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Concanavalin A stimulation of O_2 consumption in electropermeabilized neutrophils via a pertussis toxin-insensitive G protein

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Electropermeabilized human neutrophils were used to investigate the possible role of G-proteins in the respiratory burst elicited by concanavalin A (Con A). The Con A-induced activation of the NADPH oxidase was not inhibited by either pertussis toxin or cholera toxin. However, the burst was inhibited by GDP and GDPβS providing evidence for the involvement of a G-protein(s). O_2 consumption in Con A-stimulated cells was dependent on both ATP and Mg^{2+}. ATP could be substituted by ATPγS but not by the non-hydrolyzable analog AMP-PNP, suggesting involvement of phosphotransferase reactions. It is concluded that at least two distinct types of G-proteins are capable of inducing the respiratory burst in neutrophils and that accumulation of phosphorylated intermediates may be essential for activation of the respiratory burst by the lectin.

NADPH oxidase; Cholera toxin; GTP-binding protein; (Polymorphonuclear leukocyte)

1. INTRODUCTION

Phagocytic cells such as neutrophils are able to kill invading microorganisms by generating a variety of reduced oxygen metabolites. This respiratory burst is initiated by NADPH oxidase, a membrane-bound enzyme which reduces molecular oxygen to superoxide, with the concomitant oxidation of NADPH [1,2]. Superoxide anions can then be converted to H_2O_2 by superoxide dismutase and to other toxic compounds, including hydroxy radicals. Superoxide and several of its metabolites are potent bactericidal agents [1,2].

In neutrophils, the respiratory burst can be triggered by a variety of soluble and particulate stimuli [2–4]. One of the most commonly studied activators is the synthetic tripeptide formyl-methionyl-leucyl-phenylalanine (fMLP), which resembles N-terminal peptides of bacterial proteins. The interaction between fMLP and specific receptors on the surface of neutrophils is believed to alter the conformation of a GTP-binding or G-protein, which in turn signals the activation of phospholipase C [4]. This enzyme subsequently cleaves phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate and diacylglycerol, a physiological activator of protein kinase C. It is generally believed that the resulting rise in intracellular Ca^{2+} and/or phosphorylation reactions catalyzed by protein kinase C mediate stimulation of the respiratory burst by chemotactic peptides [4]. However, there is increasing evidence that alternative stimulatory pathways may exist, since the burst can still be generated when cytosolic Ca^{2+} is invariant [5] and the kinase is inhibited [5–8].

In intact cells, the involvement of G-proteins in signal transduction is generally deduced from the sensitivity of the response to the bacterial toxins, pertussis toxin (PT) and cholera toxin (CT). These

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toxins catalyze the ADP ribosylation of the α-subunits of several types of G-proteins, altering their coupling to receptors [9]. Thus, the activation of the respiratory burst by fMLP [10,11], sodium fluoride [12] or platelet-activating factor [13] is greatly inhibited by pretreatment of the cells with PT. However, the increased O_2 consumption induced by other stimuli is unaffected by PT [14,15]. One such PT insensitive activator is the lectin concanavalin A (Con A), which is thought to stimulate the respiratory burst by crosslinking carbohydrate-containing receptors into clusters [16]. The insensitivity of the Con A response to PT suggests the involvement of a G-protein different from the type that mediates the response to other activators. Alternatively, the Con A response may proceed via a novel, G-protein-independent pathway.

In addition to the bacterial toxins, exogenous guanine analogues such as guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS) and guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) have been used to determine the involvement of G-proteins in signal transduction. However, these nucleotides are membrane impermeant and cannot be utilized with intact cells. Unfortunately, receptor-mediated activation of the respiratory burst is impaired in detergent-treated neutrophils and in reconstituted cell-free systems obtained thereof. In contrast, responsiveness to fMLP and other ligands is preserved in electrically permeabilized cells [5,17]. Compounds of $M_r \leq 750$, such as the guanine nucleotides, can readily enter the electroporated cells. This experimental model was used in the present experiments to investigate the possible involvement of a G-protein(s) in the stimulation of the respiratory burst of human neutrophils by Con A.

2. MATERIALS AND METHODS

2.1. *Materials*

NADPH, EGTA, *trans*-1,2-diaminocyclohexane *N,N,N′,N′*-tetraacetic acid (CDTA), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonate (Hepes), fMLP, 2-deoxy-D-glucose, Con A, ATP (K^+ salt), 5′-adenylylimido diphosphate (AMP-PNP; Li^+ salt), GTP (Na^+ salt), GDPβS (Li^+ salt), GTPγS (Li^+ salt), GDP (Na^+ salt), GMP (Na^+ salt) and medium RPMI 1640 were obtained from Sigma. Adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS; Li^+ salt) was purchased from Boehringer Mannheim. CT and PT were acquired from List Biological Laboratories. Ficoll 400 and Dextran T500 were from Pharmacia LKB Biotechnology.

2.2. *Solutions*

Bicarbonate-free RPMI 1640 medium was buffered to pH 7.3 with 25 mM Na-Hepes. The permeabilization medium contained (in mM): 140 KCl, 10 Hepes, 10 glucose, 1 $MgCl_2$, 1 ATP, 1 EGTA and sufficient $CaCl_2$ to give a final free Ca^{2+} concentration of 100 nM. Where indicated, $MgCl_2$ or ATP were omitted. NADPH (2 mM) and GTP (100 μM) were added to the medium immediately before the oxygen consumption measurements. Media were adjusted to pH 7.0 and to 290 ± 5 mOsm with the major salt.

2.3. *Cell isolation and permeabilization*

Neutrophils were isolated from fresh heparinized human blood by dextran sedimentation and Ficoll-Hypaque gradient centrifugation. Contaminating red cells were removed by NH_4Cl lysis [18]. Neutrophils were counted using a model ZM Coulter counter, resuspended in Hepes-buffered RPMI 1640 at 10^7 cells/ml and maintained in this medium at room temperature until use.

Permeabilization was performed as described [5]. Briefly, 8×10^6 cells were washed and then suspended in 0.8 ml ice-cold permeabilization medium. The cells were transferred to a BioRad pulser cuvette and permeabilized with 2 discharges of 5 kV/cm from a 25 μF capacitor. Between pulses, the cells were sedimented and resuspended in fresh, ice-cold permeabilization medium. The electroporated cells were then suspended in this medium for immediate measurement of O_2 consumption or maintained on ice until use. Where indicated, the cells were depleted of ATP prior to electropermeabilization by incubation for 5 min at 37°C in a medium containing (in mM): 140 NaCl, 5 KCl, 10 Hepes, 1 $MgCl_2$, 1 $CaCl_2$ and 5 deoxy-D glucose. For some experiments the cells were pretreated with bacterial toxins as follows: 8×10^6 intact cells were washed and suspended in 0.8 ml of a medium containing (in mM) 140 NaCl, 5 KCl, 10 Hepes, 10 glucose, 1 $MgCl_2$ and 1 $CaCl_2$ plus 625 ng/ml PT or 25–31.25 μg/ml CT. The cells were incubated in these media for 2 h at 37°C.

2.4. *O_2 consumption*

O_2 consumption was measured polarographically using a model 53 or a model 5300 biological oxygen monitor (Yellow Springs Instrument Co.) as described [5]. 4×10^6 electropermeabilized cells were suspended in 2 ml permeabilization medium at 37°C with stirring. Guanine, adenine and pyridine nucleotides were added where indicated prior to the measurements. O_2 consumption was calculated using a solubility coefficient of 0.024 ml O_2/ml at 37°C. All measurements were performed at least three times with blood from different donors and are presented as the mean ± SE or as representative traces.

3. RESULTS AND DISCUSSION

Fig.1 shows that, as in intact cells, a respiratory burst can be elicited by Con A in electropermeabilized neutrophils. The lectin (200 μg/ml) produced a maximal rate of O_2 con-

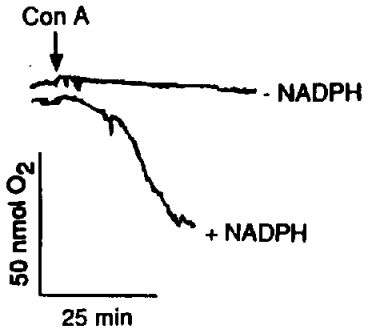

Fig.1. NADPH dependence of Con A-induced O_2 consumption in permeabilized neutrophils. Cells suspended in permeabilization medium containing 100 μM GTP in the presence (lower trace) or absence (top trace) of 2 mM NADPH. Con A (200 μg/ml) was added at the arrow. Traces are representative of three or more experiments.

sumption averaging 0.47 ± 0.05 nmol/10^6 cells per min (n = 19) after a lag time of approx. 9.41 ± 1.03 min. The magnitude of the response in electropermeabilized cells is comparable to values reported in intact cells [19]. As shown in fig.1 and in table 1, the O_2 consumption burst was dependent on the availability of exogenously added NADPH, the substrate of the plasmalemmal oxidase. Since intact cells utilize endogenous NADPH for the burst, and because the nucleotide-binding site of the oxidase is intracellular, the results of fig.1 provide confirmatory evidence that the cells were effectively permeabilized.

The sensitivity of the Con A response of permeabilized cells to PT was analyzed next. Pretreatment of intact cells with 625 ng/ml PT for the 2 h prior to permeabilization had no effect on oxygen consumption (fig.2A and table 1), whereas a comparable treatment inhibited the fMLP-induced response by 73% (fig.2B and table 1). Thus, the differential susceptibility of Con A and fMLP to inhibition by PT is preserved in the electropermeabilized cells. In neutrophils, CT has also been shown to ADP-ribosylate a phospholipase C-coupled G protein, thereby inhibiting subsequent biological responses [20,21]. In permeabilized neutrophils, we found that fMLP-induced activity in cells pretreated with this toxin (25–31.25 μg/ml)

Table 1

O_2 consumption measurements in electropermeabilized neutrophils

Stimulus	Condition	% activity	Significance
Con A	control	100	
	− ATP	19.83 ± 6.67 (8)	$p < 0.005$
	+ 500 μM AMP-PNP (− ATP)	11.16 ± 5.53 (3)	$p < 0.05$
	+ 10 μM ATPγS (− ATP)	154.73 ± 10.97 (4)	$0.10 > p > 0.05$
	− NADPH	4.40 ± 2.32 (6)	$p < 0.005$
	− Mg^{2+}	12.10 ± 6.20 (3)	$p < 0.005$
	− Mg^{2+} + 10 μM CDTA	20.86 ± 7.43 (5)	$p < 0.05$
	− GTP	110.67 ± 19.17 (3)	n.s.
	+ 100 μM GDP (− GTP)	69.14 ± 12.44 (6)	$p < 0.05$
	+ 500 μM GDP (− GTP)	23.41 ± 4.02 (5)	$p < 0.025$
	+ 100 μM GDPβS (− GTP)	25.94 ± 5.73 (12)	$p < 0.005$
	+ C. toxin (25–31.25 μg/ml)	159.07 ± 26.14 (3)	$0.10 > p > 0.05$
	+ P. toxin (625 ng/ml)	103.23 ± 7.71 (5)	n.s.
fMLP	control	100	
	+ C. toxin (25–31.25 μg/ml)	52.01 ± 14.64 (3)	$p < 0.05$
	+ P. toxin (625 ng/ml)	27.27 ± 10.29 (5)	$p < 0.01$

The control medium contained 1 mM ATP, 1 mM $MgCl_2$, 2 mM NADPH and 100 μM GTP as described in section 2. Where indicated, ATP, $MgCl_2$ or GTP were omitted. O_2 consumption rates determined from maximal slopes. Control O_2 consumption rate in the presence of 200 μg/ml Con A was 0.47 ± 0.05 nmol O_2/10^6 cells per min (n = 19) while that in the presence of 10^{-6}–10^{-7} M fMLP was 3.13 ± 0.44 (n = 7). Data are means ± SE of the indicated number of determinations (n). Percent (%) activity was calculated relative to the respective control. Significance was estimated using a paired t-test. n.s., not statistically significant

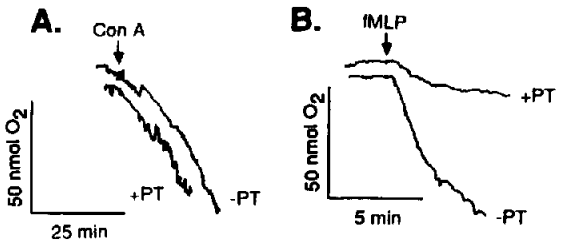

Fig.2. Effect of PT pretreatment on the activation of the respiratory burst in permeabilized neutrophils. Cells were pretreated with or without PT, electroporated and used for O_2 consumption measurements as described in section 2. (A) Cells suspended in permeabilization medium after treatment with (lower trace) or without (top trace) 625 ng/ml PT. Where indicated, the cells were stimulated with Con A (200 μg/ml). (B) Cells suspended in permeabilization medium after treatment with (top trace) or without (lower trace) 625 ng/ml PT. fMLP (10^{-7} M, final) added where indicated. A and B are representative traces from at least three experiments.

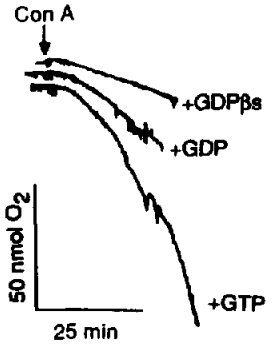

Fig.3. Inhibition of Con A-induced O_2 consumption by GDPβS and GDP. Electropermeabilized neutrophils were suspended in a medium containing NADPH in the presence of 100 μM GTP (lower trace), 500 μM GDP (middle trace) or 100 μM GDPβS (top trace). Con A (200 μg/ml) was added at the arrow. Traces are representative of at least three experiments.

was 52.0 ± 14.6% of the control response (table 1). In contrast, stimulation of O_2 utilization by Con A was not inhibited under these conditions. In fact, a moderate stimulation was recorded (table 1). Together, these observations suggest that activation of the NADPH oxidase by Con A in electropermeabilized human neutrophils is not inhibited by either CT or PT.

Stimulation of the respiratory burst by Con A in the presence and in the nominal absence of GTP produced similar O_2 consumption rates (table 1), suggesting that G-proteins are not involved in this response. However, the failure of exogenously added GTP to affect O_2 consumption could also indicate that: (i) sufficient endogenous GTP remains in the electroporated cells to support the oxidative response or (ii) that in the presence of added ATP, endogenous GDP may be converted to GTP by a nucleoside diphosphate kinase. Therefore, dependence on exogenous GTP cannot be used as the sole criterion to rule out the participation of G-proteins. To define more precisely the involvement of G-proteins in the Con A response, we determined whether O_2 consumption was inhibited by GDP or GDPβS, which stabilize the inactive form of the guanine nucleotide-binding proteins. As illustrated in fig.3, activation of the burst was inhibited by both GDP and GDPβS, at concentrations reported to inhibit G-proteins in other systems [22,23]. In the presence of 100 μM or 500 μM GDP, the rate of O_2 consumption was 69.1 ± 12.4% and 23.4 ± 4.0% of the control activity, respectively, while in the presence of 100 μM GDPβS the rate was 25.9 ± 5.7% of the control response (table 1). The inhibition was specific, inasmuch as neither GMP (data not shown) nor GTP (table 1) affected the Con A-induced burst significantly. Inhibition of the respiratory burst by both GDP and GDPβS supports the notion that Con A activates the NADPH oxidase via a PT-insensitive G-protein(s). Potentiation of the Con A response by GTPγS, another frequently used criterion of G-protein involvement, could not be tested since this nucletide was itself a powerful activator, even at concentrations as low as 1 μM (data not shown).

The involvement of a toxin-insensitive G-protein(s) in the activation of the respiratory burst by Con A indicates that this lectin activates a signalling process different from the PT-sensitive pathway triggered by fMLP. This is also supported by the finding that Con A mobilizes comparatively small amounts of Ca^{2+}, presumably due to its inability to break down significant amounts of phosphoinositides [24]. Whereas stimulation of protein phosphorylation by the increased levels of Ca^{2+} and diacylglycerol is thought to be central to stimulation by fMLP, little is known about the mode of action of Con A. There is evidence that phosphorylation of a number of proteins and in particular, a 46 kDa protein accompanies stimulation of NADPH oxidase by Con A [25,26].

However, it is not clear whether phosphorylation precedes and is essential for the respiratory burst. Information regarding the role of phosphotransferase reactions in activation can be obtained using electropermeabilized cells. In this system phosphorylation can be controlled by addition or omission of ATP. The results obtained using this approach are summarized in fig.4A and in table 1. Cells which were depleted of ATP, suspended in an ATP-free medium and then stimulated with Con A displayed an average rate of O_2 consumption which was only 19.8 ± 6.7% of the activity found in the presence of 1 mM exogenous ATP (table 1). The Con A response was also dependent on the presence of Mg^{2+} (fig.4B). In the absence of this divalent cation, the average O_2 consumption rate was only 12.1 ± 6.2% of the control rate (table 1). Dependence on both ATP and Mg^{2+} for activation of the respiratory burst by the lectin suggests the involvement of phosphotransferase reactions. This conclusion was further supported by the finding that a hydrolyzable γ-phosphate group was a required feature of the adenine nucleotide substrate. As shown in fig.4A and in table 1, the non-hydrolyzable ATP analogue, AMP-PNP was unable to support the stimulation of oxygen consumption.

Experiments using ATPγS are also consistent with a role of phosphotransferase reactions in the activation of neutrophils by Con A. This ATP analog can be used as substrate by some kinases, generating thiophosphorylated proteins or lipids which are resistant to hydrolysis by phosphatases. At a relatively low concentration (10 μM), which by itself has no effect on oxygen consumption, ATPγS potentiated the respiratory burst elicited by Con A (table 1) to a level 154.7 ± 11.0% of control activity, measured using 1 mM ATP. These results, which are similar to those obtained with fMLP [17], suggest that accumulation of biologically active thiophosphorylated proteins or lipids is responsible for stimulation of the respiratory burst in Con A-treated cells. Under physiological conditions, accumulation of phosphorylated intermediates can result from stimulation of kinase activity, from inhibition of phosphatase activity, or from a combination of these effects.

In conclusion, the experiments reported here indicate that the plant lectin Con A activates the respiratory burst in electropermeabilized human neutrophils via a G-protein that is not inhibited by PT or CT and therefore differs from that activated by fMLP and several other stimuli. Thus, at least two different types of GTP-binding proteins can mediate activation of the NADPH oxidase. The putative G-protein activated by Con A signals an ATP-dependent process, likely the stimulation of kinase activity and/or the inhibition of phosphatase activity, resulting in the formation of phosphorylated intermediates essential for the stimulation of the oxidase.

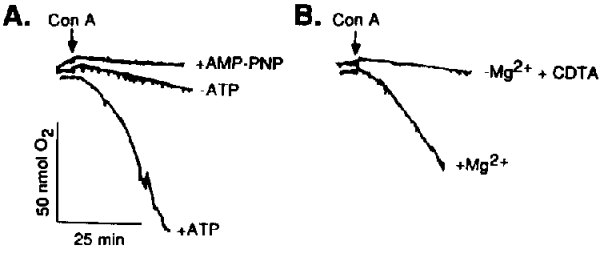

Fig.4. ATP and Mg^{2+} dependence of the Con A-induced respiratory burst. (A) Cells were ATP depleted, electropermeabilized and then used for O_2 consumption as described in section 2. The assay medium contained 500 μM AMP-PNP (top trace), 1 mM ATP (lower trace) or no added adenine nucleotide (middle trace). (B) Cells suspended in permeabilization medium containing 1 mM ATP and either 1 mM $MgCl_2$ (lower trace) or no added Mg^{2+} plus 10 μM CDTA (top trace). Con A (200 μg/ml) was added where indicated. The traces in A and B are representative of at least three experiments.

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