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

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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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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

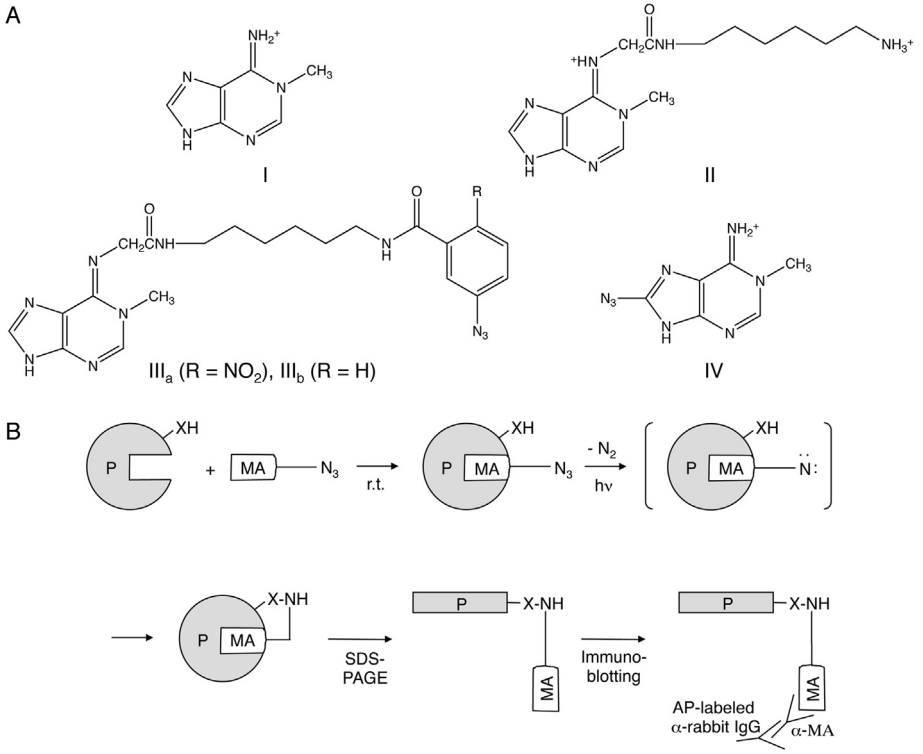

Fig. 1. The 1-MeAde derivatives used in this study (A) and the principle of immunophotoaffinity labeling of 1-MeAde binders in starfish oocytes (B). (A) I, 1-MeAde; II, Analog I; III_a, Reagent I; III_b, Reagent II; IV, Reagent III. (B) MA, 1-methyladenine; MA-N_3, photoaffinity labeling reagent containing 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.

heating at 95 °C for 30 min in 0.5 N HCl in a sealed tube. After neutralization, Reagent I was purified by first TLC on silica gel 60 F_{254} (20 × 20 cm) using Solvent A, second TLC on the same adsorbent using Solvent A, and third TLC on the same adsorbent using water-saturated 2-butanol containing 1% acetic acid (Solvent B). A photosensitive band with R_f of 0.24–0.27 in Solvent A and 0.23 in Solvent B was collected. λ_{max} in water: 317 nm and ~270 nm (shoulder).

Reagent II was synthesized by the same method, except that *N*-3-azidobenzoyloxysuccinimide was used instead of *N*-5-azido-2-nitrobenzoyloxysuccinimide.

2.3. Synthesis of Reagent III

8-Bromoadenosine (100 mg) was heated at 75 °C for 6 h with 33 mg of NaN_3 in 10 mL of dimethylformamide. After dilution with water, the mixture was applied on to a Dowex 50 (H^+) column. After washing the column successively with water and 30% ethanol, 8-azidoadenosine was eluted with 1 N NH_4OH in 30% ethanol. R_f on silica gel TLC in Solvent B, 0.69. UV: λ_{max} in nm 284 at pH 1 and 285 at pH 7 and 13. 8-azidoadenosine (40 mg) was methylated with CH_3I, essentially as described previously [20]. The mixture was diluted with water and applied on to a carboxymethyl-cellulose (H^+) column. The column was washed successively with 190 mL of water and 190 mL of 30% ethanol, and 8-azido-1-methyladenosine was eluted with 1 N NH_4OH in 30% ethanol. R_f on silica gel TLC in Solvent B, 0.13. UV: λ_{max} in nm 285 at pH 1 and 7 and 286 at pH 13. 8-azido-1-methyladenosine was hydrolyzed by heating at 75 °C for 3 h in 3.6 mL of 0.5 N HCl. After neutralization, Reagent III was purified by HPLC on Cosmosil C_{18} using 2.5% methanol containing 1% acetic acid (retention time, 16.8 min at a flow rate of 2 mL/min). R_f on silica gel TLC in Solvent B, 0.30. ^1H NMR (D_2O): δ 3.17 (s,3H), 7.59 ppm (s,1H). UV: λ_{max} in nm 287 at pH 1, 293 at pH 7, and 294 at pH 13.

2.4. Analytical procedures, SDS-PAGE, and protein staining

The concentrations of 1-MeAde derivatives were determined by measuring the absorbance at 270 nm [20]. UV–visible spectra were taken on a Millton Roy Spectronic 3000 Array recording spectrophotometer. TLC was performed on Merck silica gel 60 F_{254} precoated glass plates using Solvent A (water-saturated 2-butanol) or Solvent B (water-saturated 2-butanol containing 1% acetic acid). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was carried out as described by Laemmli [21]. Protein was stained with Coomassie Brilliant Blue R-250 (CBB). Molecular weight markers used were SDS-7 or SDS-6H (Sigma).

2.5. Animals, preparation of follicle-free oocytes, and measurement of hormonal activity

The starfish *Asterina pectinifera* (*renamed Patiria pectinifera*) collected during the breeding season near Hashirimizu, Asamushi and Ushimado, Japan were kept at 14 °C in laboratory aquaria supplied with circulating artificial seawater (products from Senju Pharmaceutical Co., Ltd., Kobe, and Rohto Pharmaceutical Co., Ltd., Osaka, Japan). *A. pectinifera* immature oocytes without follicle cells were obtained from dissected ovaries by the transfer from Ca^{2+}-free artificial seawater (CaFASW) [22] to modified van't Hoff's artificial seawater (ASW) [23], washed three times with CaFASW, and kept in ASW. The hormonal activity of 1-MeAde derivatives was estimated from germinal-vesicle-breakdown (GVBD)-inducing activity toward immature oocytes and expressed in % GVBD [20,23]. A 50%-effective concentration (EC_{50}) is defined as an agonist concentration for inducing 50% GVBD.

2.6. Preparation of oocyte crude membrane fraction and its Triton X-100/EDTA extract

Dejellied oocytes prepared from immature oocytes by washing three times with CaFASW containing 2 mM EGTA (pH 6.5) were packed by centrifugation at 120 × g for 30 s. After the addition of 2 packed volumes of homogenizing buffer (HB) containing 1 mM phenylmethanesulfonyl fluoride (PMSF), oocytes were homogenized using a Teflon homogenizer (5 strokes). The homogenates were centrifuged at 17,000 × g for 15–30 min to remove the supernatant. Ten precipitant volumes of HB containing 1 mM PMSF were then added to the precipitate, and the suspension was centrifuged again. This washing procedure was repeated three times. The precipitate was suspended in a small amount of HB containing 1 mM PMSF and stored −80 °C as the oocyte crude membrane fraction.

Oocyte crude membranes were re-suspended in an appropriate amount of 5–10 mM potassium phosphate buffer (pH 8.0) containing 1–2% Triton X-100, 1–5 mM EDTA, and 1 mM PMSF. After intermittent sonication for 2.5 min and shaking at 4 °C for 15 min, the suspension was centrifuged at 100,000 × g for 1 h. This supernatant was used as the Triton X-100/EDTA extract.

2.7. Preparation and affinity purification of antibody against a 1-MeAde hapten

A 1-MeAde hapten was prepared by coupling of Analog I (5 mg) with keyhole limpet hemocyanin (KLH) (7.7 mg) in 1.9 mL of 0.1 M $NaHCO_3$ in the presence of 2.8 mg of dimethyladipimidate·2HCl. The mixture of 0.4 mL of the solution containing 1-MeAde-KLH, 0.5 mL of saline, and 1.1 mL of Freund complete adjuvant was injected to the back of female adult rabbits. The mixture of 0.2 mL of the 1-MeAde-KLH solution, 0.7 mL of saline, and 1.1 mL of Freund incomplete adjuvant was used for the booster injections on days 21 and 32 after the first immunization. Whole blood was collected seven 7 days later, and sera were obtained by a centrifuge.

Anti-KLH antibody was precipitated from the antiserum (3 mL) by shaking at 4 °C for 2.5 d with 18 mg of KLH in 1.5 mL of Tris-buffered saline. Anti-1-MeAde antibody was then purified by affinity purification using a 1-MeAde-Sepharose 4B column (bed volume, 3 mL). The affinity-purified antibody was passed three times through a KLH-Sepharose 4B column (bed volume, 3 mL).

2.8. Immunophotoaffinity labeling of 1-MeAde binders

Reagent I was added to 10 μL of a crude membrane suspension or its Triton X-100/EDTA extract at a concentration of 30–35 μM in the dark. After 30 min at room temperature, the mixture was photo-irradiated at 0 °C for 2–5 min with a 250-W tungsten light bulb from the distance of 20 cm. After heating at 100 °C for 5 min in the Laemmli's sample buffer, the mixtures were subjected to SDS-PAGE. The photoaffinity-labeled proteins transferred to a PVDF membrane were visualized by western blotting using rabbit anti-1-MeAde antibody as a primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (EY Laboratories, Inc., San Mateo, CA) as a secondary antibody.

3. Results and discussion

3.1. Design and synthesis of photoaffinity labeling reagents

The structure-activity and the structure-affinity relationships of 1-MeAde derivatives reported so far [20,24–27] indicate the strict specificity of putative 1-MeAde receptors for a relatively small N-1 substituent, whereas bulkiness and polarity of the N^6-substituents

do not so markedly affect the MIH activity. 8-MeNH-1-MeAde and 8-Me_2N-1-MeAde serve as potent antagonists [27]. From these studies, we designed and synthesized three photoaffinity labeling reagents (Reagents I - III) for 1-MeAde binders including putative receptors for 1-MeAde. Upon photo-irradiation, these aryl azide derivatives of 1-MeAde would generate the super-active species nitrenes that are expected to react with nearby functional groups of the proteins.

3.2. Photolysis of Reagents I - III

Reagent I underwent rapid photolysis upon irradiation of visible light. Fig. 2A shows its spectral changes upon photo-irradiation in an ice-water bath with a tungsten light bulb. The absorption peak at 317 nm due to its azidonitrobenzoyl group decreased, and new shoulders at ~394 and ~262 nm increased with time of irradiation. Isosbestic points were observed at 368, ~285, and ~250 nm, indicating that Reagent I was photolyzed directly to another compound without intermediates. The photolytic decomposition followed the first-order reaction kinetics with a half-life of 2.5 min under the conditions (Fig. 2B). Upon prolonged irradiation to ~2 h, the plot deviated from a straight line, probably due to further decomposition of the photolytic product. Reagents II and III also underwent rapid photolysis following the first-order kinetics upon irradiation of UV light (data not shown). With Reagent II, the absorption peak at 266 nm decreased and a new broad shoulder ~320 nm increased. Isosbestic points were observed at 303 and ~244 nm. With Reagent III, the absorption peak at 290 nm decreased, and isosbestic points were observed at 265 and ~247 nm. All of these results offer evidence for the presence of a photosensitive azide group in Reagents I - III.

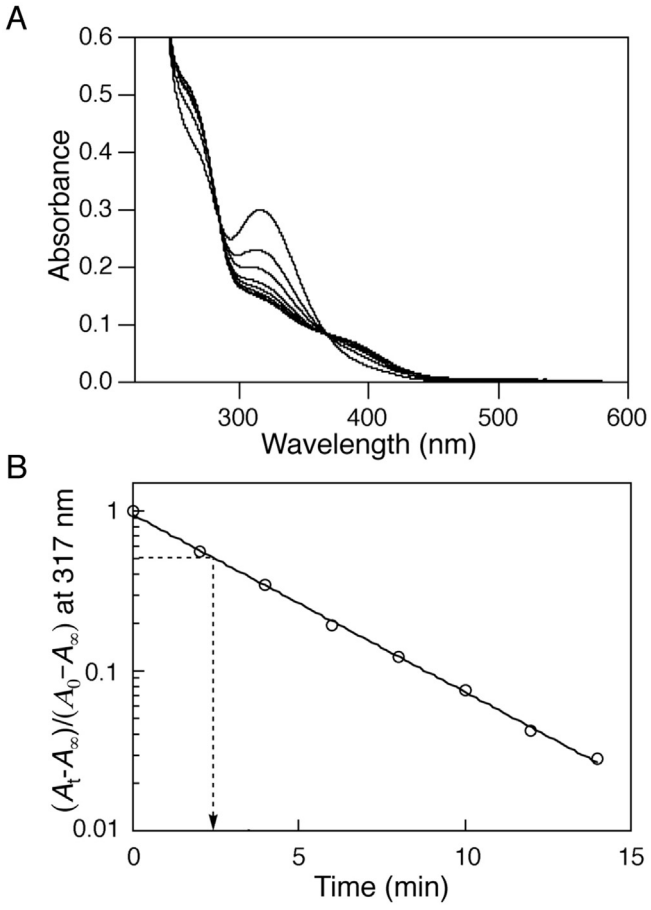

Fig. 2. Photolysis of Reagent I. Aqueous solution of Reagent I (35 μM) in a quartz cuvette was photo-irradiated in an ice-water bath with a 250-W tungsten light bulb from a distance of 20 cm, and UV–visible spectra were taken every 2 min (A) UV–visible spectra (0, 2, 4, 6, 8, 10, 12, 14, and 16 min from top to bottom at 317 nm. (B) Semi-logarithmic plot of the absorbance at 317 nm against time of photo-irradiation.

3.3. GVBD-inducing activity of Reagents I-III toward starfish oocytes

Reagents I-III were tested for GVBD-inducing activity toward *A. pectinifera* oocytes. Since EC_{50} varies significantly depending upon starfish individuals, one of the typical results is shown in Table 1.

All the three reagents served as agonists in the induction of GVBD, although their EC_{50} values were much higher than that for 1-MeAde. Since the starfish oocytes that undergo GVBD by 1-MeAde [23] or its analogs [20] extrude two polar bodies [23] and are fertilizable upon insemination (elevation of fertilization membrane and cleavage) [20], the GVBD-inducing activity may be considered as a measure of MIH activity. It was thus concluded that all of these reagents interact with 1-MeAde receptors and function as MIH agonists. It is intriguing that photolyzed Reagents I - III also served as agonists with EC_{50} values rather lower than those before photolysis (data not shown), although photolytic products from them have not yet been identified.

3.4. Immunophotoaffinity labeling of 1-MeAde binders with membrane and soluble fractions of oocytes and testis

Crude membrane and soluble fractions of oocytes and testis were subjected to immunophotoaffinity labeling with Reagent I. As shown in Fig. 3A(b), a single band with M_r of 47.5 K was detected with the oocyte crude membrane fraction, but not with the soluble fraction. This might suggest that the 1-MeAde binder is located on the oocyte membrane. When tested with testicular fractions, a similar band with M_r of 47.5 K was detected with the membrane fraction, but not with the soluble fraction. This is suggestive of the presence of a similar 1-MeAde binder in testis. Male starfish sheds gamete upon injection of MIH that was identified as 1-MeAde [6] and induces spawning of sperm when added to a piece of testicular tissues [23,28]. It seems reasonable to assume that female and male starfish have similar, membrane-bound 1-MeAde receptors in oocytes and testis, respectively.

We synthesized *N*-(5-azido-2-nitrobenzoyl)ethanolamine that is related to Reagent I but lacks the 1-MeAde moiety. This reagent and anti-1-MeAde antibody did not constitute an effective labeling system for the 47.5 K band in the oocyte crude membrane fraction (data not shown). It is therefore evident that the 1-MeAde moiety is required for the immunophotoaffinity labeling of the 47.5 K band.

Other azide group-containing compounds, Reagents II and III, were also examined for the effectiveness as a photoaffinity labeling reagent for 1-MeAde binders using the Triton X-100 extract of oocyte crude membranes. When these reagents were used instead

Table 1
GVBD-inducing activity of Reagents I-III.

Run no.	Compound	EC_{50} (μM)
1	1-MeAde	0.09
	Reagent I	14
2	1-MeAde	0.08
	Reagent II	80
3	1-MeAde	0.04
	Reagent III	1.7

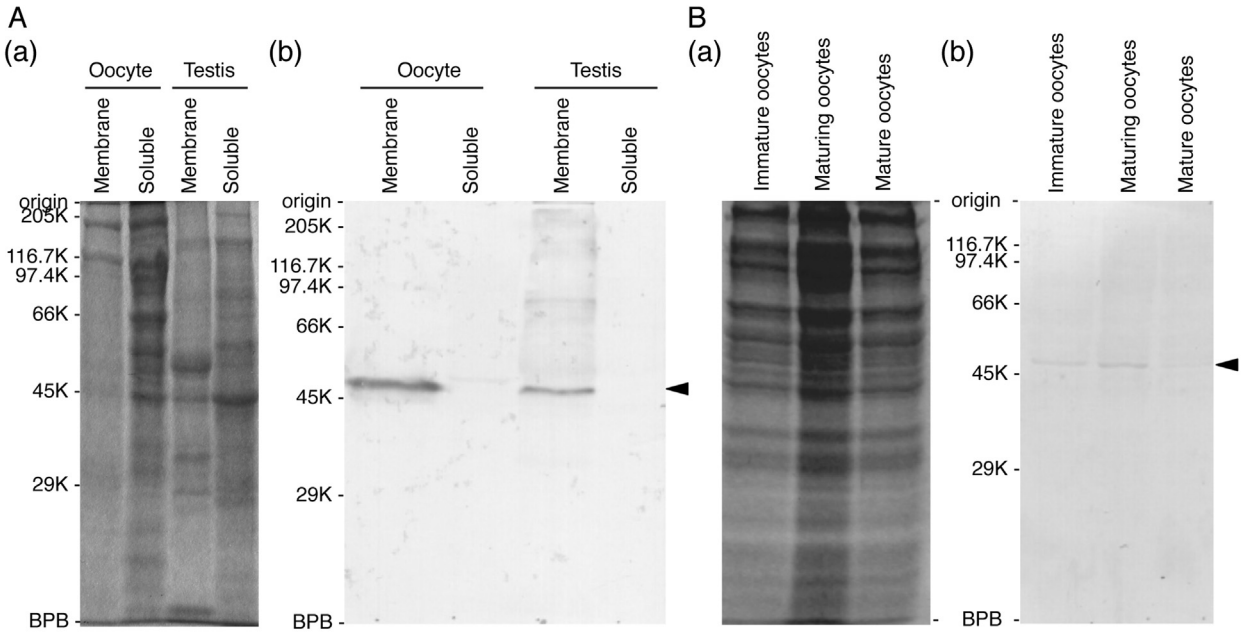

Fig. 3. Immunophotoaffinity labeling of 1-MeAde binders with membrane and soluble fractions of oocytes and testis (A) and with whole oocyte cells (B). (A, B) Appropriate amounts of soluble and precipitant fractions of oocytes and testis (A) or oocytes (ca.100 cells) untreated (immature oocytes) or treated at room temperature with 10 μM 1-MeAde for 20 min (maturing oocytes) or 60 min (mature oocytes) (B) were incubated at room temperature for 25–30 min with 30–35 μM Reagent I in 12 μL of HB. The mixtures were then photoirradiated in an ice-water bath for 5 min (A) or 2 min (B) with a 250-W tungsten light bulb from a distance of 20 cm. After heating at 100 °C for 5 min in the Laemmli's sample buffer, the mixtures were subjected to SDS-PAGE on 7.5% gel. Photoaffinity-labeled 1-MeAde binders were detected by western blotting, as described in the text. (a) Protein staining with CBB. (b) Western blotting. *Arrow-heads* indicate the position of the 47.5 K binder.

of Reagent I and UV light was irradiated for 0.5–1 min instead of visible light, the 47.5 K 1-MeAde binder was not detected with Reagent III and detected only faintly with Reagent II (data not shown). Ineffectiveness of Reagent III might be due to the absence of an appropriate functional group in the 1-MeAde binder near the super-active nitrene species formed from its azide group on C-8.

3.5. Immunophotoaffinity labeling of 1-MeAde binders with whole oocyte cells

Since the specific binding of 1-MeAde to the isolated cortices suggested the location of the putative 1-MeAde receptors on the surface of oocytes [17–20], immunophotoaffinity labeling was performed with whole cells of immature, maturing, and mature oocytes. When immature and maturing oocytes were photoaffinity-labeled with Reagent I, followed by western blotting with anti-1-MeAde antibody, a single band with M_r of 47.5 K was detected (Fig. 3B(b)). In contrast, this band was very faint with mature oocytes, suggesting the internalization of the 1-MeAde binder upon oocyte maturation. This might be as expected if this 1-MeAde binder is a cell-surface receptor for MIH and undergoes down-regulation in the hormonal signal transduction.

3.6. Extraction and some properties of the 47.5 K 1-MeAde binder

Extraction of the 47.5 K 1-MeAde binder from the oocyte membrane fraction was attempted using a Triton X-100 with or without EDTA. As shown in Fig. 4B, the addition of 1 mM EDTA to 1% Triton X-100 markedly increased the efficiency of extraction, although the significant amount of 47.5 K 1-MeAde binder still remained in the residual precipitate.

When the Triton/EDTA extract was subjected to immunophotoaffinity labeling after heat treatment at 100 °C for 10 min, the band with M_r of 47.5 K was no more detected (Fig. 4C). This result suggests that the 47.5 K 1-MeAde binder is proteinaceous.

Upon the preliminary incubation of the Triton X-100/EDTA extract with 1-MeAde or Analog I, the intensity of the photoaffinity label of the 47.5 K 1-MeAde binder was much lowered by Analog I,

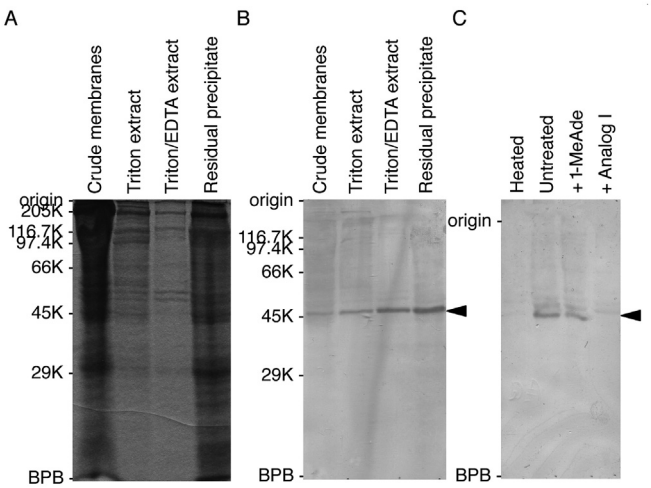

Fig. 4. Extraction from oocyte membrane fractions and heat stability of the 1-MeAde binders (A,B) and inhibition of photoaffinity labeling by Analog I (C). Crude membrane fraction (1 mL) washed with HB was extracted by sonication for 2.5 min successively with 1% Triton X-100 and 1% Triton X-100 containing 1 mM EDTA, 1 mL each. Each extract was separated by ultracentrifugation at 100,000×*g*, and the residual precipitate was suspended in the same volume of HB. They were subjected to SDS-PAGE, protein staining with CBB (A), and immunophotoaffinity labeling (B), as described in the text. The Triton X-100/EDTA extract heated at 100 °C for 10 min was analyzed by immunophotoaffinity labeling (C). Immunophotoaffinity labeling with the Triton X-100/EDTA extract was also carried out after pre-incubation with 1 mM 1-MeAde or Analog I at room temperature for 30 min (C). *Arrow-heads* indicate the position of the 47.5 K binder.

but almost not by 1-MeAde (Fig. 4C). This might suggest that Analog I is bound to the binder in more irreversible manner than 1-MeAde itself.

In summary, we designed and synthesized photoaffinity labeling reagents for 1-MeAde binders of starfish. For immunochemical detection of photoaffinity-labeled 1-MeAde binders, rabbit anti-1-MeAde antibody was raised. A 47.5 K 1-MeAde binder was detected both in oocytes and testis crude membrane fractions by immunophotoaffinity labeling. This heat-labile binder was labeled with whole oocytes cells as well and underwent down regulation after maturation. The binder might be a possible candidate of MIH receptors of starfish oocytes, a possibility that should be tested in the near future.

Conflict of interest

The authors declare no conflicts of interest.

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