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Ca^{2+} regulation of thyroid NADPH-dependent H_2O_2 generation

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A thyroid particulate fraction contains an NADPH-dependent H_2O_2-generating enzyme which requires Ca^{2+} for activity. A Chaps solubilized extract of the thyroid particulate fraction partially purified by DEAE chromatography did not show a dependence on Ca^{2+} for activity. Preincubation of the particulate fraction with Ca^{2+} yielded a preparation insensitive to Ca^{2+}. The non-particulate fraction obtained after incubation of the particles in the presence of Ca^{2+} was able to inhibit, in the presence of EGTA, the Ca^{2+}-desensitized particulate fraction and the enzyme isolated on DEAE. It is concluded that the reversible Ca^{2+} activation of the NADPH-dependent H_2O_2 generation was modulated in porcine thyroid tissue by (a) calcium-releasable inhibitor protein(s).

NADPH; Hydrogen peroxide; Calcium; (Thyroid)

1. INTRODUCTION

Biosynthesis of thyroid hormones is catalyzed by a thyroid peroxidase in the presence of H_2O_2 [1]. Hydrogen peroxide formation in thyroid tissue has been evidenced by direct methods [2,3]. Since reduced pyridine nucleotides stimulate iodination in thyroid particulate fractions [4,5], they were proposed as hydrogen donors in H_2O_2 generation. NAD(P)H-dependent H_2O_2 generation has been cytochemically localized at the apical cell surface, close to the site of thyroglobulin iodination [6,7], either in isolated rat [8] or porcine [9] thyroid follicles, and in rat thyroid slices [10]. In resting porcine open thyroid follicles incubated in a Ca^{2+} containing medium, the release of H_2O_2 is promptly and strongly stimulated by addition of the ionophore A23187 [9]. Furthermore, in dog calf and hog thyroid slices hydrogen peroxide production is controlled by TSH [2,3,11,12]. This effect is not mimicked by $(Bu)_2$ cAMP [9] or by forskolin [12]. Thus Ca^{2+} might be the second messenger of TSH action on these H_2O_2-generating systems.

We have previously detected, in a porcine thyroid particulate fraction, an NADPH-dependent H_2O_2-generating system which also requires Ca^{2+} for activity [13]. The presence of such an H_2O_2 generator has been recently confirmed and studied by Nakamura et al. [14] in a preparation of thyroid fragmented plasma membranes. However, the mode of Ca^{2+} action remains unknown. In this report we provide data on the mechanism involved in the control of the NADPH-dependent H_2O_2-generating enzyme by Ca^{2+} in the porcine thyroid tissue.

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2. MATERIALS AND METHODS

2.1. *Materials*

NADPH and lyophilized horseradish peroxidase (grade I) were obtained from Boehringer (Mannheim); (3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate (Chaps), phenylmethylsulfonyl fluoride (PMSF), leupeptin (synthetic, hemisulfate), cytochrome *c*, trypsin inhibitor from soybean, carbonic anhydrase, ovalbumin, serum albumin were purchased

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from Sigma (St. Louis, MO); scopoletin was from Serva (Heidelberg); DEAE-Trisacryl M, GF 05, AcA 44 were from IBF Biotechnics (Villeneuve-la-Garenne, France).

2.2. *Thyroid particulate fraction*

Hog thyroid glands were obtained immediately after slaughter and transferred to the laboratory on ice. Fat and connective tissues were removed, the glands were cut into small pieces and homogenized in 2.5 vols of 50 mM sodium phosphate buffer containing 0.25 M sucrose and 1 mM EGTA, pH 7.2; first in a Sorvall Omni-mixer (30 s, 11000 rpm) and second, with an Ultra-Turrax (30 s, 8000 rpm). The homogenate was then diluted with 0.5 vol. of the same buffer, filtered through 6 layers of cheesecloth and centrifuged at 1100 × *g* for 15 min. The pellet was washed once and the final pellet resuspended in homogenization buffer (1 ml/g of initial tissue). This particulate fraction was stored frozen at −20°C.

2.3. *Solubilization of particulate proteins for DEAE-Trisacryl chromatography*

Aliquots of particulate fraction (500 ml) were thawed, pelleted by centrifugation at 3000 × *g* for 15 min at 4°C and resuspended in 80 ml of 10 mM Tris-HCl, pH 8.0. The Chaps concentration was adjusted to 12 mM.

The suspension was incubated for 15 min at 30°C and any insoluble material was removed by centrifugation at 150000 × *g* for 1 h at 4°C.

2.4. *DEAE-Trisacryl chromatography*

The 12 mM Chaps extract containing approx. 15% of the total activity was loaded onto a 2.6 × 25 cm DEAE-Trisacryl column equilibrated in 10 mM Tris-HCl, pH 8.0, containing 5 mM Chaps.

The column was washed with this buffer and proteins were eluted at 1 ml/min with 650 ml linear gradient of 0–500 mM NaCl in the same buffer at 6°C.

Fractions (4.5 ml) were collected at 4°C and their NADPH-dependent H_2O_2-generating activity assayed.

2.5. *Concentration of DEAE-Trisacryl fractions*

Active fractions (generally 10–15) eluted between 100 and 150 mM NaCl were pooled, diluted with 4 vols of 10 mM Tris-HCl, 5 mM Chaps, pH 8.0, and mixed with 20–30 ml of DEAE-Trisacryl exchanger equilibrated in the same buffer.

The exchanger was poured into the column (1.6 × 20 cm) and washed with 50 μl of the same buffer. Proteins were eluted with 25 μl of 50 mM sodium phosphate buffer, 5 mM Chaps, 180 mM NaCl, pH 7.5.

2.6. *NADPH-dependent H_2O_2 generating activity assays*

Particulate or Chaps-solubilized proteins were incubated at 30°C in 50 mM sodium phosphate buffer, pH 7.2, containing 1 mM NaN_3 and various concentrations of $CaCl_2$ or EGTA. The reaction was started by the addition of 0.1 mM NADPH and 100 μl aliquots were collected at time intervals and mixed with 10 μl of 1.2 N HCl to stop the reaction and destroy the remaining reduced pyridine nucleotide.

Initial rates of H_2O_2 formation were determined from 8 aliquots of each assay performed with particles or Chaps extracts and 4 aliquots of assays done on the column fractions.

The amount of H_2O_2 present in each aliquot was determined in a Perkin Elmer MPF 43A spectrofluorimeter as previously described [13].

Initial rates of H_2O_2 production are expressed as the number of nmol of H_2O_2 formed/h per ml of each fraction, i.e. particle suspension, Chaps extract or column fraction.

2.7. *Preparation of Ca^{2+}-dependent inhibitor factor and of Ca^{2+}-desensitized particles*

Fresh thyroid particulate material prepared in the presence of EGTA was incubated at 30°C for 12 min in 50 mM phosphate buffer, pH 7.2, in the presence of 0.25 M sucrose, 1 mM EGTA, 2 mM $CaCl_2$, 0.1 mM PMSF and 40 μg/ml leupeptin (1 ml for 1 g initial fresh tissue). The mixture was centrifuged at 7700 × *g* for 30 min at 4°C. The supernatant (Ca^{2+} extract) obtained was stored at −20°C. The pellet was washed once in 50 mM phosphate buffer, pH 7.2, 0.25 M sucrose and 1 mM EGTA. The final pellet was resuspended in the same buffer (1 ml per g of initial fresh tissue).

The Ca^{2+} extract was applied to a Trisacryl GF 05 column equilibrated in 50 mM phosphate buffer, pH 7.2, immediately before use. The excluded protein peak, which contained molecules with molecular masses greater than 3 kDa, was used.

2.8. *Filtration of Ca^{2+} extract on AcA 44 column*

Ca^{2+} extracts were concentrated by dialysis under vacuum or as follows: Ficoll powder was sprinkled on the dialysis tube membrane containing the Ca^{2+} extract. 2 ml obtained from 15 ml of Ca^{2+} extract were loaded onto the AcA 44 column (1.6 × 60 cm) equilibrated in 50 mM phosphate buffer, 0.1 M KCl, 0.1 mM $CaCl_2$, 0.1 mM PMSF and leupeptin (4 μg/ml), pH 7.2. Fractions of 2 ml were collected.

2.9. *Protein determination*

Proteins were measured by the method of Lowry et al. [15] with bovine serum albumin as standard.

3. RESULTS

3.1. *Effect of Ca^{2+} on the rate of H_2O_2 formation catalyzed by thyroid particles at various pH values*

The rate of H_2O_2 generation catalyzed by the membranous NADPH-dependent enzyme was measured with 0.1 mM NADPH at different pH values in the presence or absence of Ca^{2+}. Fig.1 shows that the basal activity was maximal at pH 6.5 and low at pH 7.4, whereas the Ca^{2+}-stimulated enzyme was more efficient at pH 7.4. Consequently, at this pH the NADPH-dependent activity was fully Ca^{2+}-dependent. The presence of different concentrations of Ca^{2+} modified the V_m of the H_2O_2-generating activity but not the K_m for NADPH (K_m = 12.3 ± 1.9 μM, average of 9 determinations).

3.2. *Ca^{2+} desensitization of thyroid particles*

Fresh thyroid particles, prepared in the presence

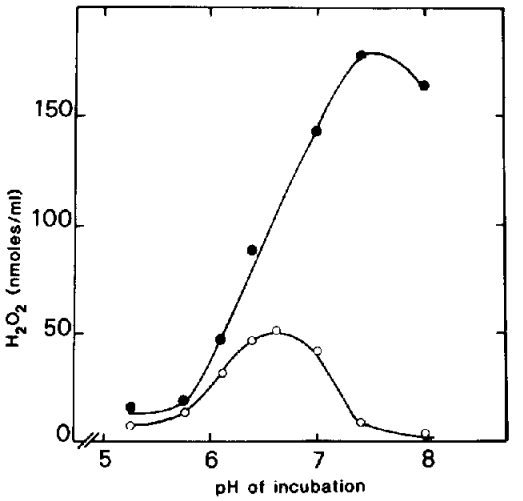

Fig.1. Initial rates of H_2O_2 generation catalyzed by (a) particulate enzyme(s) at various pH values. 0.5 mg/ml of the fresh particulate fraction were added to 1 ml of 50 mM sodium phosphate buffer at various pH values in the presence of 1.1 mM EGTA (○) or 0.5 mM $CaCl_2$ (●). Other incubations and H_2O_2 assay by the scopoletin technique are described in section 2.

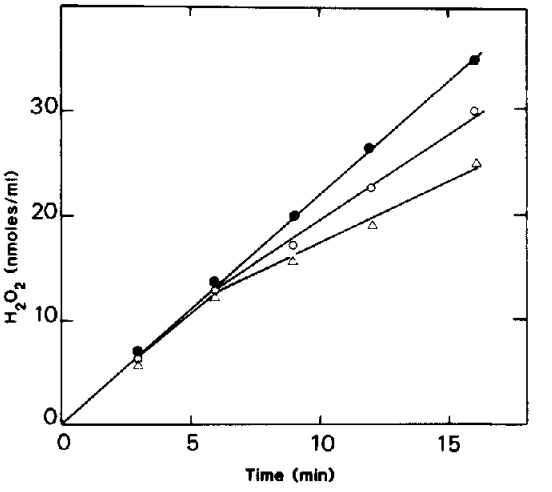

Fig.2. Ca^{2+} desensitization of NADPH-dependent H_2O_2 generation associated with the particulate material. NADPH-dependent H_2O_2 formation was measured with 100 μl of Ca^{2+}-preincubated particulate material prepared as described in section 2. Incubations were performed in the presence of 2 mM (△) or 1 mM (○) EGTA or 0.5 mM $CaCl_2$ (●).

of 1 mM EGTA were incubated in the presence of 2 mM $CaCl_2$, washed and pelleted by centrifugation as described in section 2 and resuspended in buffer containing 1 mM EGTA.

Fig.2. shows that the Ca^{2+}-preincubated particles catalyzed NADPH-dependent formation of H_2O_2 at pH 7.4 even in the presence of excess EGTA, whereas untreated particles were inactive under these conditions (fig.1).

3.3. *Evidence for Ca^{2+} regulation of NADPH-dependent H_2O_2 generation via the release of an inhibitor protein*

3.3.1. Effect of Ca^{2+} extract on Ca^{2+}-desensitized particles

The possibility that the Ca^{2+}-desensitized particulate material could result from the Ca^{2+}-dependent release of an inhibitor bound to the NADPH-dependent H_2O_2-generating system was tested. The effect of the supernatant obtained by centrifugation of the particulate fraction preincubated with Ca^{2+} was tested on the Ca^{2+}-desensitized particulate material in the presence of EGTA. There was an inhibition whose amplitude varied depending on the particulate preparation used; possibly due to the freshness of the particulate material (not shown).

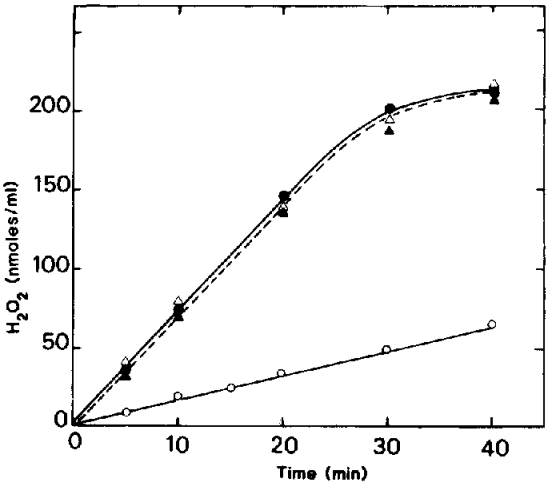

Fig.3. Evidence for the release of a Ca^{2+}-dependent inhibitor factor during Ca^{2+} treatment of particulate material. NADPH-dependent H_2O_2 formation was catalyzed by DEAE-Trisacryl purified system in the absence (△, ▲) or in the presence (○,●) of Ca^{2+} extract obtained from a Trisacryl GF05 column. Incubations were carried out with 20 μl of H_2O_2-generating system, 500 μl Ca^{2+} extract under conditions described in section 2, in the presence of 0.5 mM $CaCl_2$ (closed symbols) or 1 mM EGTA (open symbols). (Dotted line) Ca^{2+} extract was replaced by boiled Ca^{2+} extract.

3.3.2. Effect of Ca^{2+} extract on the solubilized enzyme

The Chaps-solubilized and DEAE-purified enzyme did not display any sensitivity to Ca^{2+} concentration. The enzyme remained active even when incubated with 1 mM EGTA as illustrated in fig.3. The loss of Ca^{2+} dependence of the catalytic function of the NADPH-dependent enzyme could also be interpreted as a consequence of the loss during chromatography, of some inhibitory component, whose interaction with the enzyme would normally be modulated by the Ca^{2+} concentration both in particles and Chaps extracts. This hypothesis was tested by adding the supernatant to the DEAE-purified NADPH-dependent H_2O_2-generating system. Fig.3 shows that the H_2O_2 formation fell in the presence of EGTA, but could be reestablished by addition of Ca^{2+}. These results could not be obtained with a boiled supernatant but were observed with the supernatant material excluded from a Trisacryl GF05 column suggesting that the inhibitor has a molecular mass greater than 3 kDa and may be a protein. The Ca^{2+} extract produced a dose-dependent inhibition of the NADPH-dependent H_2O_2 formation catalyzed by the DEAE-purified H_2O_2-generating system in the presence of excess EGTA (fig.4). There was a residual activity of approx. 40%. The inhibition was completely reversible by addition of Ca^{2+} to the medium (fig.4, inset).

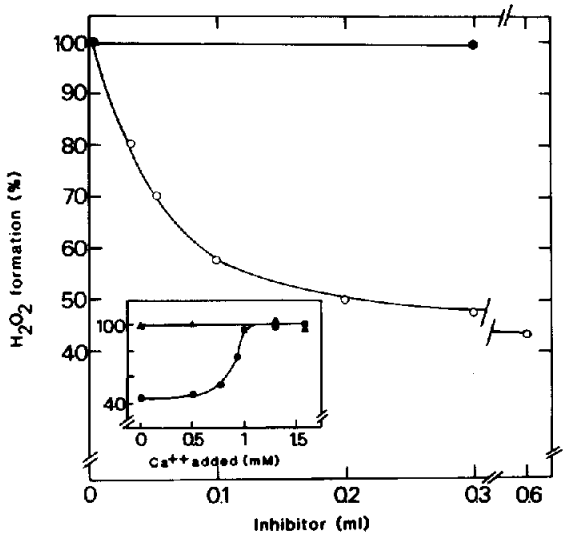

Fig.4. Inhibition of NADPH-dependent H_2O_2 generation by Ca^{2+} extract. NADPH-dependent H_2O_2 formation catalyzed by DEAE-Trisacryl purified H_2O_2-generating system was performed in the presence of 1 mM EGTA (○) and 1.5 mM Ca^{2+} (●). (Inset) Effect of Ca^{2+} on the inhibition of the NADPH-dependent H_2O_2 formation by Ca^{2+} extract. Incubations were carried out in the presence of DEAE-Trisacryl purified H_2O_2-generating system, 1 mM EGTA, varying concentrations of added $CaCl_2$, and in the absence (▲) or in the presence (●) of 0.3 ml of inhibitor. In all experiments, the Ca^{2+}-released inhibitor was passed through a Trisacryl GF05 column just before use.

3.3.3. Determination of the molecular size of the Ca^{2+}-dependent inhibitor

The Ca^{2+} extract was chromatographed on an AcA 44 column. The inhibitory activity in each fraction was tested using the NADPH-dependent H_2O_2-generating enzyme previously purified on DEAE-Trisacryl in the presence of EGTA with or without an excess of Ca^{2+}. Fig.5 shows that there are two protein peaks which inhibited the enzyme activity only in the presence of EGTA. The apparent molecular masses of these entities were estimated by comparing their respective V_e/V_o

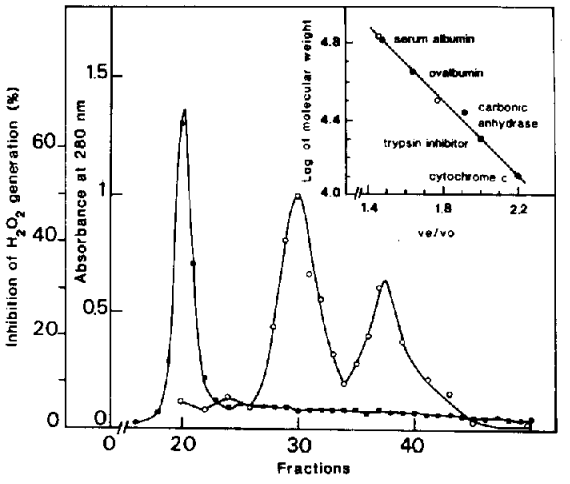

Fig.5. Gel filtration of Ca^{2+} extract on AcA 44 column. Ca^{2+} extract was filtered under conditions described in section 2. Fractions were monitored for absorbance at 280 nm (■) and tested for inhibition of H_2O_2 generation (○). Each fraction was incubated and in the presence of DEAE-Trisacryl purified H_2O_2-generating system, and either in the presence of 1 mM EGTA or in the presence of 1 mM EGTA and 1.5 mM $CaCl_2$. The initial rate of H_2O_2 formation measured in the presence of an excess of Ca^{2+} was used as reference to calculate the percentage of inhibition of the initial rate measured in the presence of EGTA. (Inset) Apparent molecular mass as a function of the elution volume/void volume ratio. Protein standard (●), Ca^{2+}-release inhibitory entities (○).

values to the V_e/V_o ratio of protein standards (fig.5, inset). They were found to be respectively approx. 69 and 33 kDa. These values were confirmed when the Ca^{2+} extract was chromatographed on FPLC Superose 12 column instead of AcA 44 (not shown). The relative proportions of these two entities would vary with different Ca^{2+} extracts.

4. DISCUSSION

This report shows that the NADPH-dependent H_2O_2-generating enzyme from thyroid particles could be active in the absence of Ca^{2+} under certain conditions: i.e. when unfrozen particles were washed with a Ca^{2+}-containing buffer or when the Chaps solubilized enzyme was chromatographed on an anion exchanger. In both cases, addition of a Ca^{2+} extract to these preparations restored the Ca^{2+} dependence of the H_2O_2-forming activity. These experiments demonstrated the presence in the Ca^{2+} extract of inhibitor factors whose interaction with the NADPH-dependent enzyme is under Ca^{2+} control. Gel filtration analysis of the Ca^{2+} extract showed that two proteins are able to produce a Ca^{2+}-reversible inhibition of the enzyme. Assuming that they are globular proteins, their apparent molecular masses were found to be 33 and 69 kDa. Although their Ca^{2+}-binding ability has not yet been determined, they could be Ca^{2+}-binding proteins.

We postulated that the inhibitor components dissociate from the enzyme in the presence of Ca^{2+}, and that they reassociate in the absence of this cation (EGTA). These interactions do not require ATP and Mg^{2+}, indicating that protein kinase activity is not involved. Phorbol ester (TPA) has been reported to increase the generation of H_2O_2 obtained by stimulation of thyroid slices by subthreshold doses of ionophore A23187 [12]. Furthermore, ATP has been shown to enhance twice the activity of the Ca^{2+}-stimulated H_2O_2-generating enzyme [14]. Therefore the implication of protein kinase C and/or other protein kinases in the control by Ca^{2+} of H_2O_2 generation cannot be excluded. For example, a phosphorylation could modulate the equilibrium between free and protein bound Ca^{2+} and/or between (a) free and enzyme-associated inhibitor(s).

Purification and identification of these entities are currently in progress and will facilitate studies on their possible phosphorylation and Ca^{2+}-binding properties. These studies will also allow us to determine whether such a mechanism is strictly specific for hog thyroid or also involved in other animals.

In conclusion, we propose that in hog thyroid tissue, Ca^{2+}, the putative second messenger for TSH action on H_2O_2 production required for thyroid hormone synthesis, would regulate the H_2O_2-generating enzyme according to the following model reaction:

Inhibitor protein-NADPH oxidase (inactive form)

$$+ Ca^{2+} \downarrow \quad \uparrow + EGTA$$

Free inhibitor protein + NADPH oxidase (active form).

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