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Oxygen metabolism in plant/bacteria interactions: characterization of the oxygen uptake response of plant suspension cells

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In recent years the accumulation of reactive oxygen species (ROS) has been studied in plant cell suspension systems treated with bacterial pathogens. However, the associated utilization of molecular oxygen has not been well characterized. Using a multi-electrode oxygen analyser, the rates of oxygen consumption by tobacco cells during bacterial interactions were monitored. Heat-killed (HK) bacteria, which initiate an immediate ROS response in plant cells, were used as an elicitor to avoid complications of oxygen consumption by viable bacteria. An increase in oxygen uptake by the tobacco cells occurred within 4 min after addition of HK-bacteria and lasted for about 10 min, returning to a steady state at approximately twice the initial basal rate. The initial burst in oxygen uptake coincided with production of H_2O_2. Calculation of the total oxygen consumption by the plant cells indicated that less than 5 % of the increased oxygen uptake was utilized in ROS production. Use of respiratory inhibitors indicated that respiration, especially the cytochrome pathway, played a significant role in this response. Results from the use of K-252, a protein kinase inhibitor, and DPI, an inhibitor of membrane bound NADPH oxidases, indicated that triggering of the oxygen uptake response may involve protein phosphorylation and is at least partially activated by the membrane bound NADPH oxidase activity. The involvement of mitochondrial respiration in the oxygen uptake response described here indicates that early events in plant recognition of pathogens involves more of the cellular machinery than previously hypothesized.

Keywords: reactive oxygen species; hydrogen peroxide, luminol, respiratory burst.

INTRODUCTION

Increased respiration in diseased plant tissues was described many years ago [19, 26, 30, 41]. More recently, immediate increases in oxygen uptake have been measured in suspension cells treated with bacterial, fungal, and plant cell wall-derived elicitors [12, 16, 38] and whole bacteria [6]. In these studies, increased oxygen uptake has been closely correlated with an increase in reactive oxygen species (ROS). This stimulated ROS increase has been compared to the "oxidative" or "respiratory" burst measured in animal and human neutrophils [9, 14, 17, 23, 31, 34].

In addition to ROS production there are, of course, other metabolic processes that involve oxygen. The major oxygen utilization pathways are mitochondrial

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Abbreviations used in text: DPI, diphenyleneiodonium chloride; KCN, potassium cyanide; HK-bacteria, heat killed bacteria; K-252, a protein kinase inhibitor; LDC, luminol-dependent chemiluminescence; ROS, reactive oxygen species; SHAM, salicylhydroxamic acid.

respiration (by either the cytochrome or alternative oxidase pathways) and photorespiration, while the major oxygen production pathway in green plants is photosynthesis. Because of these competing metabolic pathways and other processes that may utilize molecular oxygen, it is difficult to attribute increased oxygen utilization to any one physiological process. The use of dark-grown suspension cells greatly simplifies the system by eliminating photorespiration and photosynthesis.

In the current study, we used a multi-electrode oxygen analyser to more precisely characterize and quantify the oxygen utilization of tobacco suspension cells prior to and after treatment with heat-killed (HK) bacteria. Previous work with live bacteria showed a two phased oxygen uptake response. The first phase occurred immediately after adding the bacteria, whether compatible or incompatible. It is this phase that we address in this study using HK bacteria. The multi-electrode analysis techniques allowed simultaneous comparison of up to 16 cell suspensions over several hours. Cell suspensions were shaken continuously in an open system to assure that oxygen concentration in the media was not a limiting factor. The cells responded to the HK-bacteria with immediate

increases in oxygen uptake and ROS production. Results from the use of various inhibitors indicated that ROS production directly accounted for only a small fraction of the increased oxygen uptake and that mitochondrial respiration appeared to be primarily responsible for increased oxygen utilization. In addition, there is evidence that the DPI-inhibitable mechanism has a regulatory effect on the increased mitochondrial respiration in tobacco.

MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma-Aldrich Chemicals Inc. (St. Louis, MO, U.S.A.) with the exception of K-252, which was purchased from Fluka Chemical Corp. (Ronkonkoma, NY, U.S.A.). Diphenyleneiodonium chloride (DPI), K-252, and salicylhydroxamic acid (SHAM) were dissolved in dimethyl sulfoxide (DMSO) and added to suspension cells for final concentrations of 10 μM, 1·6 μM, and 1 mM, respectively. Final concentration of DMSO added to cell suspensions did not exceed 0·01 % and had no effect on oxygen uptake or Luminol-dependent chemiluminescence (LDC) assays. KCN was prepared in 0·5 mM MES, pH 6·1 and added to suspensions for a final concentration of 100 μM. Antimycin A was prepared in ethanol and added to suspension cells for a final concentration of 10 μM and less than 0·05 % ethanol.

Plant cells

Callus and suspension cells of tobacco (*Nicotiana tabacum* cv. *Hicks*) were prepared as previously described [6]. Two or 3 day old tobacco cells were washed and resuspended in 20 ml assay medium (175 mM mannitol, 0·5 mM CaCl$_2$, 0·5 mM K$_2$SO$_4$ and 0·5 mM MES, pH 6·1) for a final concentration of 0·1 g ml^{-1}. Cells were equilibrated on a rotary shaker in 50 ml; beakers at 180 rpm and 27 °C for 0·5 to 1 h. Separate but identical 50 ml beakers containing 20 ml of cells were prepared for oxygen uptake and ROS assays.

HK-bacteria

Pseudomonas syringae pv. *syringae* 61 NalR, which causes the hypersensitive response on tobacco plants, was routinely grown on King's B agar [29] augmented with naladixic acid (50 μg ml^{-1}). Bacteria were washed and suspended in water as previously described [6, 7]. Heat-killed bacteria were prepared by autoclaving bacterial suspensions for 15 min. Addition of about 200 μl to the 20 ml of suspension cells resulted in a final concentration of HK-bacteria in treatments equivalent to 1 × 10^8 cfu ml^{-1}, based on the concentration of viable bacteria prior to autoclaving.

Oxygen uptake measurements

Oxygen uptake was measured by oxygen electrodes with computerized data acquisition as previously described [6]. This method allowed for simultaneous and continuous monitoring of oxygen concentration in up to 16 beakers of suspension cells. Tobacco cells were shaken in beakers to assure continuous aeration of the media. Changes in the steady state concentration of oxygen in beakers with cells was compared to beakers with no cells (100 %) to estimate the rate of basal respiration as previously described [6]. The basic concept underlying the technique is that changes in the rate of oxygen uptake result in a new steady state oxygen concentration in the buffer. Preliminary calibration under identical conditions and shaker speed but without cells allows calculation of the oxygen flux from the air, which, as described by Ficks Law, increases as oxygen concentration decreases in the buffer. Therefore at any oxygen concentration in the buffer, the rate of oxygen flux from air is known and should equal the oxygen uptake by the cells. Electrodes were routinely soaked in Tergazyme (Alconox Inc., U.S.A.) for 10 min to eliminate any biological contaminants, and rinsed extensively with assay buffer prior to calibration in aerated assay buffer.

Suspension cells were treated with KCN, antimycin A and/or SHAM to inhibit mitochondrial respiration prior to elicitation with HK-bacteria. For decades these inhibitors have been useful for the study and characterization of the cytochrome and alternative oxidase pathways. Over the years, some guidelines for their use have been developed. For example, SHAM is arguably the best available inhibitor of alternative oxidase. However, at concentrations above 1 mM, it is reported to inhibit the cytochrome pathway and at lower concentrations it has been reported to stimulate peroxidases [35]. For this reason, we followed the criteria set forth in the literature [20, 32, 35] and used a concentration that was inhibitory to basal respiration in the presence of KCN, but not in its absence (Fig. 4). This concentration of SHAM did not have an effect on the oxygen uptake response of tobacco cells. A final concentration of 100 μM KCN was typically used and was sufficient to completely inhibit basal respiration when combined with 1 mM SHAM. This concentration of KCN showed minimal effects on peroxidase activity as measured by the guaiacol [15] or LDC assays [5]. Addition of HK-bacteria was postponed 10–15 min after pretreatment of cells with inhibitors to allow oxygen uptake rates to reach steady state.

ROS measurement

The LDC assay was used to measure H_2O_2 as an estimate of ROS accumulation [37]. Aliquots of suspension cells were dispensed into tubes, placed into the measuring chamber of an EG&G Berthold Autolumat 953 lumino-meter (Bad Wildbad, Germany), and automatically mixed with luminol and horseradish peroxidase for imme-diate measurement of luminescence. Standard curves were prepared with dilutions of H_2O_2 in assay buffer.

RESULTS

Oxygen uptake and ROS response to HK-bacteria

An increase in oxygen uptake by tobacco suspensions was detected about 3·5 min after addition of HK-bacteria [Fig. 1(a)]. The rate of oxygen uptake increased by 220 nmole min^{-1} g^{-1} over the first 10 min following treatment, generally more than tripling the basal rate. This increased rate was not sustained, and decreased over the following 15–30 min. Treated tobacco cells typically continued to respire at a rate approximately twice that of untreated cells for at least 3 h after elicitation. The basal respiratory rate of untreated tobacco cells was typically about 90 nmole min^{-1} g^{-1} and decreased slightly over the duration of the experiment. Continuous shaking of the cell cultures assured that the media was consistently aerated throughout the experiment and oxygen concen-trations were not limiting.

Tobacco cell suspensions treated with HK bacteria revealed a pattern of ROS accumulation similar to the initial oxygen uptake response [Fig. 1(a)]. Increased H_2O_2 levels could be detected by 4 min after treatment with elicitor. Concentrations increased rapidly, typically reaching concentrations of 30–35 μM H_2O_2, or about 320 nmole g^{-1}, within 15–20 min after elicitation and declined to less than 2 μM by 60 min.

Effect of DPI and K-252 on oxygen metabolism

The ROS response of tobacco cells to HK-bacteria was completely inhibited by pretreatment with 1·6 μM K-252 [27], a protein kinase inhibitor (data not shown) or by 10 μM DPI [18], an inhibitor of NAD(P)H oxidases [Fig. 1(b)]. Both compounds have been demonstrated to inhibit ROS production in plant cells [2, 11, 33].

Addition of DPI to suspension cells being monitored for ROS production at various times after HK-bacteria treatment allowed the estimation of (1) when termination of ROS production occurred, and (2) the rate constant of H_2O_2-scavenging. Pretreatment of cells with DPI com-pletely inhibited the ROS response to HK-bacteria. Addition of DPI at 7 or 12 min after HK-bacteria resulted in an immediate decrease in H_2O_2 levels
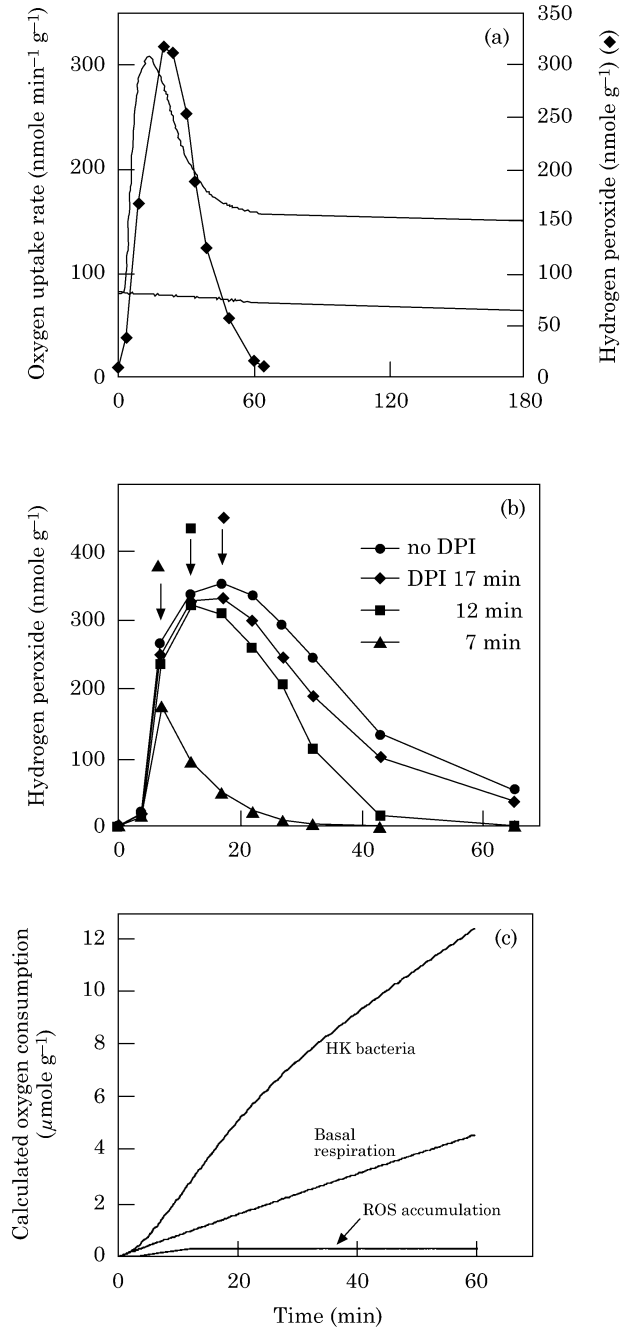

FIG. 1. (a) Oxygen uptake response and ROS production in tobacco cell suspensions treated with HK-bacteria. Oxygen uptake rates were monitored by oxygen electrodes while ROS accumulation (◆) was measured by the LDC assay. (b) Effect of DPI on ROS production by tobacco suspension cells treated with HK-bacteria. DPI (10 μM) was added to cells prior to or at different times (arrows) after HK-bacteria addition as indicated in the figure legend. ROS concentration was measured by LDC and standard errors were less than 3 nmole g^{-1}. (c) Total oxygen consumption for treated cells is calculated and compared to oxygen consumption by untreated cells and the amount of oxygen consumed for ROS production.

compared to cells without DPI [Fig. 1(b)]. However, after about 17 min the addition of DPI no longer affected the level of H_2O_2 in the treated cells. This indicated that ROS accumulation by these suspension cells had ceased by this time, lasting for less than 20 min after the addition of HK-bacteria.

Since DPI blocked further H_2O_2 production yet did not appear to affect scavenging, the decrease in H_2O_2 levels in the presence of DPI could be used to estimate the scavenging rate. A rate constant of 0·09 min^{-1}, or about 10 % min^{-1}, was estimated for this concentration of tobacco cell suspensions with the aid of SAAM II software (SAAM Institute Inc., Seattle, WA, U.S.A.). Taking this rate of scavenging into account, the amount of oxygen consumption theoretically utilized for ROS production was calculated [Fig. 1(c)]. This was based on the assumption that a net of one molecule of oxygen was required for the production of one molecule of H_2O_2. Surprisingly, only a minor fraction of the increased oxygen uptake was apparently consumed in ROS production.

Although ROS production appeared to account for a small fraction of the increased oxygen uptake, the ROS inhibitors DPI and K-252 had a dramatic inhibitory effect on the elicited oxygen uptake (Fig. 2). Pretreatment with 10 μM DPI or 1·6 μM K-252 had little effect on the basal respiration of the cells but inhibited the oxygen uptake response by 85 and 100 %, respectively. Maximum inhibition was achieved with 10 and 100 μM DPI (Fig. 2 insert), therefore 10 μM was routinely used. It was apparent that the inhibition of oxygen consumption by these compounds was disproportionate to the amount of oxygen required directly for ROS production.

Effect of respiratory inhibitors on oxygen uptake rates

To examine the contribution of mitochondrial respiration to the elicited oxygen uptake response, tobacco cell suspensions were pretreated for 10–15 min with KCN or antimycin A, two inhibitors of the cytochrome pathway. Both inhibitors had a slight effect on basal respiration, causing a slight increase (Fig. 3). These increases were not affected by prior treatment of cells with DPI or K-252 (data not shown), ruling out the possibility that the cytochrome inhibitors were stimulating an oxygen uptake response similar to that induced by HK-bacteria. The increase in basal respiration is likely due to a shift of electrons to the alternative, cyanide–resistant oxidase pathway as described previously [3, 42].

Both 100 μM KCN and 10 μM antimycin A inhibited the oxygen uptake response by 30–45 % (Fig. 3). Higher concentrations of the cytochrome pathway inhibitors did not cause further inhibition, as shown for KCN (Fig. 3, insert). Assuming the primary effect of these compounds is the inhibition of the mitochondrial cytochrome oxidase, these results indicate that the mitochondrial cytochrome
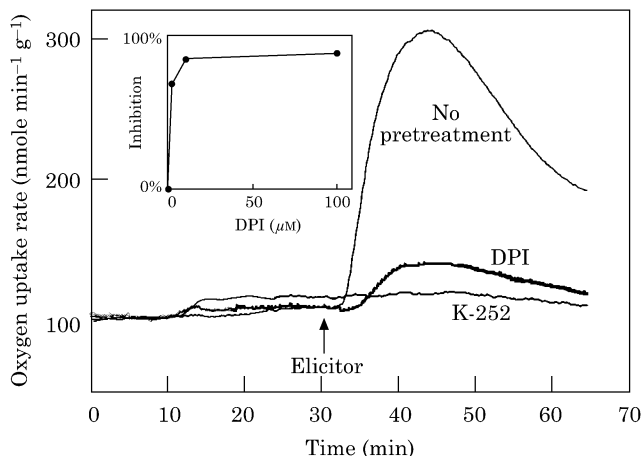

Fig. 2. Effect of DPI (10 μM) and K-252 (1·6 μM) on the oxygen uptake response of tobacco suspension cells treated with HK-bacteria. (Inset) Inhibition of the oxygen uptake response by different concentrations of DPI. Oxygen uptake was monitored by oxygen electrodes.
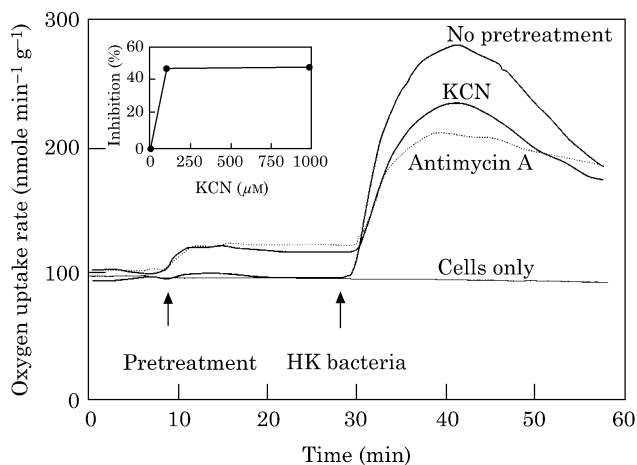

Fig. 3. Effect of cytochrome oxidase inhibitors, KCN (100 μM) or antimycin A (10 μM) on the oxygen uptake response of tobacco suspension cells treated with HK-bacteria. Oxygen uptake rates were monitored by oxygen electrodes. (Inset) Inhibition of the oxygen uptake response by different concentrations of KCN. Oxygen uptake rates were monitored by oxygen electrodes.

oxidase pathway was stimulated upon recognition of the HK-bacteria and was at least partially responsible for the oxygen uptake response.

Tobacco suspensions pretreated with 1·0 mM SHAM, an alternative oxidase inhibitor, experienced a slight transient increase in respiration, returning to basal rates within a few minutes (Fig. 4). Upon treatment with HK-bacteria, the cells showed no inhibition of oxygen uptake when compared to cells that were not pretreated with SHAM. These results indicate that the alternative
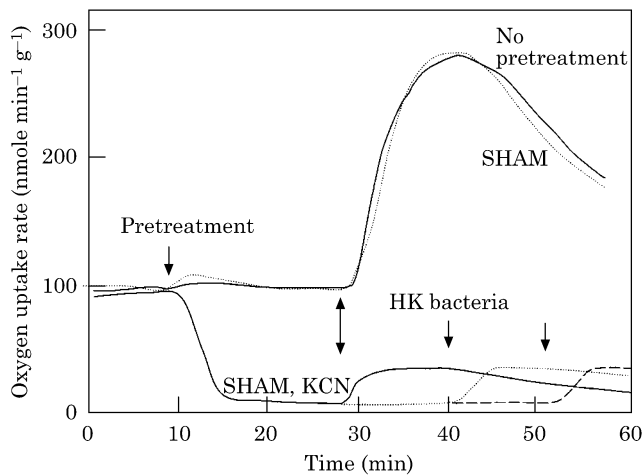
FIG. 4. Effect of the alternative oxidase inhibitor, SHAM (1 mM), and KCN (100 μM) on the oxygen uptake response of tobacco suspension cells treated with HK-bacteria. HK-bacteria were added at the indicated time intervals (arrows). Oxygen uptake rates were monitored by oxygen electrodes.

FIG. 5. Effect of DPI (10 μM) and respiratory inhibitors on the oxygen uptake response of tobacco suspension cells treated with HK-bacteria. Suspension cells were treated with DPI in combination with (1) KCN, (2) SHAM alone, or (3) KCN and SHAM together. Oxygen uptake rates were monitored by oxygen electrodes.

oxidase is either not involved or, at least, is not required for the oxygen uptake response.

Tobacco suspensions pretreated simultaneously with both 1·0 mM SHAM and 10 μM KCN immediately reduced oxygen uptake rates, indicating nearly complete inhibition of basal respiration via both the cytochrome and alternative oxidase pathways (Fig. 4). Subsequent treatment of the cells with HK-bacteria showed that the oxygen uptake response was inhibited by 85–90 %. The magnitude of the oxygen uptake response did not diminish when HK-bacteria were added 20, 30 or 40 min after pretreatment.

Addition of DPI along with SHAM and KCN completely blocked basal respiration and the elicited oxygen uptake response (Fig. 5). Treatment of tobacco cells with SHAM and DPI was not significantly different from treatment with DPI alone (Fig. 2), supporting the nonessential nature of the alternative oxidase in the tobacco oxygen uptake response. The combination of DPI and KCN, however, completely blocked the oxygen uptake response, reaffirming the importance of the NADPH oxidase and cytochrome oxidase pathway to the oxygen uptake response (Fig. 5).

Alternatively, inhibitors were added to cell suspensions 10 min after the elicitation of cells with HK-bacteria (Fig. 6). Addition of SHAM alone post-elicitation was slightly stimulatory to the oxygen uptake response, while KCN alone was somewhat inhibitory. In contrast, the simultaneous addition of KCN and SHAM after elicitation caused an immediate cessation of oxygen uptake. The data support the above conclusion that mitochondrial respiration was responsible for the majority of the elicited oxygen uptake response.
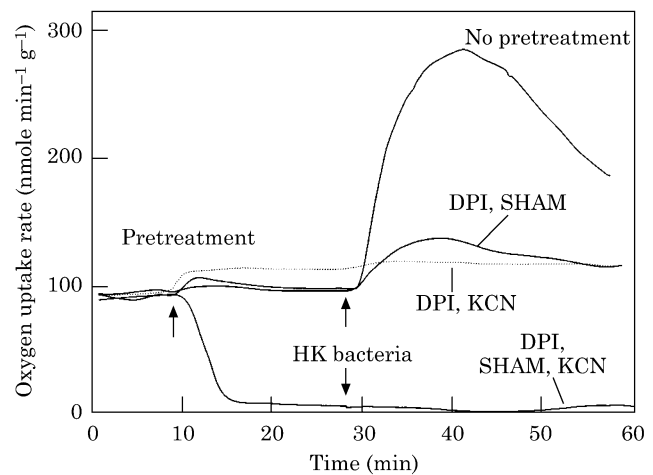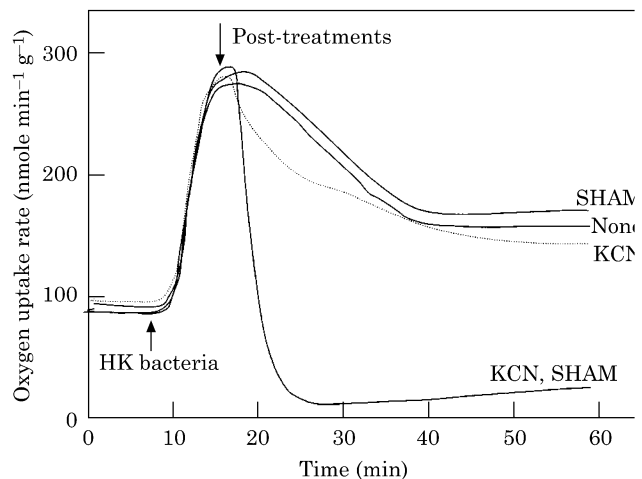

FIG. 6. Effect of respiratory inhibitors on the oxygen uptake response of tobacco suspension cells after elicitation of the oxygen uptake response by HK-bacteria. Suspension cells were treated with HK-bacteria followed 10 min later with, KCN and/or SHAM. Oxygen uptake rates were monitored by oxygen electrodes.

DISCUSSION

A primary source of ROS in stressed plants is hypothesized to be a membrane-bound NAD(P)H oxidase/superoxide synthase [13, 22, 34, 36]. Plant proteins that are immunologically related to cytosolic components of mammalian NAD(P)H oxidase [21, 33, 39] and plant homologues of membrane components of the mammalian oxidase [25, 28, 40] have been isolated. DPI is a potent inhibitor of the oxidase and is thought to compete for NAD(P)H binding sites [18]. DPI can also affect the

accumulation of ROS by stimulating H_2O_2-scavenging by peroxidases [4, 24], effectively mimicking the inhibition of ROS production by NAD(P)H oxidase. However, the rate of DPI-stimulated H_2O_2-scavenging by peroxidase is proportional to the concentration of DPI [4]. In the current study, inhibition of ROS accumulation was not proportional to the concentration of DPI (Fig. 2, insert), indicating that the inhibitor was more likely to be prohibiting the production of ROS rather than stimulating scavenging of H_2O_2 by peroxidase.

The addition of DPI to untreated tobacco cells had no effect on basal respiration rates, but did reduce the elicited oxygen uptake response by about 80 % (Fig. 2) and completely inhibited ROS accumulation [Fig. 1(b)]. The inhibition of oxygen consumption by DPI was disproportionately greater than the amount of oxygen directly utilized in ROS production, even when adjusted for potential losses due to scavenging [Fig. 1(c)]. Therefore, the activation of the oxidase appears to be necessary for triggering a significant portion of the increased oxygen uptake. However, because DPI does not completely block the oxygen uptake response, one or more parallel responses may also be actively triggering increased oxygen uptake.

The majority of the oxygen uptake response appeared to be due to increased mitochondrial respiration, primarily due to stimulation of the cytochrome oxidase pathway. When KCN or antimycin A, the cytochrome oxidase inhibitors, were added to untreated tobacco cells, the basal respiration increased slightly suggesting that the alternative oxidase pathway was activated and partially compensating for the loss of the cytochrome oxidase (Fig. 3). Upon treating these cells with HK-bacteria, the oxygen uptake response was reduced 30–45 % in the presence of KCN or antimycin A. Pretreatment of cells with SHAM alone had no affect on the oxygen uptake response to HK-bacteria (Fig. 4). This suggested that when the cytochrome oxidase pathway was functioning fully, the alternative oxidase was not required for the elicited oxygen uptake response. However, if the cytochrome oxidase was incapacitated, the alternative oxidase partially compensated.

The diagram in Fig. 7 presents the possible relationship between active oxygen production and increased mitochondrial respiration. Previous studies have hypothesized that elicitors bind to plant membrane receptors and trigger early recognition events such as the production of ROS, an increase in oxygen uptake and, in some cases, ion fluxes such as the K^+/H^+ exchange response [1, 8]. Both ROS production and the K^+/H^+ exchange response have been demonstrated to require protein phosphorylation [10, 11, 16, 43]. In the current study, pretreatment with the protein kinase inhibitor K-252 completely inhibited the oxygen uptake response (Fig. 2) and ROS production (data not shown) by elicited tobacco cells. The data

suggest that K-252 inhibits upstream of the DPI-inhibitable oxidase, completely blocking ROS production and the triggering of the oxidase-activated portion of the oxygen uptake response (Fig. 7). As discussed above, DPI alone does not completely block the oxygen uptake response, indicating the existence of a parallel trigger responsible for the remainder of the oxygen uptake response. This parallel mechanism would not necessarily directly utilize oxygen, but appears to require protein phosphorylation and ultimately lead to increased respiration.

Increased NAD(P)H oxidase activity could affect mitochondrial respiration by increasing oxidative stress in the cell and/or by depletion of reductant, presumably NADPH (Fig. 7). Triggering of the pentose phosphate pathway leads to increased metabolites, such as pyruvate, that are utilized in the TCA cycle and fuel increased respiration. The key regulatory parameters are the decrease in NADPH and ATP, which will trigger increased activity of the pentose phosphate pathway and respiration, respectively. The increased ATP production resulting from stimulated electron transport may be utilized by other early cellular responses to pathogens.

Robertson *et al.* carried out one of the few studies trying to relate oxygen uptake with ROS production [38]. In that study, elicitation of French bean cells resulted in increased oxygen uptake rates and active oxygen accumulation that were comparable in timing to those reported in our study. In addition, they monitored decreased levels of ATP and $NADH/NAD^+$ during the first few minutes after elicitation. The authors suggested that these decreases were not related to increased aerobic respiration and that the monitored oxygen uptake was directly utilized in ROS production. Another interpretation of these results is that the decreased NADH/NAD ratio was actually due to increased respiration. Because a drop in either of these ratios is a strong regulatory signal for mitochondrial respiration, it seems reasonable that the requirement for ATP and NADH could cause the decrease in these ratios and that respiration probably did increase. In this previous study, the authors found that KCN (1 mM) had little effect on basal respiration, suggesting that the alternate oxidase was compensating for the inhibition of the cytochrome oxidase or that there was little need for ATP under control conditions. However, they found that the elicited oxygen increase was significantly inhibited by 1 mM KCN, as would be expected if cytochrome oxidase were required for ATP production. As the authors concluded, this transient oxidative stress is likely to be an important metabolic signal to the plant and determine the outcome of the interaction.

It must be stressed that the hypothetical model presented in Fig. 7 is not meant to be complete. For example, further research is needed to demonstrate the relationship of (1) the ascorbate–glutathione pathway
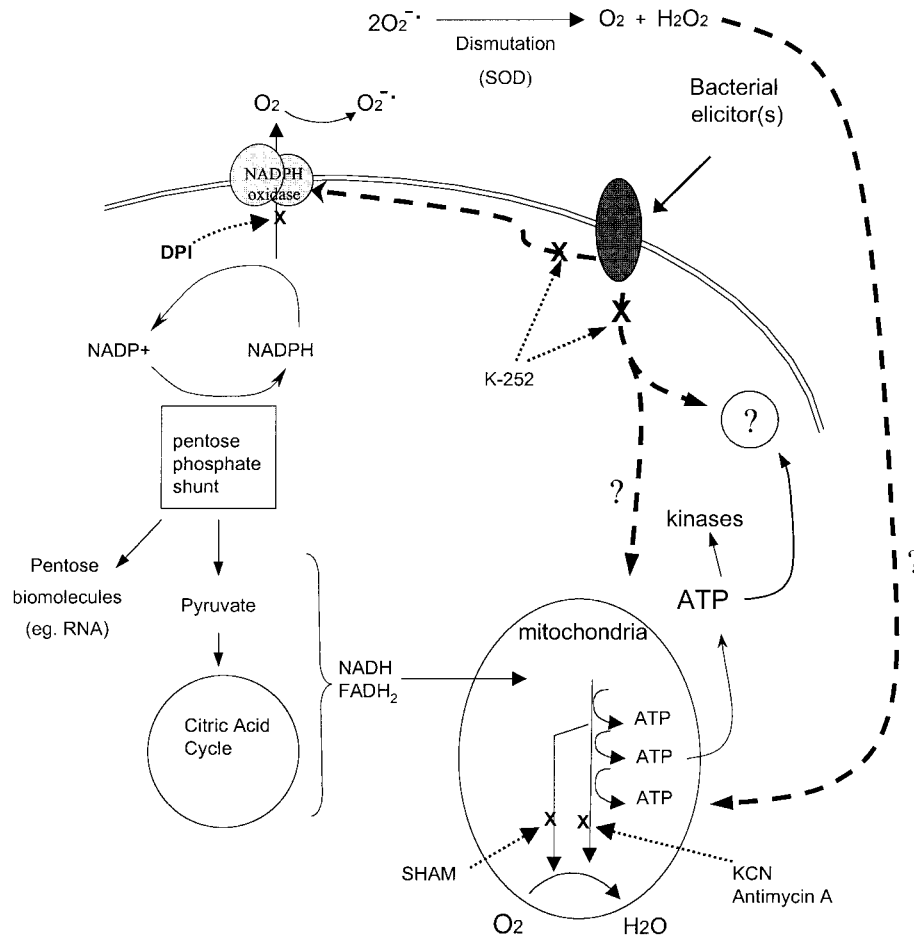
FIG. 7. Proposed model for oxygen uptake response. One or more signals are received from the bacteria which induces a signal transduction pathway involving protein phosphorylation and subsequent activation of the NAD(P)H oxidase and other parallel responses requiring and/or triggering mitochondrial respiration. The utilization of NAD(P)H triggers the pentose phosphate shunt that, in turn, recycles $NADP^{+}$ and produces metabolites that ultimately result in increased NADH available for mitochondrial respiration. The ribose produced by the pentose phosphate pathway can also be used in nucleic acid production. The NAD(P)H oxidase may also induce an oxidative stress that could result in increased mitochondrial respiration.

that utilizes NAD(P)H to scavenge H_2O_2, (2) the effect of increased glycolysis, and (3) the role of peroxidases in generating active oxygen. Also, this model is not meant to represent all plant cells. Preliminary work in our laboratory with potato suspension cells suggests that components in this model have a greater or lesser effect on the oxygen uptake response in some plant cells.

The timing and magnitude of the oxygen increase observed in this study suggest that within minutes of host/pathogen contact many biochemical pathways may be activated and putting demands on energy sources and other cellular metabolites. This can have an important impact when considering individual pathways that are dependent on other metabolic processes. It also clearly points out the need for a more integrative approach to disease physiology and the need for integrated metabolic modeling.

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