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Destabilization of neutrophil NADPH oxidase by ATP and other trinucleotides and its prevention by Mg^{2+}

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Abstract

Neutrophil NADPH oxidase (O_2^- generating enzyme) activated in a cell-free system was deactivated by dilution. When ATP was included in dilution the deactivation was further accelerated. The deactivation by dilution was biphasic, and the half-life of the enzyme was significantly shortened by ATP in each phase. ADP and AMP had little effect on the enzyme longevity while GTP and CTP had a similar effect to ATP. Staurosporine, a wide-range inhibitor of protein kinases, had no effect on ATP-induced deactivation, suggesting that the effect was not due to a protein phosphorylation. Mg^{2+} addition largely prevented the deactivation by ATP. Chemical crosslinking of the activated oxidase prevented the deactivation by dilution and ATP, suggesting that the deactivation is caused by dissociation of the oxidase complex. Estimation of actin filament (F-actin) showed that the F-actin level was markedly reduced by addition of ATP. The ATP effect on the deactivation was not prominent in a semi-recombinant system which does not contain cytosol. These results suggest that ATP-induced deactivation is largely due to the chelation of Mg^{2+} and are consistent with the concept that Mg^{2+} stabilizes the oxidase complex by stabilizing F-actin.

Keywords: Neutrophil; NADPH oxidase; Superoxide; ATP; Magnesium ion; F-actin

1. Introduction

Superoxide generation by neutrophils exerts an important role in the host defense system against microbial infection [1]. The enzyme responsible for O_2^- generation, called NADPH oxidase (or respiratory burst oxidase), is dormant in resting cells and becomes active upon cell activation [2]. The oxidase can also be activated in a cell-free system including plasma membrane and cytosol in the presence of an anionic amphiphile. The oxidase consists of membrane cytochrome b_{558} and cytosolic proteins ($p47^{phox}$, $p67^{phox}$, and rac) [3], which assemble into an active complex on the membrane upon activation [4]. Recently $p40^{phox}$ has also been thought to be a regulator protein [3].

NADPH oxidase complex is very labile and the lability has precluded the isolation of the active enzyme. In previous studies we showed that the oxidase either from activated cells or a cell-free system collapses rapidly with a half-life ($t_{1/2}$) of 2 min at 37°C [5,6]. The stability strictly depends on the cytosol concentration and is dramatically improved by chem-

Abbreviations: PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); $t_{1/2}$, half-life; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; F-actin, actin filament; TRITC, tetramethyl rhodamine isothiocyanate

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ical crosslinking [6], suggesting that the deactivation is caused by dissociation of the components from the complex.

ATP is required for the oxidase activation in neutrophils [7] and the activation is thought to involve $p47^{phox}$ phosphorylation probably by protein kinase C [8]. In contrast, cell-free activation induced by an anionic amphiphile is independent of the phosphorylation [4]. Although ATP enhances the cell-free activation [9,10], it was shown later that ATP is enzymatically converted to GTP, which actually enhances the activation [10].

Recently, we found that a cytoskeletal protein actin enhances NADPH oxidase in a cell-free system [11] and several actin-depolymerizing agents accelerate the oxidase deactivation [12]. We also showed that actin polymerization occurs during the oxidase activation and the content of actin filament (F-actin) correlates with the enzyme longevity.

In the course of our studies, we occasionally found that ATP accelerated the deactivation of the oxidase. As such an effect was unexpected, we examined in more detail the effect of ATP on the longevity of the oxidase and performed several experiments to clarify the mechanism by which ATP destabilizes the oxidase.

2. Materials and methods

2.1. Materials

ATP, CTP, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). ADP, AMP, GTP, ATPγS, and tetramethyl rhodamine isothiocyanate (TRITC)–phalloidin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). UDP was purchased from Wako Pure Chemicals (Osaka, Japan). All other reagents were the best grade commercially available.

2.2. Neutrophil preparation and subcellular fractionation

Human blood was obtained from healthy volunteers with informed consent. The separation of neutrophils and the fractionation of cytosol and plasma membrane were performed as previously described [6].

2.3. Cell-free activation and assay for O_2^- generation

The standard conditions for the cell-free activation were as follows. Plasma membrane (7.5 μg protein) and cytosol (105 μg protein) were mixed per 50 μl of 10 mM PIPES buffer (pH 7.0) containing 240 μM SDS, and incubated for 10 min at 25°C. Aliquots (10 μl) of the cell-free mixture were mixed with the buffer (240 μl) containing 200 μM NADPH and 80 μM cytochrome *c*, and the mixture was transferred into two cuvettes, one of which contained superoxide dismutase (80 μg/ml). O_2^- generation was immediately assayed at 25°C as previously described [13] and is expressed as nmol of O_2^- formed per min per mg of plasma membrane protein.

2.4. Effect of ATP on the stability of NADPH oxidase

An aliquot (10 μl) of the activation mixture was diluted 25-fold with 10 mM PIPES (pH 7.0) or the buffer containing ATP and/or $MgCl_2$ and incubated for a given time at 0°C. The mixture was transferred into two cuvettes and assayed for O_2^- generation as described above. The cell-free activation mixture originally contained 0.2 mM ATP and 0.8 mM $MgCl_2$ brought from cytosol and plasma membrane preparations. Consequently when the mixture was diluted 25-fold, the concentrations of ATP and $MgCl_2$ were 8 and 32 μM, respectively.

2.5. Semi-recombinant activation

The activation in a semi-recombinant system was performed as described previously [13]. Recombinant $p47^{phox}$ (7.1 μg), $p67^{phox}$ (N-terminal fragment 1–210) (3.9 μg), rac1 (C189S) (11.5 μg) preloaded with 100 μM GTPγS, were mixed with plasma membrane (8.1 μg) in 80 μl of 10 mM potassium phosphate buffer (pH 7.0) containing 0.8 mM $MgCl_2$. The mixture was supplemented with 200 μM SDS and incubated at 25°C for a given time. ATP effect on the stability was examined as described above (Section 2.4).

2.6. Chemical cross-linking

The cell-free activation mixture was stirred with or without 10 mM EDC at 0°C for 30 min. The control and cross-linked mixtures were diluted 25-fold with 10 mM PIPES (pH 7) or the buffer containing 1 mM ATP, and incubated for 10 min at 0°C.

2.7. F-actin estimation

F-actin content was measured according to the method of Redmond et al. [14] with modifications [12]. The cell-free activated mixture was diluted and incubated for 10 min at 0°C in the presence or absence of 1 mM ATP and then fixed with glutaraldehyde. The mixture was treated with TRITC–phalloidin with or without phalloidin at 0°C for 1 h. After ultracentrifugation, the pellets were extracted with methanol and the supernatant was subjected to fluorescence measurement. F-actin content was expressed as a difference in relative fluorescence intensity (ΔRFI) between the samples with and without phalloidin (the difference between the averages of three determinations for each condition). In some experiments, the cell-free activated mixture was fixed with glutaraldehyde without dilution (control).

3. Results

3.1. ATP facilitates the deactivation of NADPH oxidase

Fig. 1 shows the effect of dilution and ATP on the O_2^- generation from NADPH oxidase activated in a cell-free system. After a 60-min incubation at 0°C, the activity of non-diluted sample was 93% of the initial activity, which was reduced to 62% by the presence of ATP. When the sample was incubated in dilution, the activity decreased to 61%, which was further reduced to 19% by ATP. These results showed that either dilution or ATP caused an acceleration of the deactivation, and when used in combination they showed a synergistic effect on the deactivation.

Fig. 2A shows the time course of the deactivation in the presence or absence of ATP. As seen in the figure, ATP significantly accelerated the deactivation,

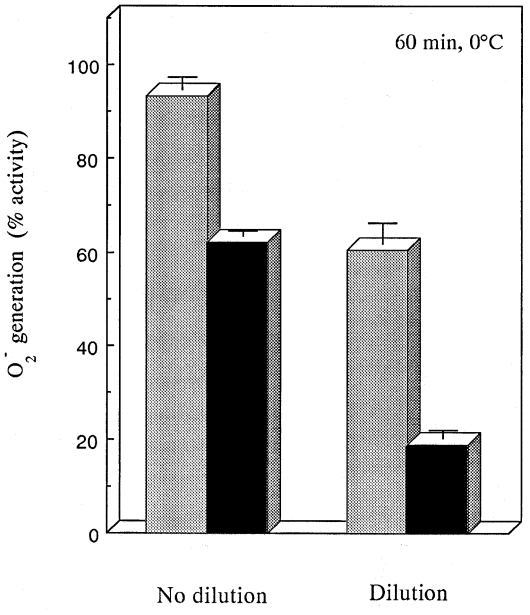

Fig. 1. The effect of dilution and/or ATP on the deactivation of NADPH oxidase. The cell-free mixture was incubated at 0°C with or without dilution for 60 min in the presence (dark bars) or absence (gray bars) of ATP. Then the mixture was assayed for O_2^- generation. The activity without incubation was taken as 100% (1132 ± 52 nmol/min/mg). Data are expressed as means ± S.D. of three determinations.

and the activity was 30% of the initial at 30 min while the control sample had 70% activity. The first-order plot of the data showed that the diluted oxidase deactivated in a biphasic manner (Table 1). The $t_{1/2}$s of the control were 17 and 147 min in the first and second phases, which were shortened to 3 and 42 min, respectively.

The deactivation by ATP was dose-dependent.

Table 1
Half-lives ($t_{1/2}$) of NADPH oxidase activity in dilution in the presence of ATP and/or Mg^{2+}[a]

Condition	Additives	$t_{1/2}$ (min)[b]	
		1st phase	2nd phase
Dilution	None	17	147
	ATP	3	42
	ATP+Mg^{2+}	29	107
	Mg^{2+}	37	340
No dilution	None	350	–

[a]The activated enzyme was diluted 25-fold with 10 mM PIPES (pH 7.0) or the buffer containing 1 mM ATP and/or 8 mM $MgCl_2$ and incubated at 0°C for 0∼60 min. The mixture was assayed for O_2^- generating activity.
[b]$t_{1/2}$ was estimated from a first-order plot of the data.

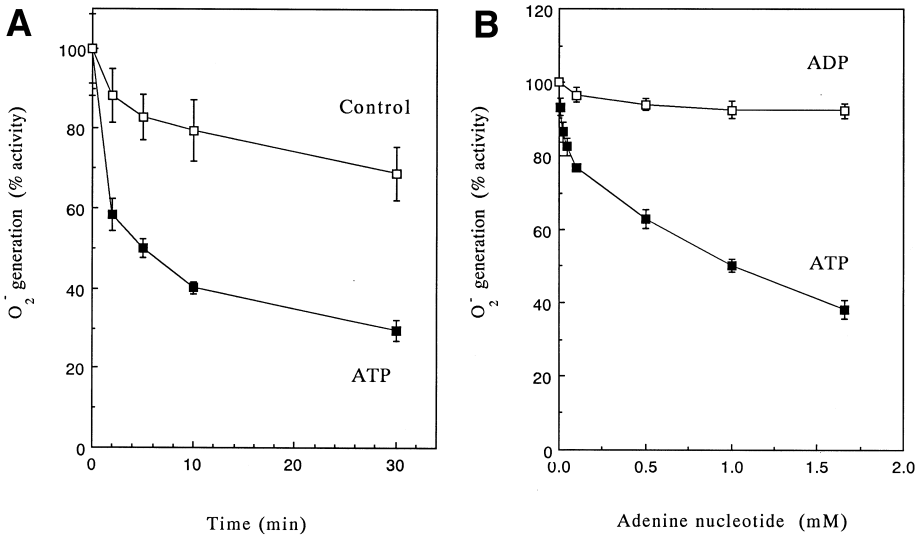

Fig. 2. ATP-accelerated deactivation of NADPH oxidase. (A) Time course. The cell-free mixture was diluted 25-fold with 10 mM PIPES buffer (pH 7.0) with or without 1 mM ATP and incubated for 0–60 min at 0°C. The mixture was assayed for O_2^- generation. The initial activity of the diluted sample with or without ATP was 1214 ± 91 or 960 ± 82 nmol/min/mg, respectively. (B) Concentration dependence. The cell-free mixture was diluted with the buffer containing 0–1.66 mM ATP or ADP. After a 10-min incubation, the mixture was assayed for O_2^- generation. The activity at a 10-min incubation without ATP is taken as 100% activity (962 ± 47 nmol/min/mg). Data are expressed as means ± S.D. of three determinations.

Fig. 2B shows the activity after a 10-min incubation in dilution with various concentrations of ATP. The percent activity from the control activity, which was incubated for 10 min without ATP, was 50% at 1 mM and 39% at 1.7 mM. At higher concentrations the activity gradually decreased (data not shown). In contrast to ATP, ADP showed no effect on the deactivation.

3.2. Protein phosphorylation is not involved in the deactivation by ATP

It was reported that $p47^{phox}$ loses its ability to activate the oxidase when it is hyperphosphorylated [15]. Although the phosphorylation is generally not assumed to be involved in a cell-free activation, it was observed that SDS stimulates the phosphorylation of $p47^{phox}$ [16]. Therefore, to test if protein phosphorylation is involved in the deactivation by ATP, we examined the effect of protein kinase inhibitor on the ATP effect. When staurosporine, a wide-range inhibitor for protein kinases, was included in the dilution mixture the deactivation rate was similar to the control (data not shown). The result showed that protein phosphorylation is not involved in the deactivation by ATP.

3.3. The structure of nucleoside triphosphate is required for the effect

Then we tested various nucleotides for the deactivation effect. Table 2 shows the activity at 10 min incubation at 0°C with several nucleotides. AMP and ADP showed no effect on the oxidase longevity. On the other hand, GTP and CTP are both effective,

Table 2
Effect of several nucleotides on the deactivation of NADPH oxidase in dilution[a]

Nucleotide	O_2^- generating activity	
	nmol/min/mg	%[b]
None	960 ± 47.5	100
ATP	384 ± 19.1	41
ATPγS	346 ± 16.1	36
GTP	317 ± 11.4	33
CTP	432 ± 40.6	45
ADP	883 ± 15.4	92
AMP	874 ± 64.9	91

[a]The activated enzyme was diluted 25-fold with 10 mM PIPES buffer (pH 7.0) containing a given nucleotide (1 mM). After a 10-min incubation at 0°C, the mixture was assayed for O_2^- generation.
[b]The percentages are based on the activity at 10 min incubation without additives (None).

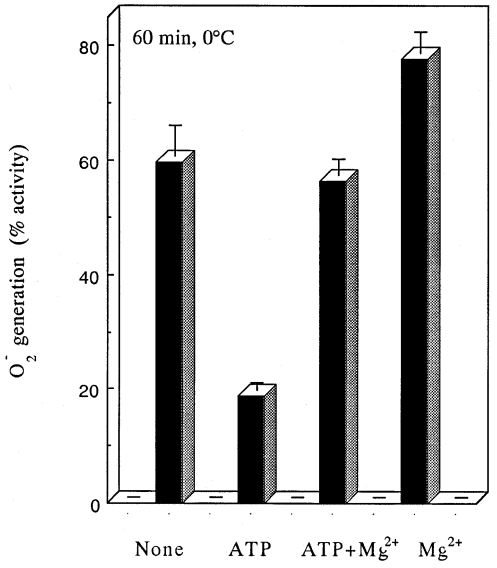

Fig. 3. Prevention of ATP-induced deactivation of NADPH oxidase by Mg^{2+}. The cell-free mixture was diluted and incubated for 60 min at 0°C in the presence and absence of 1 mM ATP and/or 8 mM $MgCl_2$. The mixture was assayed for O_2^- generation. The activity without incubation is taken as 100% activity (1204 ± 57 nmol/min/mg). Data are expressed as means ± S.D. of three determinations.

GTP being slightly more effective than ATP. Also ATPγS, a non-hydrolyzable analog of ATP, showed an effect, even stronger than ATP. The deactivation by ATP, GTP, or CTP was not blocked by UDP, an inhibitor of nucleoside diphosphate kinase [10] (data not shown), indicating that these trinucleotides are effective themselves. Taken together, the results suggest that the effect is not specific for ATP but for trinucleotides. Also nucleotide hydrolysis is not necessary, which is consistent with the result that protein phosphorylation is not involved in the deactivation.

3.4. The deactivation of NADPH oxidase by ATP is prevented by Mg^{2+}

When $MgCl_2$ was included the ATP-induced deactivation was mostly prevented. Fig. 3 shows the activity at 60 min incubation at 0°C. The ATP-containing sample shows 20% activity from the initial activity, but when $MgCl_2$ was added the activity recovered to 60%, which was the same as the control. The $t_{1/2}$s were also prolonged to the level of the control in both the first and second phases (Table 1). When $MgCl_2$ was added by itself the activity was further stabilized (80% of the initial activity), the $t_{1/2}$s being longer than those without additives (37 and 340 min for the first and second phases, respectively).

3.5. Protein crosslinking prevents the ATP-accelerated deactivation of the oxidase

To examine if the accelerated deactivation by ATP is caused by dissociation of the oxidase complex, we examined the effect of a crosslinker on the deactivation. The activation mixture was treated with EDC, and tested for stability in the presence of ATP. As shown in Fig. 4, with non-crosslinked sample, ATP caused an acceleration of the deactivation. In contrast, when the sample was crosslinked with EDC, ATP failed to accelerate the deactivation. This result suggests that the deactivation by ATP is due to the dissociation of the oxidase complex.

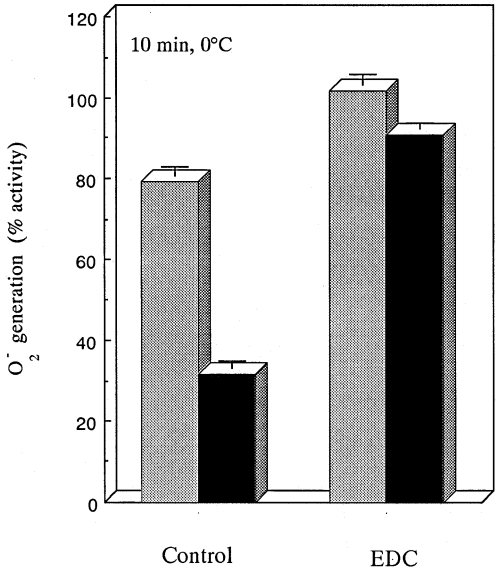

Fig. 4. Prevention of ATP-induced deactivation of NADPH oxidase by crosslinking with EDC. After the activation, the cell-free mixture was cooled to 0°C in 3 min, stirred with or without 10 mM EDC for 30 min at 0°C. Crosslinked and control mixtures were diluted with 10 mM PIPES (pH 7.0) with (dark bars) or without (gray bars) 1 mM ATP and incubated for 10 min at 0°C. The activity without incubation is taken as 100% activity (920 ± 43 nmol/min/mg). Data are expressed as means ± S.D. of three determinations.

3.6. ATP diminishes F-actin content

The results described above suggested a possibility that the ATP effect is due to the chelation of Mg^{2+}. We have recently found that Mg^{2+} stabilizes the oxidase complex by preventing F-actin depolymerization [12]. Therefore, we measured the F-actin content in the diluted samples with or without ATP in parallel with the oxidase activity (Fig. 5). As expected, the F-actin level of the sample was lowered by dilution, and further lowered by ATP addition in parallel with the oxidase activity. The result suggests that the addition of ATP results in depolymerization of F-actin.

3.7. ATP effect was not prominent in a semi-recombinant system

The results described above suggested that the ATP effect is related to F-actin depolymerization. If this is the case, the ATP effect would be diminished in a semi-recombinant system consisting of recombinant phox proteins instead of cytosol, which is rich in actin. Fig. 6 shows the time course of activity of a semi-recombinant system after dilution. The initial rate ($t_{1/2}$ = 23 min) was similar to that of the cell-free system, however, the activity later on was more labile than that of the cell-free system. As shown in the figure, ATP did not much influence the deactivation. The initial deactivation rate was slightly shifted by ATP ($t_{1/2}$ = 16 min), but the activities at 20 min with and without ATP were similar (56 and 50%, respectively). These slight differences were prevented by the addition of Mg^{2+} (data not shown). The effect of ATP in the semi-recombinant system might be due to some actin molecules brought with the plasma membrane as cortical actin. Otherwise, it might suggest that a portion of ATP effect is independent of actin.

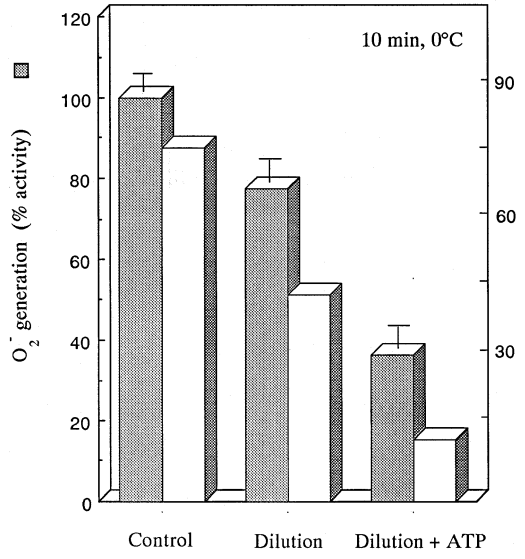

Fig. 5. Effect of ATP on NADPH oxidase activity and F-actin content. After activation with SDS, the cell-free mixture was diluted 25-fold with 10 mM PIPES buffer (pH 7.0) with or without 1 mM ATP, and incubated at 0°C for 10 min. The mixture was then assayed for O_2^- generation, and the activities are expressed as means ± S.D. of three determinations. The activity without incubation is taken as 100% activity (1210 ± 67 nmol/min/mg). F-actin estimation was performed as described in Section 2. This is a typical result of two independent experiments.

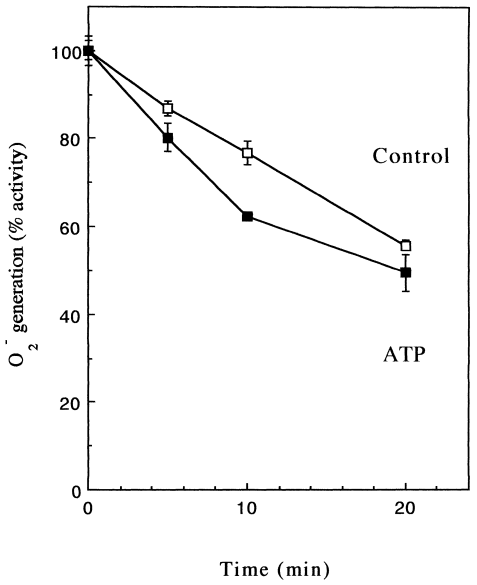

Fig. 6. Effect of ATP on the stability of NADPH oxidase activated in a semi-recombinant system. The semi-recombinant activation mixture was diluted 25-fold with 10 mM PIPES (pH 7.0) or the buffer containing 1 mM ATP and incubated at 0°C for a given time. An aliquot of the mixture was taken up and assayed for O_2^- generation. The results are expressed as means ± S.D. of three determinations. The initial activity of the diluted sample with or without ATP was 304 ± 11.2 or 420 ± 4.3 nmol/min/mg, respectively.

4. Discussion

In the present study, we showed that ATP deactivates NADPH oxidase in a cell-free system. The

study revealed that (i) the effect is time-dependent, (ii) it is more evident in the diluted mixture, (iii) ADP and AMP had little effect while GTP and CTP had similar effects to ATP, (iv) ATPγS, a hydrolyzable analog of ATP, was more effective, and (v) the effect is not suppressed by an inhibitor for protein kinases. These results indicate that the ATP effect does not require hydrolysis but requires the structure of a trinucleotide.

It was also shown that dilution itself accelerates the deactivation. We have previously reported that the enzyme stability depends on the concentration of cytosol [6]. The following reasons can now be considered for the phenomenon: (i) dilution of phox proteins, (ii) dilution of Mg^{2+} concentration, and (iii) dilution of actin concentration [12]. Dilution and ATP addition synergistically accelerated the deactivation of NADPH oxidase, therefore the action of these two factors seems to be closely related. It was also observed that the deactivation by dilution and ATP was biphasic. This implies the presence of subpopulations of the oxidase complex, which are resistant to the conditions.

One of the most important findings is that the ATP effect is largely prevented by the addition of Mg^{2+}. Recently we reported that the oxidase activity is markedly deactivated by EDTA and that Mg^{2+} prevents the deactivation [12]. Based on these experiments, we concluded that Mg^{2+} stabilizes the oxidase by preventing F-actin depolymerization, which supports the oxidase.

ATP has a high affinity for Mg^{2+} with a dissociation constant (K_d) of around 40 μM [17]. It has been shown that Mg^{2+} chelates β- and γ-phosphate groups of ATP [18]. GTP, CTP, and ATPγS have a similar affinity [19] while ADP and AMP have a much lower affinity for Mg^{2+}. These facts are consistent with the concept that the structure of trinucleotide is necessary for the ATP-induced deactivation. We consider that ATP sequesters free Mg^{2+} ions by chelation.

Mg^{2+} has usually been used in the cell-free activation of NADPH oxidase. Actually Mg^{2+} is required for the maximal activity in the cell-free system. However, Gross et al. showed that Mg^{2+} is not required in a pure reconstitution system [20]. We have recently found that Mg^{2+} stabilizes NADPH oxidase in a crude cell-free system and suggested that the effect is caused by F-actin stabilization [12].

The direct effect of ATP on actin depolymerization might also contribute to the deactivation. ATP is thought to be required for both polymerization and depolymerization of actin [21,22]. Katz et al. showed that ATP induces F-actin depolymerization at low ionic strength using the cytoskeleton from rat hepatocytes [23] although the mechanism was not described.

ATP did not much influence the stability of the oxidase in a semi-recombinant system, which does not contain cytosol, consequently not much actin. This is consistent with the concept that actin is involved in the ATP effect observed in the cell-free system. Also the oxidase activated in a semi-recombinant system was more labile than that in the cell-free system (Figs. 2A and 6), implying the presence of a stabilizing factor(s) in the cytosol, which we assume to be actin.

In the present study GTP was more effective than ATP or CTP on the deactivation suggesting that GTP has an additional mechanism to Mg^{2+} chelation, e.g. activation of a small GTPase such as rap1A [24], which is thought to regulate the oxidase [4]. Further study will be required to clarify this point.

In summary, we found that ATP and other trinucleotides accelerate the deactivation of NADPH oxidase and suggest that it is largely due to the chelation of free Mg^{2+} which stabilizes the complex of NADPH oxidase.

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