Volume 246, number 1,2, 117–119 FEB 06956 March 1989

Generation of O_2^- and tyrosine cation-mediated chemiluminescence during the fertilization of sea urchin eggs

Atsushi Takahashi, Hiroko Totsune-Nakano, Minoru Nakano*, Shinro Mashiko, Nobutaka Suzuki, Chikako Ohma+ and Humio Inaba+

*Inaba Bio-Photon Project, Research Development Corporation of Japan, Kojinkai Central Hospital, 2-1-6 Tsutsujigaoka, Sendai, Miyagi, *College of Medical Care and Technology, Gunma University, Maebashi, Gunma* and *+Research Institute of Electrical Communication, Tohoku University, Sendai, Miyagi, Japan*

Received 3 February 1989

Sea urchin eggs emit light in the visible region during their fertilization. Judging from the chemiluminescence spectra, one of the excited species generated is considered to have originated from a tyrosine cation radical-mediated reaction. Chemiluminescence probes such as luminol or a cypridina luciferin analog (2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo-[1,2-*a*]pyrazin-3-one) are useful for detecting the ovoperoxidase+H_2O_2 reaction associated with membrane hardening and O_2^- generation, respectively, during the fertilization of sea urchin eggs.

Fertilization; Superoxide; Chemiluminescence; (Sea urchin egg)

1. INTRODUCTION

It is known that ovoperoxidase of sea urchin eggs is secreted from cortical granules at fertilization and acts in the hardening of the fertilization membrane by formation of cross-links between tyrosine residues in the membrane protein [1]. One of the substrates of ovoperoxidase appears to be H_2O_2 which is synthesized by the egg in a burst after fertilization [2,3]. The generation of H_2O_2 in the egg is probably an NAD(P)H-O_2 oxidoreductase activity which was found in the egg cortex and completely accounted for ovoperoxidase [4]. The natural chemiluminescence associated with fertilization of sea urchin eggs has been reported by Foerder et al. [2], but its properties remain unknown. We report here that O_2^- and excited species involved in tyrosine cation radical-mediated reactions are generated during the fertilization of sea urchin eggs.

Correspondence address: M. Nakano, College of Medical Care and Technology, Gunma University, Maebashi, Gunma 371, Japan

2. MATERIALS AND METHODS

2.1. *Materials*

S. purpuratus eggs and seminal fluid were collected in the usual manner [3]. In all experiments, the frequency of fertilization on exposure of eggs to sperm was at least 90%.

2.2. *Methods*

The reaction mixture contained about 2×10^5 eggs, 2-fold diluted seminal fluid, and artificial seawater at pH 7.8 in a total volume of 1.5 ml (standard reaction mixture). In some cases, 10 μM luminol or 1 μM 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo-[1,2-*a*]pyrazin-3-one (MCLA) [5] were used as chemiluminescence probes to detect the peroxidase + H_2O_2 reaction or O_2^- generation, respectively. Catalase (40 μg/ml), 5×10^{-7} M superoxide dismutase (SOD) or 5 mM NaN_3 was occasionally added to the system, prior to or during incubation. Incubations were carried out at 28°C and chemiluminescence was detected with a Luminescence Reader (Aloka, BLR-101) [6]. Chemiluminescence spectra were recorded with a filter-type spectrometer [7].

3. RESULTS AND DISCUSSION

As shown in fig.1A, chemiluminescence appeared about 3 min after mixing of the eggs with the seminal fluid, gradually reached a maximum at 5–6 min and then slowly decreased. Little or no

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

chemiluminescence was detected when 5 mM NaN_3 (a peroxidase inhibitor) was added to the system just before the appearance of chemiluminescence. Addition of 5 mM NaN_3 to the system during the reaction significantly suppressed chemiluminescence. Neither SOD nor catalase affected this luminescence (not shown). As shown in fig.1, luminol-dependent luminescence, which could reflect the ovoperoxidase + H_2O_2 reaction, appeared earlier than the natural chemiluminescence, reaching a maximum at 4–5 min and subsequently decreasing. The duration of this chemiluminescence was much shorter than that of the natural chemiluminescence. The luminol-dependent luminescence was also inhibited by the addition of

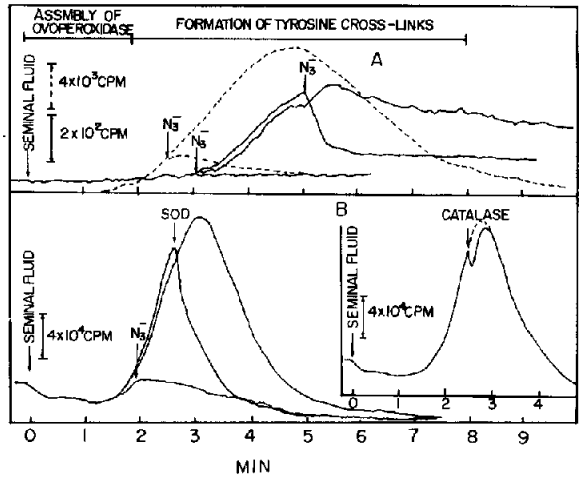

Fig.1.(A) Natural chemiluminescence (——) and luminol-dependent chemiluminescence (– – –) during fertilization of sea urchin eggs in the presence or absence of NaN_3. The standard reaction mixture with or without luminol was used. All components, except for seminal fluid, were preincubated at 28°C for 3 min and the reaction was initiated by mixing seminal fluid with the components of the mixture. Luminescence was measured in a luminescence reader at 28°C. Chemiluminescence intensities expressed in terms of cpm. Arrows indicate the times at which seminal fluid or N_3^- was added. Durations of assembly of ovoperoxidase with the egg membrane and formation of tyrosine cross-links were derived from the literature (see text). (B) MCLA-dependent chemiluminescence during fertilization of sea urchin eggs. The standard reaction mixture, in which MCLA was present, was used. All components, except for spinal fluid, were preincubated for 3 min at 28°C and the reaction was initiated by mixing seminal fluid with the components of the mixture. Chemiluminescence was measured in a luminescence reader at 28°C. Arrows indicate the times at which N_3, SOD, or catalase was added. Chemiluminescence intensities expressed in terms of cpm.

NaN_3, prior to or during the incubation. It seems likely that the duration of the luminol-dependent luminescence is highly correlated with that of the formation of tyrosine cross-links, which appears after the stage of interaction of ovoperoxidase with the egg membrane, as reported [3].

The difference between the time courses of luminol-dependent and natural luminescence may indicate that the formation of bityrosine cross-links is independent of the presumed tyrosine radical-mediated luminescence. Thus, in the horseradish peroxidase-tyrosine-H_2O_2 reaction, bityrosine is efficiently formed by the reaction of tyrosine radicals before the appearance of the obvious tyrosine radical-mediated chemiluminescence [8]. As shown in fig.1B, MCLA-dependent luminescence was inhibited by SOD and also by NaN_3, but not by catalytic amounts of catalase, indicating that ovoperoxidase is probably involved in O_2^- generation. A rapid decrease in MCLA-dependent luminescence may be due to prevention of the escape of O_2^- to the outside of the egg, probably by hardening of the membrane. Several possible H_2O_2-forming oxidase activities, including glucose, xanthine, fatty acyl and fatty acyl-CoA oxidase activities are absent from the egg cortex. However, two types of NADH oxidase activity which may produce O_2^- as a precursor of H_2O_2 are reported to be present in the egg cortex; one of these two activities is similar to that of horseradish peroxidase or of lactoperoxidase which could be completely inhibited by CN^-, the other being stimulated by Ca^{2+} and inhibited slightly by CN^- [4]. It has been found that O_2^- is generated during thyroxine-catalyzed oxidation of NADH in the horseradish peroxidase-H_2O_2 system and suggested that NAD$^\cdot$ formed in the $NADH^+$ + phenoxy radical reaction transfers its electron to O_2, yielding O_2^- as a precursor of H_2O_2 [9]. Thus, NAD$^\cdot$ produced in reaction mixtures containing an ovoperoxidase + H_2O_2 + NADH + an electron donor may be a candidate for the generation of O_2^- during the fertilization of sea urchin eggs. An alternative pathway for generating O_2^- may be that compound I of ovoperoxidase abstracts an electron from H_2O_2 to yield O_2^-. This can be supported by evidence that the horseradish peroxidase-H_2O_2 system produces O_2^-, which can be detected by the chemiluminescence of MCLA and inhibited by 5×10^{-7} M SOD, 5 mM NaN_3 or a catalytic amount of

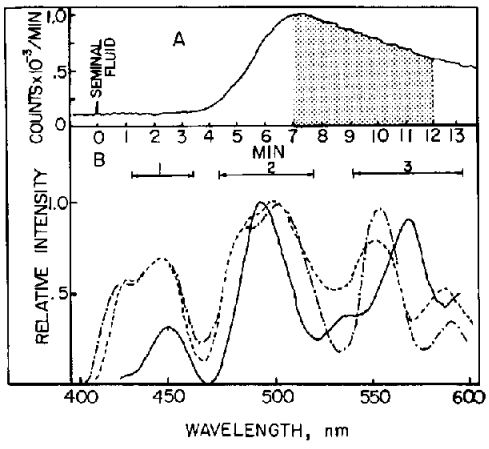

Fig.2. Typical natural luminescence during fertilization of sea urchin eggs (A) and the natural chemiluminescence spectra obtained in 3 different experiments (B). Incubation mixtures and conditions as in fig.1A. Spectral analysis was begun 7 min after mixing of the seminal fluid with eggs and continued for 5 min (stippled area in A) in a filter-type spectrometer using 25 colored filters covering the region between 330 and 600 nm. 1, luminescence 1; 2, luminescence 2; 3, luminescence 3. Three different experiments [(——)(-----)(– – –)] were carried out using different samples of seminal fluid and eggs from sea urchin.

catalase (40 μg/ml) (Sugioka, K. and Nakano, M., unpublished).

Even though catalase should rapidly decompose H_2O_2 and compete with peroxidase for H_2O_2, it should not penetrate into the egg and inhibit the chemiluminescence induced by the ovoperoxidase + H_2O_2 reaction in the egg membrane. Thus, it is not surprising that exogenously added catalase does not affect all the chemiluminescence seen during the fertilization of sea urchin eggs.

To investigate the excited species which emits the natural chemiluminescence, spectra of the emitted light were recorded during the time subsequent to the mixing of eggs with seminal fluid (fig.2A). As shown in fig.2B, the luminescence spectra displayed one or two peaks between 420 and 460 nm (luminescence 1), one or two peaks between 475 and 520 nm (luminescence 2) and two peaks between 540 and 600 nm (luminescence 3). In luminescence 2, observed in two out of three experiments, the ratio of the peak intensity at 500 nm to that at 480 nm was approx. 1.15, which is quite similar to that of the peaks at 516 and 490 nm, i.e. 1.1, reported to be due to phosphorescence originating from the tyrosine cation radical-solvated electron interaction [10] and comparable to the value for the peaks at 500 and 478 nm, namely 1.25, observed in the horseradish peroxidase-H_2O_2-tyrosine system [8]. Judging from the resolution of 20 nm of our spectrometer, luminescence 2 is considered to be the tyrosine cation-mediated luminescence. The sources of luminescence 1 and 3 are unknown, but are likely to have originated from excited CO_2 [11] and excited tryptophan or protein [12,13].

The present results indicate that ovoperoxidase participates in the natural, luminol-dependent and MCLA-dependent luminescence processes which accompany the fertilization of sea urchin eggs.

REFERENCES

[1] Foerder, C.A. and Shapiro, B.M. (1977) Proc. Natl. Acad. Sci. USA 74, 4214–4218.
[2] Foerder, C.A., Klebanoff, S.J. and Shapiro, B.M. (1978) Proc. Natl. Acad. Sci. USA 75, 3183–3187.
[3] Veron, M., Foerder, C.A., Eddy, E.M. and Shapiro, B.M. (1976) Cell 10, 321–328.
[4] Turner, E., Somers, C.E. and Shapiro, B.M. (1985) J. Biol. Chem. 260, 13163–13171.
[5] Nishida, A., Kimura, H., Nakano, M. and Goto, T. (1989) Clin. Chim. Acta, in press.
[6] Nakano, M., Noguchi, T., Sugioka, K., Fukuyama, H., Sato, M., Shimizu, Y., Tsuji, Y. and Inaba, H. (1975) J. Biol. Chem. 250, 2404–2406.
[7] Nakano, M., Sugioka, K., Ushijima, Y. and Goto, T. (1986) Anal. Biochem. 159, 363–369.
[8] Ushijima, Y., Nakano, M., Takyu, C. and Inaba, H. (1985) Biochem. Biophys. Res. Commun. 128, 936–941.
[9] Takayama, K. and Nakano, M. (1977) Biochemistry 16, 1921–1926.
[10] Vladimilov, Y.A., Roshchupkin, D.I. and Fesenko, E.E. (1970) Photochem. Photobiol. 11, 227–246.
[11] Nakano, M. and Sugioka, K. (1977) Arch. Biochem. Biophys. 181, 371–383.
[12] Ushijima, Y. and Nakano, M. (1980) J. Appl. Biochem. 2, 138–151.
[13] Yoshimoto, T., Yamamoto, S., Sugioka, K., Nakano, M., Takyu, C., Yamagishi, A. and Inaba, H. (1980) J. Biol. Chem. 255, 10199–10204.