37°C. The amount of superoxide anion radicals was determined 10 min after cell stimulation from the increase in absorbance of reduced cytochrome c using $\varepsilon_{550} = 21,000\ M^{-1}\ cm^{-1}$.[14] Control experiments in the presence of SOD (100 U ml^{-1}) did not show any reduction of cytochrome c.

Chemiluminescence Measurements

All chemiluminescence measurements were carried out using the microtiter plate luminometer MicroLumat LB 96 P (EG&G Berthold, Wildbad, Germany).

25,000 cells were incubated with Pholasin (7×10^{-8} mol l^{-1}, final concentration) at 37°C. In some cases SOD (100 U ml^{-1}), potassium cyanide (KCN) (10^{-4} mol l^{-1}), methionine (from 10^{-5} to 10^{-3} mol l^{-1}), taurine (from 10^{-5} to 10^{-3} mol l^{-1}) or cytochalasin B (10^{-5} mol l^{-1}) were also preincubated with neutrophils. Neutrophils were stimulated with 50 µl fMLP (10^{-6} mol l^{-1}) or 50 µl PMA using an injection device after 2 min incubation time. The resulting chemiluminescence was measured during the next 13 min. The total measuring volume was 250 µl. All concentrations indicated are final ones.

Determination of Myeloperoxidase Concentration

For the measuring of the MPO concentration, a MicroScreener LB 9260 P (EG&G Berthold, Wildbad, Germany) has been used. 3×10^6 neutrophils were stimulated at 37°C with fMLP (10^{-6} mol l^{-1}) fMLP/cytochalasin B (10^{-6} and 10^{-5} mol l^{-1}, respectively) and PMA (10^{-7} mol l^{-1}) in HBSS.

All concentrations are final ones. The supernatant obtained after gentle centrifugation was stored at −20°C until the determination of MPO concentration was performed using a commercially available ELISA test kit.

Determination of Elastase Release

For inducing elastase release, 5.5×10^6 cells ml^{-1} were incubated at 37°C in HBSS at pH 7.4 and stimulated with fMLP (10^{-6} mol l^{-1}), fMLP and cytochalasin B (10^{-6} and 10^{-5} mol l^{-1}) as well as PMA (10^{-7} mol l^{-1}) for different times. The stimulation was stopped by adding ice-cold HBSS buffer. Cells were spun down and the activity of elastase in supernatant was measured by the release of *p*-nitroaniline from MeO–Suc–Ala–Ala–Pro–Val–*p*-nitroanilide as substrate.[15] *p*-Nitroaniline was detected at 405 nm using an extinction coefficient of 8800 M^{-1} cm^{-1}[16] with a MicroScreener LB 9260 from EG&G Berthold Bad Wildbad, Germany.

RESULTS

Pholasin Luminescence of Stimulated Neutrophils

fMLP Stimulated Cells

Typical examples of Pholasin luminescence determined with fMLP stimulated neutrophils are given in Fig. 1. This stimulator induced a strong luminescence peak decreasing after about 3 min (-■-). SOD was used to remove superoxide anion radicals. This enzyme inhibited most of luminescence induced (-○-, panel B).

KCN was used to exclude the influence of MPO on Pholasin luminescence. KCN binds to native MPO and prevents further conversion of this enzyme to the oxidativley active compounds I, II or III. The Pholasin luminescence induced by O_2^- is unaffected by KCN as shown using the xanthine xanthine oxidase system (data not shown). The MPO inhibitor KCN had only a very small effect on Pholasin luminescence in fMLP stimulated neutrophils (-□-, panel A).

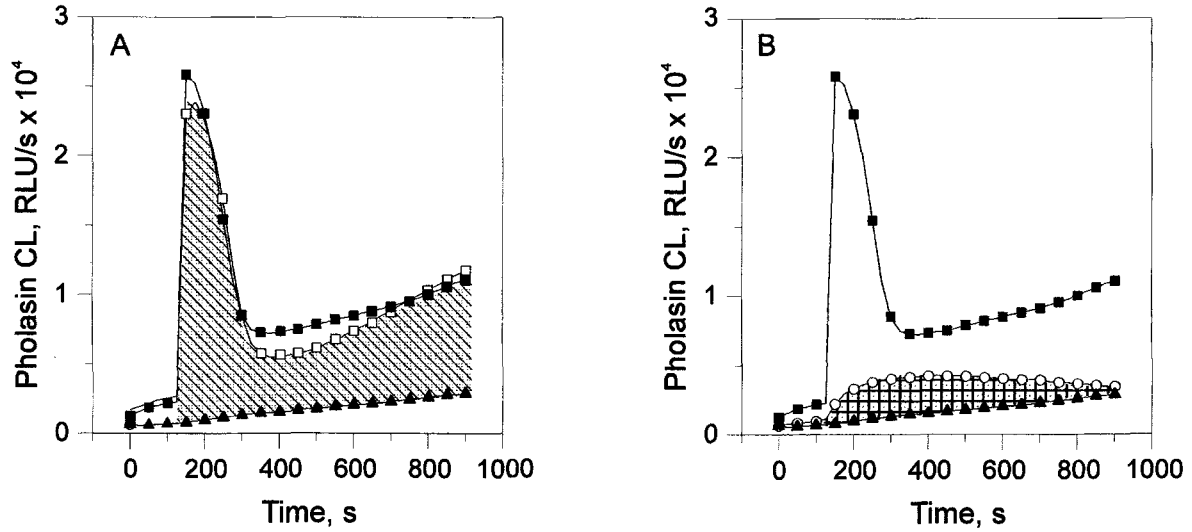

FIGURE 1 Pholasin luminescence of fMLP stimulated neutrophils. Marked areas correspond to luminescence caused by O_2^- (panel A) and generated by MPO reactions (panel B). Cells were incubated with Pholasin (7×10^{-8} M, final concentration) (-■-), Pholasin and SOD (100 U ml^{-1}, final activity) (-○-), Pholasin and KCN (10^{-4} M, final concentration) (-□-) or with Pholasin and both substances (-▲-). Luminescence was induced by injection of 50 µl fMLP (10^{-6} M, final concentration) using an injection device 2 min after the start of measurements. Chemiluminescence was followed over 13 min. The total measuring volume was 250 µl. Typical examples of luminescence curves of five independent measurements are shown.

The combination of SOD and KCN (-▲-) abolished this luminescence nearly completely. The contribution of superoxide anion radicals to Pholasin luminescence is highlighted in panel A by the marked area. Since MPO was blocked by KCN, only $O_2^{\cdot-}$ contributes to the luminescence intensity. A burst of $O_2^{\cdot-}$ production occurred during the first 3 min after cell stimulation. Neutrophils were still active in releasing superoxide anion radicals at later times, however, with a lower intensity.

In a similar way, the contribution of MPO to light emission of neutrophils in the presence of Pholasin is shown by the marked area in panel B of Fig. 1. Here, in the presence of SOD, a contribution of $O_2^{\cdot-}$ can be excluded. The contribution of MPO is rather small in comparison to the effects of superoxide anion radicals. A small increase of MPO-induced luminescence was observed during the first 2 min after stimulation. This light emission decreased slowly at longer incubation.

The contribution of MPO-induced Pholasin oxidation was, however, much higher in neutrophils stimulated by fMLP/cytochalasin B (Fig. 2) or PMA (Fig. 3).

fMLP/Cytochalasin B Stimulated Cells

The addition of cytochalasin B to fMLP stimulated cells led to a change in Pholasin luminescence response compared to exclusively fMLP stimulated cells (Fig. 2, -■-). The luminescence yield was about 4-fold enhanced and the kinetics of luminescence generation were changed. The luminescence increased immediately after the addition of fMLP to the cells. A luminescence maximum was found about 5 min after stimulation and the luminescence declined more slowly than in fMLP stimulated cells.

SOD (-○-) decreased the luminescence, but to a smaller extent than in fMLP stimulated cells. The signal increased slowly, but it showed a luminescence maximum at the same time as in the absence of SOD. KCN (-□-) diminished considerably the luminescence signal over all incubation. Immediately after cell stimulation,

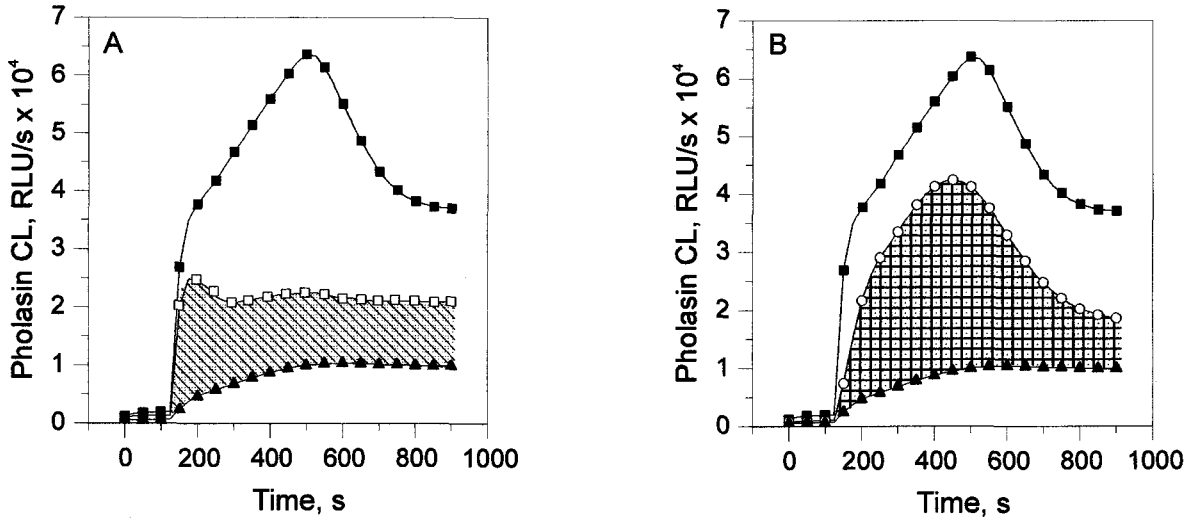

FIGURE 2 Pholasin luminescence of fMLP/cytochalasin B stimulated neutrophils. Cells were treated with cytochalasin B (10^{-5} M, final concentration), Pholasin (7×10^{-8} M, final concentration) (-■-), and in some cases with SOD (100 U ml^{-1}, final activity) (-○-), KCN (10^{-4} M, final concentration) (-□-) or both substances (-▲-). All other experimental conditions were the same as in Fig. 1. Marked areas show the influence of $O_2^{\cdot-}$ (panel A) or MPO (panel B) on Pholasin luminescence. Typical examples of luminescence curves of five independent measurements are shown.

there was a short time period of a few seconds where the KCN treated cells behaved in a similar manner to untreated samples. The combination of SOD and KCN (-▲-) inhibited most of the luminescence signal.

The marked area in panel A of Fig. 2 corresponds to luminescence caused by superoxide anion radicals. This luminescence yield is about 2-fold enhanced compared to fMLP stimulated cells. MPO influenced the Pholasin luminescence in fMLP/Cytochalasin B stimulated cells more dramatically (marked area in panel B). The luminescence yield is about 10-fold enhanced when exclusively compared to fMLP stimulated cells. Cytochalasin B is known to enhance superoxide anion production as well as MPO degranulation in fMLP stimulated neutrophils.[12]

PMA Stimulated Cells

Typical examples for Pholasin luminescence in PMA stimulated cells are shown in Fig. 3 (-■-). Two different phases in the kinetics of luminescence are clearly visible. Immediately after cell stimulation the luminescence signal increased, then decreased slightly, before increasing again more dramatically at longer incubation times. SOD (-○-) diminished strongly the first part of luminescence. The second part of luminescence was only slightly affected by SOD. KCN (-□-) showed no influence on the first part of luminescence but inhibited the later events of luminescence. The combination of KCN and SOD (-▲-) inhibited most of the luminescence.

The marked area in panel A of Fig. 3 corresponds to Pholasin luminescence induced by $O_2^{\cdot-}$. Luminescence marked in panel B is caused by MPO reactions. In Fig. 3 is clearly visible that superoxide anion radicals contribute to Pholasin luminescence maximally during the first 2 min. The luminescence yield caused by $O_2^{\cdot-}$ is about 2-fold enhanced compared to fMLP stimulated cells. MPO-driven reactions contribute also to Pholasin luminescence in PMA stimulated cells, but during the first minutes after stimulation only to a small extent. MPO is secreted to a greater extent after longer incubation time. The luminescence yield caused by

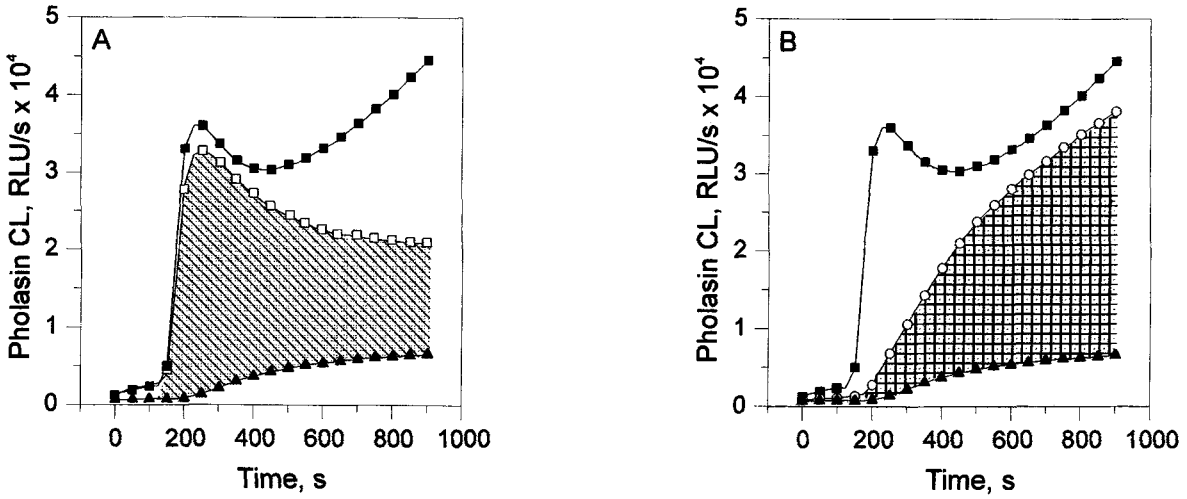

FIGURE 3 Pholasin luminescence of PMA stimulated neutrophils. Pholasin (7×10^{-8} M, final concentration) (-■-) and in some experiments also SOD (100 U ml^{-1}, final activity) (-○-), KCN (10^{-4} M, final concentration) (-□-) or both substances (-▲-) were incubated with the cells before addition of PMA (10^{-7} M, final concentration). Luminescence was detected over 15 min. Typical examples of luminescence curves of five independent measurements are shown. Marked areas indicate the influence of $O_2^{\cdot-}$ (panel A) or MPO (panel B) on Pholasin luminescence.

MPO is about 5-fold higher in PMA stimulated cells than in fMLP stimulated ones.

Production of Superoxide Anion Radicals Under Different Stimulation Conditions

In order to compare the data on Pholasin luminescence towards the production of superoxide anion radicals, $O_2^{\cdot-}$ was also determined by the cytochrome c test under different stimulation conditions.

The principle of this assay is that cytochrome c is reduced by superoxide anion radicals that results in an increased absorbance at 550 nm. The cytochrome c test is specific for the detection of $O_2^{\cdot-}$ in suspensions of stimulated neutrophils,[17] but its sensitivity is rather limited.

Table I shows the amount of $O_2^{\cdot-}$ produced by neutrophils stimulated for 10 min with fMLP, fMLP/cytochalasin B and PMA compared to unstimulated cells. Obviously, fMLP stimulated cells reduced only a low amount of cytochrome c. However, the production of $O_2^{\cdot-}$ was 10-fold enhanced in fMLP/cytochalasin B and PMA stimulated cells in comparison to the fMLP stimulated cells. Cytochrome c reduction was totally inhibited by SOD. These control measurements were carried out to verify the specificity of the cytochrome c assay for $O_2^{\cdot-}$.

Assessment of Enzyme Release from Azurophilic Granules Under Different Stimulation Conditions

The release of MPO and elastase from azurophilic granules of stimulated neutrophils was also investigated. MPO released under different stimulation conditions was determined by a commercially available ELISA test. Results for fMLP, fMLP/cytochalasin B and PMA stimulated cells are shown in Fig. 4. For quantification a standard curve with isolated MPO was recorded (data not shown).

When cells were exclusively stimulated with fMLP only a very small amount of MPO could be detected in the supernatant. The same small release was found in unstimulated cells at 37°C. PMA stimulated cells, however, released a 5-fold greater amount of MPO, and fMLP/cytochalasin B stimulated cells released about 10 times more MPO, than fMLP stimulated cells. All data are expressed as the difference to background value detected in unstimulated cells held at 4°C.

Elastase was also used as a marker enzyme of degranulation of azurophilic granules.[18] The activity of this enzyme released into the supernatant of cells was measured after different incubation times (Fig. 5). As in the case of MPO determination, when cells were stimulated with fMLP/cytochalasin B the measured activity was higher than in PMA stimulated cells. In fMLP stimulated cells, only a low elastase activity could be detected. Kinetics of the degranulation process were also investigated. Whereas, in fMLP/cytochalasin B stimulated cells, the release of the enzyme occurred immediately after stimulation, elastase was secreted with a delay in PMA stimulated cells. Similar kinetics of enzyme release were found for Pholasin luminescence in fMLP/cytochalasin B and

TABLE I Superoxide anion radicals released from 10^6 neutrophils stimulated for 10 min at 37°C as determined by the cytochrome c test

Stimulator	E_{550}	$[O_2^{\cdot-}]$ ($\mu mol\,l^{-1}$)
unstimulated cells	0.01	0.5
FMLP ($10^{-6}\,mol\,l^{-1}$)	0.078	3.6
fMLP ($10^{-6}\,mol\,l^{-1}$)/cytochalasin B ($10^{-5}\,mol\,l^{-1}$)	0.728	34.5
PMA ($10^{-7}\,mol\,l^{-1}$)	0.787	37.3

PMA stimulated cells. Thus, Pholasin luminescence (Figs. 1b, 2b, 3b) reflects very well the release of MPO from azurophilic granules.

Effect of HOCl Produced by Stimulated Neutrophils

When soluble stimulators are used for activation of neutrophils, the powerful oxidizing species HOCl is also released into the extracellular environment.[6]

HOCl is able to induce Pholasin luminescence.[18,10] To clarify whether HOCl produced by neutrophils contributes to Pholasin luminescence, the HOCl scavengers, methionine and taurine, were incubated with cells prior to stimulation. Both scavengers are known to quench the Pholasin luminescence induced by the reagent HOCl.[8] The influence of the scavengers methionine and taurine on Pholasin luminescence in stimulated neutrophils is shown in Table II. Cells were additionally treated with SOD prior to stimulation, since SOD is known to enhance the HOCl production of cells up to 500%.[19] SOD is also known to promote the formation of compound I of MPO, which is responsible for the chlorinating activity.[5] However, both methionine and taurine showed only a negligible influence on Pholasin luminescence of neutrophils, upon all kinds of stimulation. Thus, HOCl produced from neutrophils does not contribute to the Pholasin luminescence.

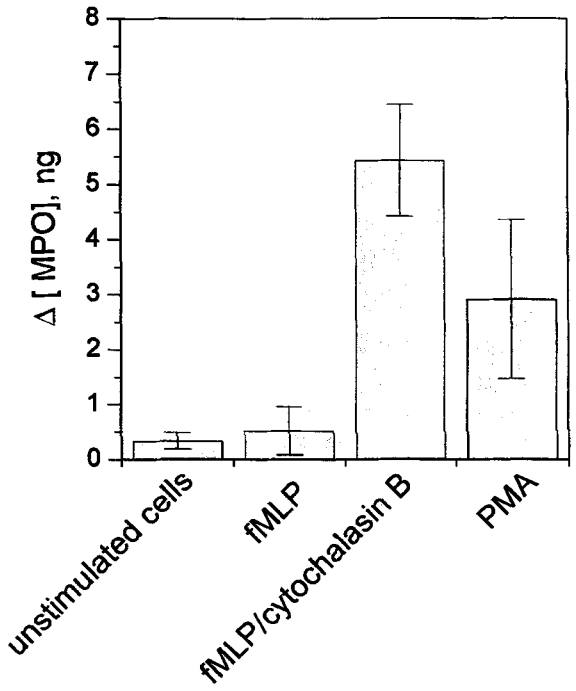

FIGURE 4 Determination of the amount of MPO in supernatant of stimulated neutrophils. 100 µl of supernatant was used for ELISA test. The control sample corresponds to unstimulated cells incubated at 37°C. Column two corresponds to MPO release from cells stimulated with fMLP (10^{-6} M, final concentration). In a third sample, cells were treated with cytochalasin B (10^{-5} M, final concentration) and stimulated with fMLP (10^{-6} M, final concentration). In column four, cells were stimulated with PMA (10^{-7} M, final concentration). Background absorbance measured with samples of unstimulated cells at 4°C were subtracted. Means and SD of three independent measurements are shown.

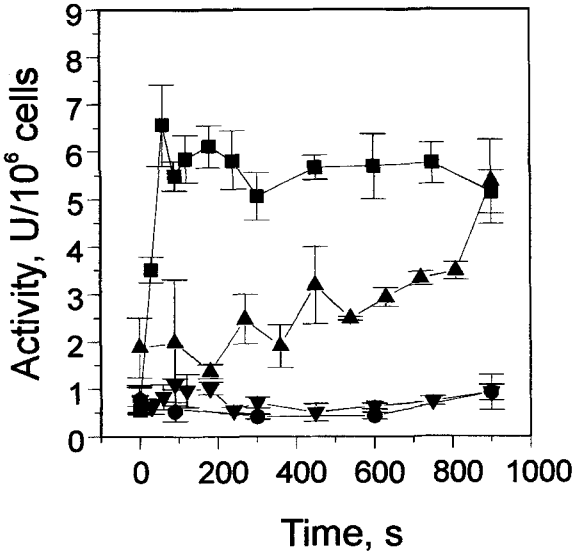

FIGURE 5 Determination of elastase activity in the supernatant of stimulated neutrophils. 5.5×10^6 cells were stimulated. As stimulators fMLP (10^{-6} M, final concentration) (-◆-), fMLP (10^{-6} M, final concentration) and cytochalasin B (10^{-5} M, final concentration) (-■-) and PMA (10^{-7} M, final concentration) (-◆-) were used in comparison to unstimulated cells (-●-). Means and SD of three different cell populations are shown.

TABLE II Effects of the HOCl scavengers, methionine and taurine, on Pholasin luminescence in stimulated neutrophils. Cells were incubated with methionine or taurine, SOD (100 U/ml, final activity) and Pholasin (7×10^{-8} M, final concentration). Luminescence was followed over 15 min and the luminescence yield was determined. Means and SD of integral intensities of three independent measurements are shown. The luminescence intensities without HOCl scavengers were set to 100%

Stimulator	Luminescence yield, %	
	Methionine, (10^{-3} mol l^{-1})	Taurine, (10^{-3} mol l^{-1})
fMLP (10^{-6} mol l^{-1})	85.7±20	101.7±26
fMLP (10^{-6} mol l^{-1})/cytochalasin B (10^{-5} mol l^{-1})	96.6±10	130.8±10
PMA (10^{-7} mol l^{-1})	84±10	71±1

DISCUSSION

The highly sensitive Pholasin luminescence induced after the stimulation of human neutrophils depends mainly on superoxide anion radicals and secreted MPO. Using either SOD or the MPO inhibitor, KCN, it is possible to evaluate the contribution of MPO or superoxide anion radicals, respectively, to the light emission. Thus, Pholasin in combination with KCN or SOD allows the specific detection of the generation of superoxide anion radicals and the release of MPO, respectively. Pronounced effects of hypochlorous acid generated from MPO on Pholasin luminescence of neutrophils can be excluded under all stimulation conditions because of the lack of effects of the HOCl scavengers, methionine and taurine. Although reagent HOCl is able to stimulate Pholasin light emission,[8,10] HOCl produced by the $MPO/H_2O_2/Cl^-$ system does not oxidise Pholasin as shown by a lack of effects of methionine and taurine.[8] Thus, compound I (and maybe compound II) of MPO contributes to Pholasin oxidation.

To obtain data on Pholasin luminescence, only a low number of cells are required. We used routinely 25,000 cells per well. The signals were high enough that it is in principle possible to use even lower amounts of cells. One of the first papers on the application of Pholasin to measure the oxidative activity of stimulated neutrophils reported that measurement on a single cell is possible.[13] On the other hand, established assays to measure superoxide anion radical production or degranulation of enzymes from azurophilic granules require about 10^6 or more cells per measurement.[17,20,21]

Degranulation was assessed by the detection of MPO in the supernatant using an ELISA test kit, or by measurement of elastase activity. Both methods revealed that cells stimulated for 10 min with fMLP/cytochalasin B or PMA released about 10- or 5-fold more enzymes than fMLP-stimulated or control cells. Similar data were obtained using Pholasin in combination with SOD (Figs. 1b, 2b, and 3b). Comparison of the kinetics of enzyme release also revealed similar results between the measured elastase activity and Pholasin luminescence for all applied stimulators.

The cytochrome c test revealed large differences in the production of superoxide anion radicals in stimulated neutrophils compared to Pholasin luminescence. High levels were found in fMLP/cytochalasin B and PMA stimulated cells. The production of O_2^- in fMLP stimulated cells was only about 10% of these values. Pholasin in combination with KCN detects superoxide anion radicals (Figs. 1a, 2a, and 3a). Although the luminescence levels for superoxide anion radicals are lower in fMLP stimulated neutrophils than in fMLP/cytochalasin B or PMA stimulated cells, the differences between these values are not as large as those obtained by the cytochrome c test.

The reasons for this different behaviour can be numerous. Different cell numbers were used in both test systems. Consequently, the total

amount of superoxide being produced is quite different. Furthermore, superoxide anion radicals are not a final product of oxidative activity of neutrophils. These species undergo a spontaneous dismutation to hydrogen peroxide and oxygen. Superoxide anion radicals react also with native MPO to yield compound III of the enzyme[7] or with hypochlorous acid to form hydroxyl radicals.[22] In any test system either cytochrome c or Pholasin compete with other reactions for superoxide anion radicals. Considering that totally different concentrations of reactants were employed in each assay system, it is possible that these systems differ in their ability to reflect the generation of superoxide anion radicals. In each case, the signals were inhibited by SOD in order to verify the dependence of the signal on superoxide anion radicals.

In PMA as well as in fMLP/cytochalasin B stimulated cells MPO contributes to Pholasin luminescence. This is a new observation. In some publications Pholasin luminescence was used to determine the oxidative activity of neutrophils. Müller *et al.* found a strong dependence of Pholasin luminescence from superoxide anion radicals but only a small influence of MPO.[23] These authors used fMLP and PMA as stimulators and followed the chemiluminescence only for 5 min.

Our experiments have shown that when cells are treated with SOD prior to stimulation, Pholasin luminescence is an excellent marker for MPO degranulation. Therefore, a new, very fast and sensitive method has been developed to observe the degranulation of azurophilic granules from neutrophils.

Acknowledgements

This work was supported by the German Research Council (Grants AR 283/4-1, SFB 294, SFB 197 and the Postgraduate Training Programme "Mechanisms and Applications of Non-Conventional Oxidation Reactions").

References

[1] Henry, J.P. and Monny, C. (1977) "Protein–protein interactions in the *Pholas dactylus* system of bioluminescence", *Biochemistry* **16**, 2517–2525.
[2] Henry, J.P. and Michelson, A.M. (1973) "Studies in bioluminescence VIII. Chemically induced luminescence of *Pholas dactylus luciferin*", *Biochimie* **55**, 83–93.
[3] Roberts, P.A., Knight, J. and Campbell, A.K. (1987) "Pholasin—a bioluminescent indicator for detecting activation of single neutrophils", *Analytical Biochemistry* **160**, 139–148.
[4] Edwards, S.W. (1994) *Biochemistry and Physiology of the Neutrophil* (Cambridge University Press, Cambridge).
[5] Kettle, A.J. and Winterbourn, C.C. (1997) "Myeloperoxidase: a key regulator of neutrophil oxidant production", *Redox Report* **3**, 3–15.
[6] Harrisson, J.E. and Schultz, J. (1976) "Studies on the chlorinating activity of myeloperoxidase", *Journal of Biological Chemistry* **251**, 1371–1374.
[7] Winterbourn, C.C., Garcia, R. and Segal, A.W. (1985) "Production of the superoxide adduct of myeloperoxidase (compound III) by stimulated neutrophils and its reactivity with H_2O_2 and chloride", *Biochemical Journal* **228**, 583–592.
[8] Reichl, S., Arnhold, J., Knight, J., Schiller, J. and Arnold, K. (2000) "Reactions of Pholasin with peroxidases and hypochlorous acid", *Free Radical Biology and Medicine* **28**, 1555–1563.
[9] Reichl, S., Petković, M., Schiller, J. and Arnhold, J. (2001) "Reactions of Pholasin® with peroxidases in the presence of superoxide anion radicals", In: Albrecht, S., Zimmermann, T. and Brandl, H., eds, Chemiluminescence at the turn of the millennium—an indispensable tool in modern chemistry, biochemistry and medicine (Schweda-Werbedruck-Verlag, Dresden) 152–155.
[10] Wittko-Sarsat, V., Nguyen, A.T., Knight, J. and Descamps-Latscha, B. (1992) "Pholasin: a new probe for detection of chloramines derived from human phagocytes", *Free Radical Biology and Medicine* **13**, 83–88.
[11] Baggiolini, M., Boulay, F., Badwey, J.A. and Curnutte, J.T. (1993) "Activation of neutrophil leukocytes: chemoattractant receptors and respiratory burst", *FASEB Journal* **7**, 1004–1010.
[12] Dahlgren, C., Johannson, A., Lundquist, H., Bjerrum, O.W. and Borregaard, N. (1992) "Activation of oxygen radical generating system in granules of intact human neutrophils by a calcium ionophore (ionomycin)", *Biochimica et Biophysica Acta* **1137**, 182–188.
[13] White, J.M., Huang, C.-K., Hill, J.M., Naccache, P.H., Becker, E.L. and Sha'afi, R.I. (1984) "Effect of phorbol 12-myristate 13-acetate and its analogue 4α-phorbol 12,13-diacetonate on protein phosphorylation and lysosomal enzyme release in rabbit neutrophils", *Journal of Biological Chemistry* **259**, 8605-8611.
[14] Hoffstein, S.T., Gennaro, D.E. and Manzi, R.M. (1985) "Surface contact inhibits neutrophil superoxide generation induced by soluble stimuli", *Laboratory Investigations* **52**, 515–522.

[15] Nakajima, K., Powers, J.C., Ashe, B.M. and Zimmermann, M. (1979) "Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the al-protease inhibitor reactive site", *Journal of Biological Chemistry* **254**, 4027–4032.

[16] Erlanger, B.F., Kokowsky, N. and Cohen, W. (1961) "The preparation and properties of two new chromogenic substrates of trypsin", *Archives of Biochemistry and Biophysics* **95**, 271–278.

[17] Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) "Biological defence mechanisms. The production by leukocytes of superoxide, a potential bactericidial agent", *Journal of Clinical Investigations* **52**, 741–744.

[18] Barrett, A.J. (1981) "Leukocyte elastase", *Methods in Enzymology* **80**, 581–588.

[19] King, C.C., Jefferson, M.M. and Thomas, E.L. (1997) "Secretion and inactivation of myeloperoxidase by isolated neutrophils", *Journal of Leukocyte Biology* **61**, 293–302.

[20] Mayo, L.A. and Curnutte, J.T. (1990) "Kinetic microplate assay for superoxide production by neutrophils and other phagocytic cells", *Methods in Enzymology* **186**, 567–575.

[21] Wiedow, O., Muhle, K., Streit, V. and Kameyoshi, Y. (1996) "Human eosinophils lack human leukocyte elastase", *Biochimica et Biophysica Acta* **1315**, 185–187.

[22] Ramos, C.L., Pou, S., Britigan, B.E., Cohen, M.S. and Rosen, G.M. (1992) "Spin trapping evidence for myeloperoxidase-dependent hydroxyl radical formation by human neutrophils and monocytes", *Journal of Biological Chemistry* **267**, 8307–8312.

[23] Müller, T., Davies, E.V. and Campbell, A.K. (1989) "Pholasin chemiluminescence detects mostly superoxide anion radicals from activated human neutrophils", *Journal of Bioluminescence and Chemiluminescence* **3**, 105–113.