Biochemical and Biophysical Research Communications 485 (2017) 41–46

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Immunophotoaffinity labeling of binders of 1-methyladenine, the oocyte maturation-inducing hormone of starfish

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ARTICLE INFO

Article history:
Received 23 January 2017
Accepted 3 February 2017
Available online 4 February 2017

Keywords:
Immunophotoaffinity labeling
1-Methyladenine
Receptor
Oocyte maturation
Starfish

ABSTRACT

Starfish oocytes are arrested at the prophase stage of the first meiotic division in the ovary and resume meiosis by the stimulus of 1-methyladenine (1-MeAde), the oocyte maturation-inducing hormone of starfish. Putative 1-MeAde receptors on the oocyte surface have been suggested, but not yet been biochemically characterized. Immunophotoaffinity labeling, *i.e.,* photoaffinity labeling combined with immunochemical detection, was attempted to detect unknown 1-MeAde binders including putative maturation-inducing hormone receptors in starfish oocytes. When the oocyte crude membrane fraction or its Triton X-100/EDTA extract was incubated with N^6-[6-(5-azido-2-nitrobenzoyl)aminohexyl]carboxamidomethyl-1-methyladenine and then photo-irradiated, followed by western blotting with antibody that was raised against a 1-MeAde hapten, a single band with M_r of 47.5 K was detected. The band was lost when extract was heated at 100 °C. A similar 47.5 K band was detected in the crude membrane fraction of testis as well. Upon labeling with whole cells, this band was detected in immature and maturing oocytes, but only faintly in mature oocytes. As judged from these results, this 1-MeAde binder might be a possible candidate of the starfish maturation-inducing hormone receptors.

1. Introduction

Photoaffinity labeling is a useful method to detect ligand-binding proteins including enzymes and hormonal receptors [1–3]. Although difficulty in radioactive synthesis of photoaffinity probes limits its general application, immunochemical detection of photoaffinity-labeled proteins overcomes this disadvantage [4,5]. To detect the 1-methyladenine (1-MeAde) binders in starfish oocytes including putative maturation-inducing hormone (MIH) receptors, immunophotoaffinity labeling, *i.e.,* photoaffinity labeling combined with immunochemical detection, was attempted.

Fully-grown oocytes in the starfish ovary remain arrested at the prophase stage of the first meiotic division. Reinitiation of meiosis is triggered by 1-MeAde, the oocyte MIH of starfish [6], which is produced and released by the ovarian follicle cells in response to the gonad-stimulating substance, a peptide hormone excreted from the radial nerve [6]. It was reported that the gonad-stimulating substance is closely related to the vertebrate relaxin [7].

Upon exposure of oocytes to 1-MeAde, the maturation-promoting factor (MPF) is activated in the cytoplasm and induces oocyte maturation [8]. An MPF was identified as the complex of Cdk1 kinase with cyclin B [9,10]. The involvements of pertussis toxin-sensitive G-protein, phosphoinositide-3-kinase, phosphoinositide-dependent kinase (PDK) 1, Akt (protein kinase B), and Cdc25 phosphatase in starfish or other animal oocyte maturation were suggested [11–16]. Putative 1-MeAde receptors have

Abbreviations: Analog I, N^6-(6-aminohexyl)carboxamidomethyl-1-methyladenine; ASW, modified van't Hoff's artificial seawater; CaFASW, Ca^{2+}-free artificial seawater; CBB, Coomassie Brilliant Blue R-250; EC_{50}, 50%-effective concentration; EGTA, *O,O'*-bis(2-aminoethyl)ethyleneglycol-*N,N,N',N'*-tetraacetic acid; GVBD, germinal-vesicle breakdown; HB, homogenizing buffer or 20 mM HEPES buffer (pH 7.4) containing 5 mM EGTA and 5 mM $MgCl_2$; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid; KLH, keyhole limpet hemocyanin; 1-MeAde, 1-methyladenine; MIH, maturation-inducing hormone; MPF, maturation-promoting factor; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; Reagent I, N^6-[6-(5-azido-2-nitrobenzoyl)aminohexyl]carboxamidomethyl-1-methyladenine; Reagent II, N^6-[6-(3-azidobenzoyl)aminohexyl]carboxamidomethyl-1-methyladenine; Reagent III, 8-azido-1-methyladenine; SDS, sodium dodecyl sulfate; Solvent A, water-saturated 2-butanol; Solvent B, water-saturated 2-butanol containing 1% acetic acid; TLC, thin-layer chromatography.

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http://dx.doi.org/10.1016/j.bbrc.2017.02.009
0006-291X/

not yet been characterized biochemically, although the specific binding of 1-MeAde to the isolated cortices of starfish oocytes was reported independently by Yoshikuni et al. [17,18], Tadenuma et al. [19], and by ourselves [20]. Characterization of the receptors would help us to understand the entire signal transduction pathway from 1-MeAde to MPF in starfish oocytes. For this purpose, labeling of 1-MeAde binders is a necessary step.

In this study, we designed and synthesized nitrene-forming photoaffinity labeling reagents for 1-MeAde binders (Fig. 1A) and used them for labeling the binders in starfish oocytes by a photoaffinity labeling technique. We raised rabbit anti-1-MeAde antibody using a 1-MeAde derivative as hapten and used it for the detection of the photoaffinity-labeled proteins by western blotting. The principle of this labeling method is shown in Fig. 1B. Evidence for the presence of a similar 1-MeAde binder in oocytes and in testis as well as for heat stability and down-regulation of the binder are also provided here.

2. Materials and methods

2.1. Synthesis of Analog I

N^6-Carboxymethyladenosine (0.55 g) [20] and 2.5 g of hexamethylenediamine were allowed to react at room temperature for 2 h in 40 mL of water at pH 4–5 with gentle stirring in the presence of 0.4 g of water-soluble carbodiimide or 1-(3-dimethyaminopropyl)-3-ethylcarbodiimide. The carbodiimide was then added six times, 0.1 g each time. Four days later, the reaction was terminated by adding 80 mL of water, and the supernatant was loaded onto a florisil column (bed volume, 103 mL). After washing with water, N^6-(6-aminohexyl)carboxamidomethyladenosine was eluted with 80%(v/v) acetone containing 1%(v/v) acetic acid. Its purity was established by thin-layer chromatography (TLC) on silica gel. R_f in water-saturated 2-butanol (Solvent A), 0.11.

To 0.5 g of this compound in 15 mL of 50%(v/v) 1,4-dioxane were added 0.25 g of 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile and 0.18 mL of triethylamine. The same amounts of the nitrile and amine were added twice on days 2 and 4. Six days later, the mixture was evaporated to dryness under reduced pressure, washed with ether, and then extracted with water for N^6-(6-*tert*-butoxycarbonylaminohexyl)carboxamidomethyladenosine. The product was purified by silica gel column chromatography using Solvent A. R_f on silica gel TLC in Solvent A, 0.62.

This compound was methylated with CH_3I, essentially as described previously [20]. R_f of N^6-(6-*tert*-butoxycarbonylaminohexyl)carboxamidomethyl-1-methyladenosine on silica gel TLC in Solvent A, 0.32. The product was hydrolyzed to Analog I by heating at 95 °C for 45 min in 0.5 N HCl in a sealed glass tube. After neutralization, Analog I was purified by HPLC on Cosmosil C_{18} using 1% acetic acid (retention time, 4.6 min at a flow rate of 2 mL/min). Its purity was established by silica gel TLC in Solvent A (R_f, 0.03). ^{1}H NMR (D_2O): δ 1.08 (m,2H), 1.15 (m,2H), 1.31 (m,2H), 1.43 (m,2H), 2.79 (t,2H), 3.04 (t,2H), 3.80 (s,3H), 4.68 (s,2H), 8.12 (s,1H), 8.37 ppm (s,1H). UV: λ_{max} in nm 269 at pH 1, 273 at pH 7, and 275 at pH 13.

2.2. Synthesis of Reagents I and II

To N^6-(6-aminohexyl)carboxamidomethyladenosine (11 mg) and 23 mg of *N*-5-azido-2-nitrobenzoyloxysuccinimide were allowed to react at room temperature for 50 h in 5 mL of *N,N*-dimethylacetamide in the dark with gentle stirring. CH_3I (0.4 mL) was then added, and the mixture was kept at room temperature for 47 h in the dark. N^6-[6-(5-azido-2-nitrobenzoyl)aminohexyl]carboxamidomethyl-1-methyladenosine obtained was hydrolyzed by

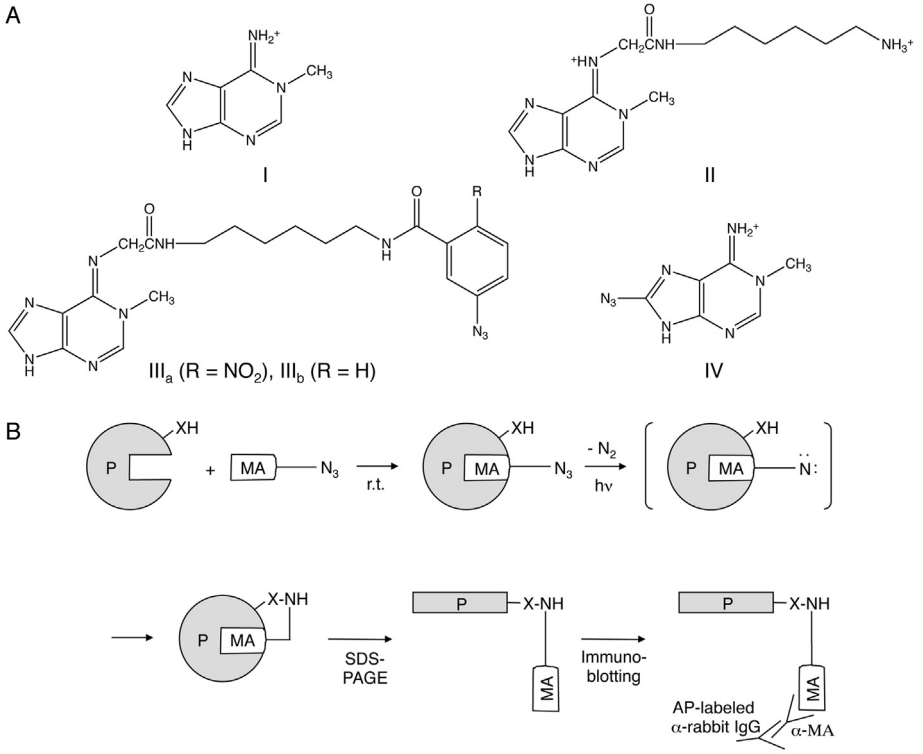

Fig. 1. The 1-MeAde derivatives used in this study (A) and the principle of immunophotoaffinity labeling of 1-MeAde binders in starfish oocytes (B). (A) I, 1-MeAde; II, Analog I; III_a, Reagent I; III_b, Reagent II; IV, Reagent III. (B) MA, 1-methyladenine; MA-N_3, photoaffinity labeling reagent containing an azide group; P, 1-MeAde binders including putative 1-MeAde receptors; -XH, generic functional group reactive with a nitrene; α-MA, anti-1-MeAde antibody; AP, alkaline phosphatase.