The Ca^{2+} /NADPH-dependent H_2O_2 generator in thyroid plasma membrane: inhibition by diphenyleneiodonium

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The thyroid plasma membrane contains a Ca^{2+} -regulated NADPH-dependent H_2O_2 -generating system which provides H_2O_2 for the peroxidase-catalysed biosynthesis of thyroid hormones. The electron transfer from NADPH to O_2 catalysed by this system was studied by using diphenylene iodonium (DPI), an inhibitor of flavo- and haemo-proteins. The prosthetic group of the H_2O_2 generator was removed by incubation with 5 mM CHAPS at 40 °C, and an active holoenzyme was reconstituted with FAD, but not with FMN. The H_2O_2 -generating system also had an intrinsic Ca^{2+} -dependent NADPH:ferricyanide reductase activity which is probably linked to its flavodehydrogenase component (or domain). Both activities, H_2O_2 production and ferricyanide reductase activity, were inhibited by DPI, with

similar K_i (2.5 nmol/mg of protein). DPI only inhibited a system reduced with NADPH in the presence of Ca^{2+} . NADPH could not be replaced by NADP^+ , NADH or sodium dithionite, suggesting the need for specific mild reduction of a redox centre in a particular conformation. Ferricyanide protected both activities against inhibition by DPI; the NADPH:ferricyanide reductase activity was completely protected at a ferricyanide concentration 20 times lower than that needed to protect the H_2O_2 formation, implying at least two target sites for DPI. One might be the flavodehydrogenase component; the other was beyond, on the entity which transfers the electrons to O_2 . This second site has not been identified.

INTRODUCTION

Thyroid hormones are synthesized on the apical surface of the follicular cells during, or after, the fusion of exocytic vesicles with the apical membrane. The two final reactions, iodination of thyroglobulin tyrosyl residues and their coupling into the hormonal form, are catalysed by thyroid peroxidase in the presence of H_2O_2 . The H_2O_2 level controls the relative rate of the two reactions [1]. Excess H_2O_2 is harmful to the cells, so that its production must be well regulated *in vivo*. H_2O_2 generation is controlled by thyrotropin in intact cells [2,3].

The thyroid particulate fraction contains an NADPH-dependent H_2O_2 -generating system [4] whose activity requires sub-micromolar concentrations of Ca^{2+} [5,6]. The most extensively studied NADPH oxidase, neutrophil NADPH oxidase, initially generates superoxide anion [7], the one-electron reduced form of molecular oxygen which spontaneously dismutates to form H_2O_2 and O_2 . It has been suggested that the initial electron acceptor in this electron chain is a FAD-linked protein that, in turn, reduces a low-potential cytochrome *b* which interacts with oxygen (for reviews see [8,9]). Recent studies indicate that cytochrome b_{558} is a heterodimeric flavocytochrome bearing the complete electron-transporting apparatus of neutrophil NADPH oxidase [10–13]. In contrast, the thyroid NADPH oxidase produces H_2O_2 directly rather than via initial production of O_2^- [14,15]. But the molecular nature of the membrane-associated electron-transport chain that generates H_2O_2 in thyroid membranes is unknown. Apart from the almost universal observation that the nucleotide dehydrogenases are flavoproteins, there are no results implicating such a protein in the thyroid H_2O_2 generator. Nor is there any indication as to whether or not a *b*-type cytochrome is involved in the reduction of molecular oxygen.

On the other hand, iodonium compounds inhibit the activity of a variety of flavoproteins, mitochondrial NADH dehydrogenase [16], bacterial nicotine oxidase [17], macrophage NO synthase [18] and xanthine oxidase [19]. These compounds also interact with the haem prosthetic groups of cytochrome *P*-450 [20] and neutrophil cytochrome b_{558} [19]. They have been extensively used to explore the function of the redox components of the neutrophil oxidase [19,21,22].

This report describes the involvement of a flavoprotein having FAD, rather than FMN, as prosthetic group in the electron transport catalysed by membrane bound thyroid NADPH-dependent H_2O_2 generator. The effects of diphenyleneiodonium (DPI) on the O_2 reductase and NADPH dehydrogenase activities of the H_2O_2 generator were investigated. DPI was found to be inhibitory when the thyroid membrane was in a reduced state, produced by NADPH in the presence of Ca^{2+} . The results indicate that DPI has at least two target sites.

MATERIALS AND METHODS

NADP(H), NADH and freeze-dried horseradish peroxidase (HRP) (grade 1) were purchased from Boehringer (Mannheim, Germany); scopoletin was from Serva (Heidelberg, Germany); CHAPS, FAD and FMN were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); 2-amino-biphenyl was from Aldrich-Chemie (Steinheim, Germany); $\text{K}_3\text{Fe}(\text{CN})_6$ was from Prolabo (Paris, France); and sodium dithionite was from Merck (Darmstadt, Germany). Reagents for enhanced chemiluminescent (ECL) detection of HRP-labelled antibodies were purchased as a kit from Amersham.

DPI was synthesized by the method of Gatley and Sherratt [23] as modified by Doussière and Vignais [19]. It was dissolved in

dimethyl sulphoxide and standardized at pH 7.4 by using $\epsilon_{264\text{nm}} = 15.1 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [16].

The preparation of plasma membranes of pig thyroid glands was adapted from Nakamura et al. [6] as previously described [14]. Membranes were quick-frozen in liquid N_2 and stored at -80°C .

A cytochrome *c* reductase activity not related to H_2O_2 production was removed from thyroid membranes by extraction with CHAPS before some experiments [15]. Membranes (5–10 mg/ml) were incubated for 30 min at 0°C in 50 mM sodium phosphate, pH 7.25, containing 0.5 mM CaCl_2 , 0.4 mM EGTA, 1 mM NaN_3 (buffer A) plus 1 mM dithiothreitol, 10 mM CHAPS and 10 μM FAD. The membrane fraction was collected by centrifugation at 10000 *g* for 15 min at 4°C . The pellet was washed once with buffer A and resuspended in buffer A.

The bovine neutrophil membranes were prepared as previously described [24].

Release of flavin from the H_2O_2 -generating system

The membrane fraction (2 ml; maximal concn. 34 μg of protein/ml) was placed in 2 ml Eppendorf tubes and incubated for 10 min at 40°C in buffer A plus 1 mM dithiothreitol and 5 mM CHAPS. The reaction was stopped by placing the tubes in crushed ice. In some assays, 10 μM FAD or FMN was then added at 0°C for 5 min to reconstitute the holoenzyme. The membrane fraction was then collected by centrifugation at 10000 *g* for 15 min at 4°C . The pellet was washed once with 2 ml of buffer A plus 1 mM CHAPS and finally resuspended in 50 μl of buffer A plus 10 mM CHAPS before testing for H_2O_2 -forming activity.

Treatment of the membrane fraction with DPI

Membrane fraction (1 ml; 60–300 μg of protein/ml) was routinely preincubated for 10 min at 30°C in 2 ml Eppendorf tubes containing buffer A plus 10 μM FAD, 1 mM dithiothreitol and 10 μl of DPI (at different concentrations in dimethyl sulphoxide). Dithiothreitol was omitted from the preincubation medium of the assays performed in the presence of 0.1 mM ferricyanide to avoid any reaction between the ferricyanide and the dithiothreitol [25]. The preincubation medium was depleted of O_2 in some experiments by bubbling argon through it for 10 min, and the assays of NADPH oxidase inhibition by DPI were performed under argon.

Preincubation with DPI was initiated by adding 0.1 mM NADPH and stopped by centrifugation at 10000 *g* for 15 min at 4°C in a Hettich 30 RF centrifuge. The membrane pellet was washed once with 1.5 ml of buffer A plus 1 mM CHAPS. The final pellet was resuspended in 50 μl of buffer A plus 10 mM CHAPS, and residual activities were immediately measured.

In some experiments, 1–20 mM dithionite was added in preincubation medium. The membrane fraction was collected by centrifugation and washed with $2 \times 2 \text{ ml}$ of buffer A plus 1 mM CHAPS for complete elimination of dithionite before testing residual activity.

Measurement of enzymic activities

H_2O_2 formation was measured by incubating samples of membrane fraction at 30°C in 1 ml of 50 mM sodium phosphate, pH 7.2, containing 1 mM NaN_3 , 0.4 mM EGTA, 0.5 mM CaCl_2 and 0.5 mM CHAPS. The reaction was started by adding 0.1 mM NADPH, and 100 μl samples were collected at time intervals and mixed with 10 μl of 1.2 M HCl to stop the reaction by destroying

the remaining NADPH. The amount of H_2O_2 in each sample was measured by the decrease in scopoletin fluorescence in the presence of HRP in a Perkin–Elmer MPF43A spectrofluorimeter as previously described [5]. Initial rates of H_2O_2 formation were determined on eight samples from each test and expressed as nmol of H_2O_2 /h per ml of membrane.

NADPH oxidation was followed by monitoring the decrease in A_{340} at 30°C in a Uvikon 930 double-beam spectrophotometer. Samples of membrane fraction were incubated in 1 ml of buffer A, and the reaction was started by adding 0.1 mM NADPH. The reaction mixture was magnetically stirred during measurement. Blank reference samples contained all reactants except the membrane fraction, permitting the direct subtraction of the rate of chemical NADPH oxidation. The absorption coefficient at 340 nm used for NADPH was $6.22 \text{ mM}^{-1} \cdot \text{cm}^{-1}$.

NADPH:ferricyanide reductase activity was measured by NADPH oxidation as described above, except that the assay medium contained 0.1 mM ferricyanide and membrane fraction that had been washed with 10 mM CHAPS for 30 min at 0°C to extract the membrane NADPH:ferricytochrome *c* reductase. Absorbances were measured against a blank containing all reactants except the membrane fraction; this again permitted the direct subtraction of the rate of chemical NADPH oxidation in the presence of ferricyanide. Under these conditions, NADPH oxidation correlated well with ferricyanide reduction (measured by monitoring changes in A_{405} , absorption coefficient = $1.04 \text{ mM}^{-1} \cdot \text{cm}^{-1}$) and was preferred for its greater sensitivity.

SDS/PAGE and Immunoblot analysis

The membrane suspension, thyroid membranes (200 μg) or bovine neutrophil membranes were treated as previously described [24], and their constituents were separated by SDS/PAGE [26] on slab gels by using a 4% stacking gel and a running-gel acrylamide concentration of 11%. The proteins were electrotransferred to nitrocellulose sheets [27] for immunodetection. Two antisera [24], directed against the C-terminal regions of the α and β subunits of cytochrome b_{558} , were incubated (dilution 2000-fold) with nitrocellulose sheets in PBS containing 0.1% Tween 20, for 2 h at 22°C . (PBS consisted of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 , pH 7.4.) The sheets were washed exhaustively and incubated with goat anti-rabbit antibodies conjugated to HRP (diluted 2000-fold) in PBS containing 0.1% Tween 20 for 1 h 30 min at 22°C . They were again washed, and the HRP activity was revealed by chemiluminescence using the kit from Amersham.

Determination of protein content

Protein concentration was determined by the method of Bradford [28].

RESULTS

Determination of the prosthetic group of the thyroid NADPH oxidase

The direct effect of free flavins on the rate of H_2O_2 generation was assayed, and neither 1 μM FAD nor 1 μM FMN altered the rate of H_2O_2 formation catalysed by NADPH oxidase of the thyroid membranes (results not shown). Thus flavins are likely to be involved in the electron transfer from NADPH to oxygen only as putative prosthetic groups of the H_2O_2 generator. Preincubation of the membrane preparation without flavins for 10 min at 40°C with 5 mM CHAPS caused 70% of the activity to be lost (Table 1). At 10 μM , FAD, but not FMN, protected

Table 1 FAD as prosthetic group in electron transport of the NADPH-dependent H₂O₂ generator

Thyroid membranes (34 µg of protein/ml of preincubation medium) were preincubated for 10 min at 40 °C in buffer A containing 1 mM dithiothreitol plus the additives listed below. In Expts. 5 and 6, thyroid membranes were first preincubated for 10 min at 40 °C with 1 mM dithiothreitol and 5 mM CHAPS; then 10 µM FAD or 10 µM FMN was added and the preincubation prolonged for 5 min at 0 °C. The membrane fraction was washed with 2 ml of buffer A in the absence of flavin (Expt. 2) or in the presence of 1 µM FAD (Expts. 3 and 5) or 1 µM FMN (Expts. 4 and 6) and then H₂O₂ formation was measured. Data are the mean initial H₂O₂-formation rates, ± S.E.M. of at least 3 experiments.

Expt. no.	Conditions of preincubation	H ₂ O ₂ formation	
		(nmol/h per ml of membranes)	(%)
1	Without preincubation	3250 ± 107	100
2	5 mM CHAPS	589 ± 107	18
3	5 mM CHAPS + 10 µM FAD	2276 ± 160	70
4	5 mM CHAPS + 10 µM FMN	535 ± 54	16
5	5 mM CHAPS and then 10 µM FAD was added	1607 ± 160	50
6	5 mM CHAPS and then 10 µM FMN was added	600 ± 53	18

the enzyme form, since residual activity increased from 15–20 % to 70 % of the initial activity. Addition of 10 µM FAD at the end of the preincubation with CHAPS partially restored the activity, whereas FMN had no effect; the activity of the membranes when FAD was added after preincubation with CHAPS was 70 % that of membranes preincubated with CHAPS plus FAD. These results are consistent with FAD being a non-covalently bound prosthetic group of thyroid NADPH oxidase that can be removed by incubation with a detergent. The removal of FAD depended on the protein and CHAPS concentrations and increased with temperature. No FAD was released when the membranes were preincubated with CHAPS at 0 °C (results not shown).

Adding back FAD did not completely reconstitute the active enzyme. Like most apoproteins, the FAD-depleted NADPH oxidase is much less stable than the corresponding holoprotein, and was partially denatured at 40 °C. This is probably why there was still 30 % inactive enzyme after re-incorporation of FAD.

The following experiment also indicated that FAD is the prosthetic group of the H₂O₂ generator. Particulate fraction was digested with α-chymotrypsin in the presence of 12 mM CHAPS [29]. The 150000 g supernatant was loaded on to a DEAE-Trisacryl column equilibrated in 10 mM Tris/HCl, pH 8, containing 5 mM CHAPS. The proteins were eluted with a linear gradient of NaCl in the equilibration buffer [30]. The NADPH-dependent H₂O₂ formation catalysed by the fractions eluted between 100 and 150 mM NaCl was increased by 250 % in the presence of 1.8 µM FAD, but FMN was without effect (results not shown).

Conditions required for inhibition of H₂O₂ formation by DPI

H₂O₂ formation by the membrane fraction was initiated by adding NADPH and monitored routinely for 10–15 min. Inhibition by DPI was concentration-dependent, but not instantaneous. There was a time-dependent increase in the inhibition (results not shown), as for other systems [16,22,31].

The effects of DPI were assessed after the membranes had been preincubated at 30 °C for various times with NADPH, plus Ca²⁺,

Figure 1 Residual H₂O₂-forming activities as a function of preincubation time of the thyroid membranes with DPI

The membrane fraction (120 µg membrane protein/ml) was preincubated at 30 °C without DPI (△) or with 0.25 µM DPI (□) or 5 µM DPI (■) in buffer A plus 10 µM FAD, 1 mM dithiothreitol and 0.1 mM NADPH. The reaction was stopped by centrifugation, the membrane pellet was washed with buffer A plus 1 mM CHAPS, and residual H₂O₂-forming activity was measured.

plus different DPI concentrations, collected by centrifugation, and washed. Only then was the residual H₂O₂-forming activity measured. The DPI concentrations used gave maximal inhibition after about 5–10 min of preincubation (Figure 1). Under these conditions, the NADPH oxidase in control membranes retained 80–85 % of its initial activity. A standard 10 min preincubation at 30 °C was therefore used in all subsequent trials.

DPI only blocked H₂O₂ formation when it was preincubated with membranes in the presence of NADPH plus Ca²⁺ (Table 2, Expts. 2 and 5). DPI plus NADPH without Ca²⁺ (Expt. 3) or DPI plus Ca²⁺ without NADPH (Expt. 4) did not significantly inhibit H₂O₂ formation. NADP⁺, the oxidized form of the substrate and a competitive inhibitor of NADPH (result not shown) (*K_i* = 0.2 mM), could not replace NADPH (Expt. 6). NADH was unable to replace NADPH (Expt. 7), according to the reduced-nicotinamide-nucleotide specificity of the H₂O₂ generator [6]. The reduced H₂O₂ generator (NADPH plus Ca²⁺) was inhibited by DPI, even in an O₂-depleted medium with an argon atmosphere (results not shown), so an oxygen-modified oxidase structure is not required for DPI inhibition. These results imply that DPI is bound to its target protein when electrons are transferred or when the target protein is reduced.

The effect of DPI on thyroid membrane fraction reduced with 1–20 mM dithionite was checked during the preincubation step. The membranes were reduced either before addition of DPI (Expt. 9) or after (results not shown). DPI had no inhibitory effect whatever the order, showing that reduction of the H₂O₂ generator by dithionite could not replace the specific reduction obtained with NADPH. Adding NADPH to dithionite-reduced membranes during the preincubation step (Expt. 10) did not restore sensitivity to DPI, suggesting that the H₂O₂ generator is still fully reduced by dithionite. Moreover, the oxidized form of the substrate did not induce inhibition by DPI in the dithionite-reduced system (Expt. 11).

The dithionite-reduced membranes were washed extensively before the measure of H₂O₂ formation, and this production was not the result of chemical reduction of O₂ by dithionite during the preincubation step, since H₂O₂ formation catalysed by preincubated membranes required NADPH plus Ca²⁺ (results not shown).

Table 2 Effect of the redox state of the H₂O₂ generator on its inhibition by DPI

Membrane fraction (120 µg of protein/ml) was preincubated for 10 min at 30 °C in buffer A plus 10 µM FAD and 1 mM dithiothreitol plus the additives listed below. Inhibition was started by adding the reducer or NADP⁺. Expts. 2–4 were preincubated for 2.5 min, instead of 10 min, to avoid losing activity (the Ca²⁺-depleted preparation is unstable at 30 °C). The membrane fraction was then centrifuged, washed once (or twice for experiments with dithionite), and its residual NADPH-dependent H₂O₂-generating activity measured. The additives were 0.1 mM NADPH, 0.1 mM NADH, 2 mM NADP⁺ and 10 mM dithionite. In Expt. 3, Ca²⁺ was omitted from buffer A and 1 mM EGTA was added. Data are the mean initial H₂O₂-formation rates ± S.E.M. of at least 3 experiments.

Expt. no.	Conditions of preincubation	H ₂ O ₂ formation	
		(nmol/h per ml of membranes)	(%)
1	NADPH + Ca ²⁺	3000 ± 104	100
2	NADPH + Ca ²⁺ + 2 µM DPI	1024 ± 90	34
3	NADPH + EGTA + 2 µM DPI	2589 ± 72	86
4	Ca ²⁺ + 2 µM DPI	2890 ± 60	96
5	NADPH + Ca ²⁺ + 1 µM DPI	570 ± 60	19
6	NADP ⁺ + Ca ²⁺ + 1 µM DPI	2929 ± 90	98
7	NADH + Ca ²⁺ + 1 µM DPI	2674 ± 98	89
8	Dithionite + Ca ²⁺	2791 ± 300	93
9	Dithionite + Ca ²⁺ + 10 µM DPI	2746 ± 184	92
10	Dithionite + Ca ²⁺ + 10 µM DPI and then NADPH	2825 ± 388	94
11	Dithionite + Ca ²⁺ + 10 µM DPI + NADP ⁺	2756 ± 18	92

Figure 2 Effect of increasing concentrations of DPI on H₂O₂ formation by thyroid membrane NADPH oxidase

The membrane fraction was preincubated for 10 min at 30 °C with DPI in buffer A plus 10 µM FAD, 1 mM dithiothreitol and 0.1 mM NADPH. Two parallel experiments were carried out, one with 125 µg of protein/ml (○) and the other with 300 µg of protein/ml (●). The membranes were centrifuged and washed; residual H₂O₂-forming activity was measured and plotted against amount of DPI added per mg of membrane protein.

Effect of DPI concentration on H₂O₂ formation

Inactivation of H₂O₂ formation by DPI depended on the protein concentration. The half-maximal effect increased with the protein concentration (results not shown), as for the neutrophil NADPH oxidase complex [19]. A smooth dose–response curve was obtained when the concentration of DPI was expressed in nmol/mg of membrane protein, rather than molarity (Figure 2), suggesting that the lipophilic DPI must be dissolved in the lipid phase to

Figure 3 Effect of DPI on NADPH oxidation by thyroid membrane fraction in the presence of ferricyanide (NADPH:ferricyanide reductase activity)

The membrane fraction was first treated with 10 mM CHAPS to extract a NADPH:cytochrome *c* reductase not related to the H₂O₂-generating system. (a) Membrane fraction was preincubated for 10 min at 30 °C without (○) or with 160 nmol of DPI/mg of protein (■) in buffer A plus 10 µM FAD and 0.1 mM NADPH, centrifuged, washed, and the NADPH oxidase activity was assayed at 340 nm in the presence of 0.1 mM ferricyanide. (b) Membrane fraction was preincubated with different concentrations of DPI with (■) or without (▲) NADPH as in (a). The membranes were centrifuged, washed, and the residual NADPH oxidase activity was measured in the presence of 0.1 mM ferricyanide. Each point is the mean ± S.E.M. of four experiments.

reach its target site(s) and that the concentration of DPI in the membrane is the factor limiting inhibition of the H₂O₂ generator; 50 % inhibition was obtained with about 2–2.5 nmol of DPI/mg of protein. A 20-fold excess of DPI did not cause total inhibition; the residual activity was 10–30 %, depending on the thyroid membrane preparation. It may be due to a degraded part of the H₂O₂ generator having a very low affinity for DPI.

Inhibition of NADPH:ferricyanide reductase activity by DPI

Ferricyanide scavenges electrons provided by the Ca²⁺-activated thyroid NADPH oxidase and stops the H₂O₂ production [14]. Inactivation of the ferricyanide reductase activity by DPI was examined by measuring NADPH oxidation in the presence of 0.1 mM ferricyanide. Membranes were first washed with 10 mM CHAPS to extract a NADPH:cytochrome *c* reductase activity which also catalyses a NADPH-dependent reduction of ferricyanide [15]. The NADPH:ferricyanide reductase activity of NADPH oxidase was almost completely inhibited by DPI (160 nmol/mg of protein) (Figure 3a). Like the inhibition of H₂O₂ production by DPI, NADPH:ferricyanide reductase was inactivated by DPI only when both NADPH and Ca²⁺ were present in the medium, confirming that the system must be reduced to bind DPI (Figure 3b). When the residual membrane NADPH:ferricyanide reductase activity after DPI treatment was

Figure 4 (a) Effect of increasing concentrations of DPI on H₂O₂ formation (●) and on NADPH:ferricyanide reductase activity (■), in the presence of ferricyanide; (b) effect of increasing concentrations of ferricyanide on H₂O₂ formation (●) and on NADPH:ferricyanide reductase activity (■), in the presence of DPI

(a) Before assaying H₂O₂ formation or NADPH:ferricyanide reductase activity, the membrane fraction or the CHAPS-pretreated membrane fraction respectively was preincubated as described in the Materials and methods section with DPI in the presence of 0.1 mM ferricyanide, centrifuged and washed. (b) The membrane fraction or the CHAPS-pretreated membrane fraction was preincubated as described in the Materials and methods section with different concentrations of ferricyanide in the presence of 5 nmol of DPI/mg of protein, centrifuged and washed. H₂O₂ formation (membrane) or NADPH:ferricyanide reductase (CHAPS-treated membrane) was then assayed.

plotted against the amount of DPI added per mass of membrane protein, it gave half-inhibition at about 2 nmol of DPI/mg of protein (Figure 3b). This concentration is of the same order of magnitude as that which gave half-inhibition of H₂O₂ formation (Figure 2). Thus the dehydrogenase component is a target site for DPI, but there could be another target site beyond the dehydrogenase component, having the same or a lower affinity for DPI.

Effect of ferricyanide on the inhibition of NADPH:ferricyanide reductase activity and H₂O₂ formation by DPI

The effect of ferricyanide on the inhibition of NADPH:ferricyanide reductase activity and H₂O₂ formation by DPI was assessed by preincubating the membranes with DPI in the presence of ferricyanide. The residual H₂O₂-generating activity of membranes preincubated with NADPH, Ca²⁺ and 6–10 nmol of DPI/mg of protein, in the absence or presence of 0.1 mM ferricyanide, was largely inhibited compared with the controls without DPI (results not shown). In contrast, the ferricyanide reductase activity was completely or significantly protected under these conditions (results not shown).

The effect of DPI concentration on H₂O₂ formation was tested in the presence of a ferricyanide concentration (0.1 mM) which completely protected the NADPH:ferricyanide reductase against DPI inhibition at the DPI concentrations used (1–6 nmol/mg). Figure 4(a) shows that 0.1 mM ferricyanide partially protected H₂O₂ formation, compared with the results in Figure 2 obtained without ferricyanide. The residual H₂O₂ activities of the membranes were 60% after preincubation with 5 nmol of DPI/mg with 0.1 mM ferricyanide and 38% after preincubation with DPI alone. Figure 4(b) shows that increasing the preincubation-medium ferricyanide concentration protected membranes against DPI inactivation. Ferricyanide (1 mM) almost completely protected H₂O₂ formation catalysed by the membranes from the effect of 5 nmol of DPI/mg. This ferricyanide concentration is 20 times that needed to protect the NADPH:ferricyanide reductase activity.

DISCUSSION

The thyroid produces H₂O₂ by a plasma-membrane NADPH oxidase, and this catalysis is completely Ca²⁺-dependent. The thyroid NADPH oxidase does not generate O₂^{•-}, as assessed by superoxide dismutase-inhibitable reduction of cytochrome *c* [14]. The O₂^{•-} formation in thyroid membranes found by Nakamura et al. [32,33] using diacetyldeuterohaem peroxidase results from the oxidation of NADPH by that enzyme in the presence of H₂O₂ [15,34]. The only known systems that directly reduce molecular O₂ to H₂O₂ are the thyroid membrane and oocyte membrane oxidases [35]. These systems may need electron-transport component(s) different from those of NADPH oxidases of phagocyte and non-phagocyte cells (for reviews see [9,36]), which produced O₂^{•-}. It is difficult to investigate the components of the thyroid H₂O₂ generator, because this system is only a minor part of the thyroid membrane proteins. The specific activity of thyroid membrane oxidase is approx. 1–2% of the value for the neutrophil membrane oxidase.

There is good evidence that FAD, but not FMN, is the prosthetic group of the H₂O₂ generator. This result agreed with the study of a non-toxic goitre with a defect in the iodination reaction [37], resulting in a failure of H₂O₂ formation. *In vitro*, FAD, but not FMN, restored iodination of thyroglobulin catalysed by goitre thyroid homogenate. Moreover, DPI, which acts via a flavin component [38,39], is a potent inhibitor of the thyroid H₂O₂ generator.

DPI had no inhibitory effect unless the H₂O₂ generator was incubated with both the specific substrate NADPH and Ca²⁺, the thyroid NADPH oxidase activator, suggesting that a reduced redox centre is needed to bind DPI. The Ca²⁺ is thought to act via an autoinhibitory domain or inhibitory protein component, masking the catalytic site from the substrate and/or enabling electrons to be transferred from NADPH to O₂ [29]. The need for a reduced redox centre for DPI inactivation is consistent with a recent report showing that the inhibitory potency of DPI is directly correlated with the rate of human neutrophil NADPH oxidase turnover [22]. Iodonium biphenyl and DPI have also been shown to require reducing conditions, NADPH or sodium dithionite, in order to inhibit bovine neutrophil oxidase [19,21] or cytochrome *P*-450 [20]. In contrast, sodium dithionite-reduced thyroid membranes are not inhibited by DPI, even in the presence of Ca²⁺.

Three lines of evidence indicate that inhibition of the H₂O₂ generator by DPI requires mild reduction of a component in a particular conformation. The dithionite-reduced FAD prosthetic group of the H₂O₂ generator cannot react with DPI; consequently the reduction of this redox component is not sufficient. The DPI

Figure 5 Diagram showing the DPI target sites on the H_2O_2 -generating system of the thyroid plasma membrane: DPI binding needs a Ca^{2+} -activated NADPH oxidase and a NADPH-reduced enzyme

In the absence of Ca^{2+} , the autoinhibitory domain or inhibitory protein component (In.) prevents NADPH reaching its binding site or electron transfer from NADPH to the NADPH flavodehydrogenase entity (Fp). Sub-micromolar Ca^{2+} concentrations allow electrons to flow from NADPH through the components of the H_2O_2 generator to molecular O_2 , to generate H_2O_2 . Ferricyanide acts as an electron scavenger of the NADPH dehydrogenase entity. There are at least two DPI target sites: one is on the flavodehydrogenase component (or domain), and the other is the unknown O_2 -reacting component (or domain).

phenyl radical, which is probably formed when the membranes are reduced by dithionite in the presence of DPI, cannot attack a strategic amino acid group in the H_2O_2 generator. The substrate, NADPH, cannot induce inhibition of dithionite-reduced membranes by DPI. Our working hypothesis is that DPI^+ is electro-attracted to the electronegatively charged redox centres when electrons flow through the activated H_2O_2 generator. It may then take electrons and form a phenyl radical, which may react *in situ* with a strategic amino acid residue. It is also possible that electrostatic interactions between the redox centres and DPI are sufficient to inhibit the electron flow without covalent binding.

Ferricyanide scavenges the electrons provided by the Ca^{2+} -dependent NADPH oxidase. H_2O_2 formation and O_2 consumption are completely inhibited by excess ferricyanide, whereas Ca^{2+} -dependent NADPH oxidation is unchanged. The ferricyanide reductase activity remaining in CHAPS-treated membranes then became fully dependent on Ca^{2+} . Both activities, Ca^{2+} -dependent NADPH:ferricyanide reductase and NADPH oxidase, had similar properties: they were completely and reversibly inhibited by 10 mM CHAPS [14], and were very

sensitive to thiol reagents (results not shown). These results indicate that the NADPH-dependent ferricyanide reduction is catalysed by a component of the H_2O_2 generator. DPI inactivated the membrane NADPH:ferricyanide reductase only when membranes were incubated with NADPH plus Ca^{2+} , and the DPI concentration which gave half-inhibition is of the same order of magnitude as that which half-inhibited H_2O_2 formation. Ferricyanide protected the NADPH:ferricyanide reductase activity against DPI inhibition. But complete protection of the NADPH:ferricyanide reductase activity was obtained with a concentration which was about 20 times lower than that needed to protect H_2O_2 formation completely. This strongly suggests that there are at least two target sites for DPI (Figure 5). One site may be the NADPH flavodehydrogenase entity or domain, the site from which ferricyanide first takes electrons. The other site could be beyond the dehydrogenase component, on the entity(ies) or domains in which the electrons are transferred to molecular oxygen. The bovine neutrophil NADPH oxidase has at least two DPI-sensitive components, differing in their sensitivity to the inhibitor [19]. One, which may be the NADPH dehydrogenase component, is inhibited at a higher DPI concentration than is the other, which is presumed to be the haem-binding component of cytochrome b_{558} . In the thyroid membrane, half-inhibition of the NADPH:ferricyanide reductase and of H_2O_2 formation occur at the same concentration with the same range of DPI concentrations. Thus, either the two sites have the same sensitivity to DPI, or the flavodehydrogenase site had the highest sensitivity.

There is no evidence as yet for a cytochrome linked to the thyroid NADPH oxidase, as there is in neutrophil NADPH oxidase. Butyl isocyanide, which reacts with the haem of pig neutrophil cytochrome b_{558} [40], does not inhibit thyroid NADPH oxidase. Imidazole, which reversibly inhibits neutrophil oxidase [41] and NO synthase [42], presumably by occupying the sixth co-ordination-site haem iron (i.e. the binding site of oxygen), does not alter the rate of thyroid NADPH oxidation, even at concentration of 100 mM (results not shown). Lastly, antibodies directed against the C-terminal regions of the α and β subunits of the neutrophil cytochrome b_{558} detected no equivalent subunits in thyroid membranes. Thus the link between the flavodehydrogenase component and the O_2 -reacting component (or domain) of the thyroid NADPH oxidase remains to be determined.

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