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Benzothiazolylphenol–Substituted Ketoester is a Useful Fluorescent Probe for Detection of the Mitochondrion in Sea Urchin Sperm

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Abstract: One of the ketoesters derived from benzothiazolylphenol-substituted dioxetane, benzothiazolylphenol-substituted ketoester (TPKE), demonstrates fluorescence in a 0.1 M NaOH [1)]. In this study, the fluorescent staining of a living cell with TPKE was demonstrated by fluorescence microscopy. When sperm from two species of sea urchins—*Pseudocentrotus depressus* and *Anthocidaris crassispina*—were used as biological materials, TPKE showed a fluorescent signal in the midpiece that was composed of a single mitochondrion. The ratio of fluorescent signal intensity to background noise (S/N) was high in the sperm stained with 1.0–5.0 μg/ml TPKE in normal artificial seawater (pH 8.0). The S/N ratio decreased in acidic seawater (pH 6.0); acidic conditions repress respiratory activity in sea urchin sperm. Moreover, in the presence of the respiratory chain inhibitor antimycin A and the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone, the sperm showed faint or no fluorescence in normal artificial seawater (pH 8.0). Sea urchin sperm stained with TPKE after fixation showed faint or no fluorescence. These results suggest that TPKE is a potential fluorescent probe of living sea urchin sperm mitochondria with high respiratory activities.

Keywords: sea urchin sperm, mitochondrion, fluorescence microscopy, dioxetane, ketoester

Introduction

Chemiluminescent and fluorescent substrates are powerful tools in many assays and other applications in the modern fields of biology and medicine. Examples of high-energy chemiluminescent molecules with applications in high-sensitivity biochemical and biomedical analyses are 1,2-dioxetanes [1, 2)]. Dioxetanes have good thermal stability; however, they decompose exclusively into the corresponding ketoesters on prolonged heating in hot xylene. A typical dioxetane, namely, benzothiazolyl-phenol-substituted dioxetane (TPOX), undergoes base-induced chemiluminescent decomposition to the corresponding ketoester TPKE; this ketoester demonstrates fluorescence in a 0.1 M NaOH solution [1)]. The characteristics of the ketoester make it a candidate fluorescent probe; however, there are no reports of the application of this ketoester in cell staining and other biological assays.

The use of this ketoester in staining and/or detection conditions would require basic conditions. To examine whether this ketoester could be used as a fluorescent probe to stain cells, base-resistant biological materials are needed. We selected the sea urchin sperm as the base-resistant cell. Sea urchin generally releases sperm into sea water, which has a pH value of 7.8–8.2. The pH of artificial sea water (ASW) is generally adjusted to 8.0 in the experiments of development and fertilization in sea urchin [3)]. The intracellular pH of the sea urchin sperm increases at the initiation of the flagellar motion brought about by the Na^+/H^+ exchange across the plasma membrane [4)].

Another advantage of sea urchin sperm is their simple structure; they are classified as primitive sperm with a single torus-shaped mitochondrion surrounding the flagellum at the base of the nucleus [5,6]. Such a structure would permit easy recognition of the region that stains with fluorescent substrates under an optical microscope. These properties of sea urchin sperm make them suitable for experiments on staining with dioxetanes and their corresponding ketoesters.

The aim of the present study is to prove the potential of dioxetane-derived ketoesters as probes for fluorescence microscopy. We examined whether sea urchin sperm stained with TPKE and three other dioxetane chemiluminescent substrates, namely, TPOX, adamantyl-derivative TPOX (TPOX -Ad), and benzothiazolylresorcinol-substituted dioxetanes (TROX) (Fig. 1). We report cell staining with these substrates and the physiological conditions in which the detection of fluorescent or chemiluminescent substrates in sea urchin sperm is feasible.

Materials and Methods

Chemicals

Three dioxetanes—TPOX, TPOX-Ad, and TROX—and TPKE a ketoester-derived TPOX (Fig. 1), were prepared as described by Matsumoto *et al* [1]. These compounds were dissolved in dimethyl sulfoxide (DMSO; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and stored at 4°C. Other fluorescent substrates 4′,6-diamidino-2-phenylindole (DAPI; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and MitoTracker Green FM (M7514; Invitrogen Co., Carlsbad, CA, U.S.A.) were dissolved in DMSO to produce 50 µg/ml and 0.1mM stock solutions, respectively. The stock solution of DAPI was stored at 4°C and that of MitoTracker Green FM was stored at –20°C.

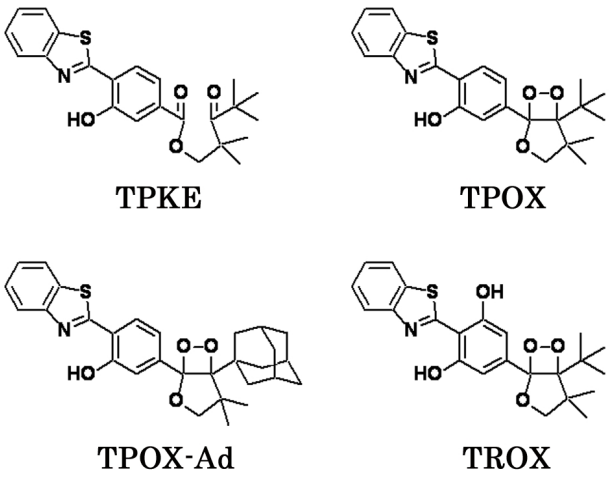

Fig. 1. Schemes of TPKE and three dioxetanes.

ASW composed of 420 mM NaCl, 9.0 mM KCl, 10 mM $CaCl_2$, 24.5 mM $MgCl_2$, 25.5 mM $MgSO_4$, 2.15 mM $NaHCO_3$, and 10 mM HEPES (N-2-hydroxylpiperazine-N′-2-ethanesulfonic acid; Dojindo Laboratory, Kumamoto, Japan). The pH was adjusted to 6.0–9.0 by addition of HCl or NaOH. All the reagents used in ASW preparation were purchased from Wako Pure Chemical Industries, Ltd. Paraformaldehyde was purchased from Merck & Co., Inc. (Whitehouse Station, NJ, U.S.A.). The respiratory inhibitor antimycin A (AMA) and the respiratory uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) were purchased from SIGMA-ALDRICH Co. (St. Louis, MO, U.S.A.). These were stored as 20 mg/ml and 15mM stock solutions in DMSO, respectively, at –20°C.

Biological materials

Two species of sea urchins, *Pseudocentrotus depressus* and *Anthocidaris crassispina,* were used for the experiment during their breeding seasons. *A. crassispina* was collected from Sagami Bay, Kanagawa, Japan. *P. depressus* was purchased from Misaki Marine Biological Station, Tokyo University. Sea urchin gametes were obtained by injecting 0.5 M KCl into the coelom to induce spawning. The sperm samples were subsequently stored on ice as "dry sperm" (approximately 20×10^9 sperm/ml) until use.

The staining of sperm was initiated with a 1000-fold dilution of dry sperm into ASW containing with a fluorescent substrates. The treatment of sperm was performed at staining process with the presence of 100 µg/ml antimycin A (AMA), 75 µM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), or 0.5% dimethyl sulfoxide (DMSO). The staining and treating process was terminated after 30 min. After centrifugation (1,000 g, for 5 min at 4°C), the suspensions were substituted twice with fresh

ASW.

Image analyses

The sperm were observed using a fluorescence microscope system (BX 51 equipped with a 100 W Hg lamp, and mirror units of U-MWU2 and U-MWIB3; Olympus Corporation, Tokyo, Japan). After staining with TPKE and three dioxetanes, fluorescent images were obtained with an exposure time of 10 sec and bright field images, with an exposure time of 1/5.5 s, by using a Penguin 150CL camera (Pixera Co., San Jose, CA, U.S.A.) connected to the fluorescence microscope system. Fluorescent and bright-field images were acquired with the same field of view.

All images were imported into, resized, and trimmed using the Adobe PhotoShop 7.0 software (Adobe Systems, Mountain View, CA, USA). All fluorescent images were converted into 8-bit gray-scale images for image analyses. The average pixel intensities of selected fluorescent regions were measured by using the Adobe PhotoShop 7.0 software. The fluorescent intensities were determined by the average of S/N values, i.e., ratio of the average signal intensity of the fluorescent region to that of the same square area of absence sperm regions as background noise.

The results were expressed as mean ± (standard deviation) S.D. of the S/N value from ten or more sperm on three or more images acquired independently under the same experimental conditions. Statistical analyses were performed using a Student's t test (two-tailed, unpaired). A p value less than 0.01 was considered to be statistically significant.

Results

Sperm morphology and TPKE staining

The midpiece of the sea urchin sperm in normal ASW (pH 8.0) is closely pressed against the sperm head, and the flagellum extends directly back from the head through the midpiece [5, 6].

Sperm of the sea urchin *P. depressus* were suspended without fixation and washing in ASW (pH 8.0) containing 10 μM TPOX.

After fixation with 1% paraformaldehyde in ASW, the sperm of *P. depressus*, were stained with DAPI and MitoTracker Green (Fig.2). DAPI and MitoTracker Green are commercial fluorescent substrates that stain nuclei and mitochondria, respectively. The blue color emitted by DAPI on UV excitation was identified to originate from a sperm head with a conical or triangular shape (Fig.2 B). The region from which green color was emitted on excitation with blue light was observed to be elliptical or round (Fig.2 C).

The shape and location of the region of green fluorescence indicated that the region stained by MitoTracker Green was the midpiece. Merged images confirmed these observations (Fig.2 D); the locations of the nucleus and mitochondrion of the sea urchin sperm are consistent with those reported in previous studies [5, 6].

Sperm after fixation with 1% paraformaldehyde in ASW showed no fluorescence and chemiluminescence with 10 μg/ml TPKE in ASW. However, the sperm without paraformaldehyde was allowed to observe the fluorescent before substitution fresh ASW as shown in Fig. 3. The whole sperm was stained; however, no specific staining with TPKE was observed. To obtain a clearer image, fluorescence was determined under different staining conditions such as different pH values of ASW and different TPKE concentration. Additionally, washing as substitution of fresh ASW was performed after the staining.

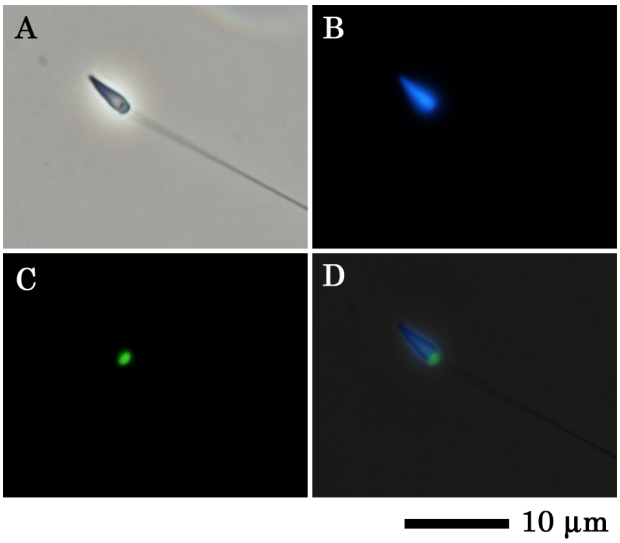

Fig. 2. Fluorescent images of the sperm of *P. depressus*. After fixation, sea urchin sperm were stained with DAPI and MitoTracker Green. The images were captured with a UV (B) or a blue (C) excitation filter. The bright fluorescence is shown in (A). The image (D) is obtained by merging images (A), (B), and (C).

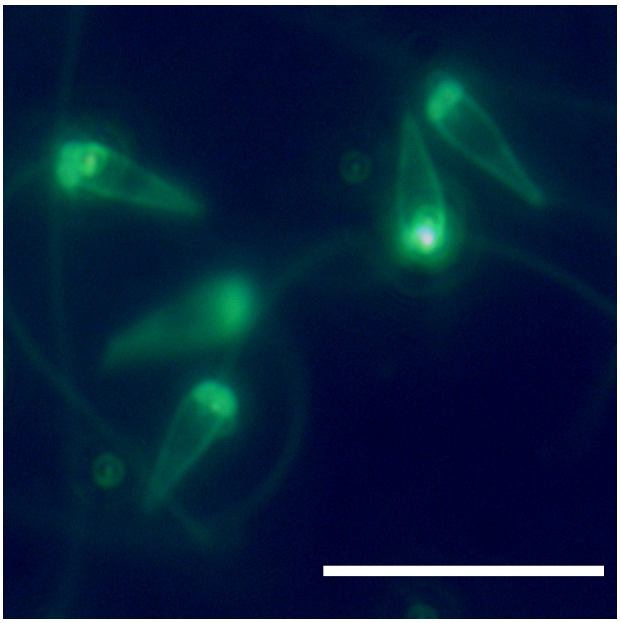

Fig. 3. Fluorescent images of sea urchin sperm stained with TPKE. Sperm of the sea urchin *P. depressus* were suspended without fixation and washing in ASW (pH 8.0) containing 10 μM TPOX. The bar represents 10 μm.

Staining process under different pH value

To compare the degrees of fluorescence of sperm when different pH values of ASW were used (pH 6.0, 7.0, 8.0 and 9.0), *P. depressus* sperm were suspended without fixation in each of the ASW containing 5.0 μg/ml TPKE. Sperm images were acquired after termination of the staining process. Typical images are shown in Fig. 4. In ASW with pH 6.0 and 7.0, sea urchin sperm showed faint fluorescent signals from the midpiece. Strong fluorescence was observed in the sperm suspension in ASW of pH 8.0. Fluorescence was detected only in the midpiece.
Occasionally, other regions demonstrated faint fluorescence.

The image analyses are shown in Fig. 5. The ratio of the signal intensities of the fluorescent regions (S/N; midpiece region/background) in ASW of pH 8.0 was significantly ($p < 0.01$) higher than in ASW of pH 6.0 and 7.0. However, the S/N values in ASW with pH 9.0 and pH 8.0 were the same; therefore, there was no significance ($p = 0.085$). It was suggested that the sea urchin sperms in basic ASW (pH 8.0 – 9.0) demonstrated strong fluorescent signals.

TPKE concentration

To determine TPKE concentration to stain *P. depressus* sperm in ASW (pH 8.0), the staining performed with various concentrations of TPKE ranging from 0–10 μg/ml (Fig. 6 and 7). Before acquiring the images, we washed the sea urchin sperm twice with fresh ASW (pH 8.0) after the staining process.

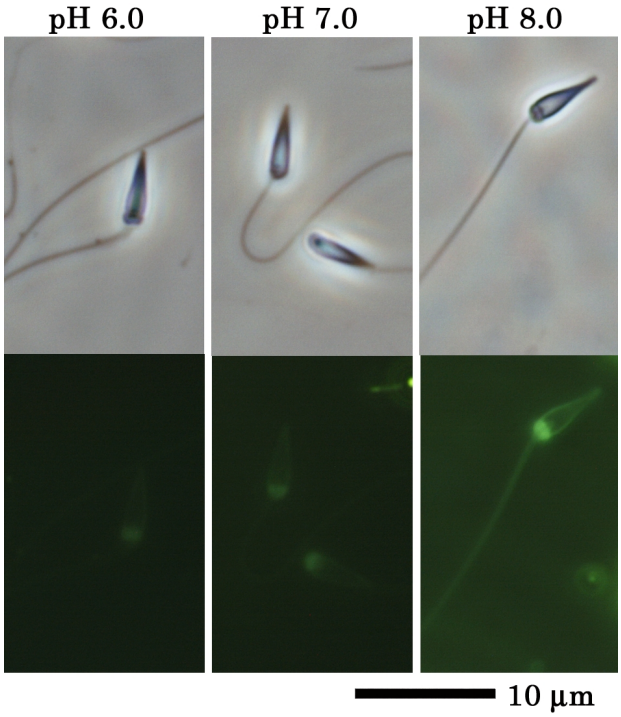

Fig. 4. Sperm stained with TPKE in ASW with different pH values. Sea urchin sperm were stained with 5.0 μM TPKE in ASW with pH adjusted between 6.0–9.0. These images were captured after each wash with fresh ASW.

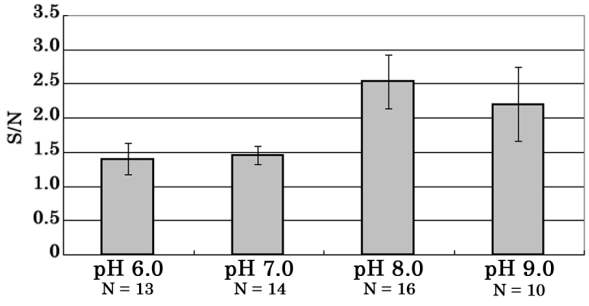

Fig. 5. The fluorescence intensity in the sperm midpiece in ASW with different pH values. The mean of S/N are represented as the fluorescent intensities. These sperm were stained with 5.0 μM TPKE. The N indicates the number of sperm counted in 3 or more images. The bar represents S.D.

Clearer fluorescent images were obtained with TPKE concentrations ranging from 1.0–10 µg/ml. In these images, strong fluorescent signals showed as the midpiece of sperm. The fluorescence intensities of sperm stained with 0.1 µg/ml or less TPKE were weak; however, a higher fluorescence intensity was observed in the case of sperm stained with 1.0–10 µg/ml TPKE. The image of sperms with 10 µg/ml TPKE demonstrated clear fluorescent signal at the midpiece, as was the case with sperms stained with 1.0 µg/ml TPKE. The S/N of sperm stained with 1.0, 5.0, and 10 µg/ml TPKE were significantly higher ($p < 0.01$, Fig. 7), however, the S/N of sperm stained with 10 µg/ml TPKE was lower than that with 1.0 and 5.0 µg/ml TPKE. Thus, appropriate staining of the sea urchin sperm could be achieved with TPKE concentrations of 1.0–5.0 µg/ml.

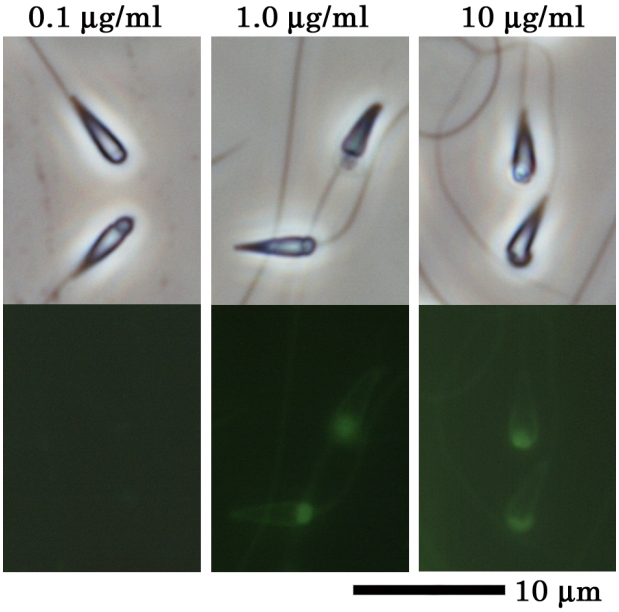

Fig. 6. Fluorescent images obtained with various TPKE concentrations. Sea urchin sperm were stained with 0–10 µM TPKE in ASW (pH 8.0).

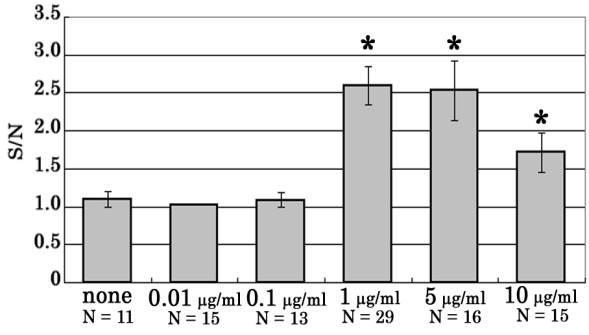

Fig. 7. The fluorescent intensity in the sperm midpiece after staining with various TPKE concentrations. The mean of S/N values are represented (The bar represents S.D). The sperms were stained with 5.0 µM TPKE. * indicates statistical significance ($p < 0.01$ compared with absence of TPKE). The n indicates the number of sperms counted in 3 or more images.

Relationship between respiratory activity and TPKE signal

As previously mentioned, the strong green fluorescent signals were identified to correspond to the midpiece of a sea urchin sperm. The midpiece is a single mitochondrion that carries out aerobic respiration. In order to examine the requirement for TPKE staining to the mitochondrial activity, sperm were suspended in ASW (pH 8.0) containing a respiratory chain inhibitor AMA and an uncoupler FCCP.

Fig. 8 shows typical images of sea urchin *P. depressus* sperm stained with 1.0 µg/ml TPKE in the presence of AMA or FCCP, and in their absence. Whether treated with AMA or FCCP, the sperm showed faint or no fluorescent signals. These sperm showed mitochondrial deformation that the mitochondrion was spherical and removed to a lateral side [6]. In contrast, clear fluorescent signals and no mitochondrial deformation were showed in the sea urchin sperm that had been stained with 1.0 µg/ml TPKE in ASW containing 0.5% DMSO as co-solvent of AMA or FCCP. The S/N of that fluorescence is no significant compared with the absence of DMSO ($p = 0.062$).

The S/N of the sperm stained with 1.0 µg/ml TPKE in the presence of AMA and FCCP showed in Fig. 9. This analysis with bar graph revealed that the S/N values of a sperm stained with 1.0 µg/ml concentration of TPKE. After fixation with 1% paraformaldehyde, and in the presence of AMA and FCCP, the S/N values of the sperm stained with 1.0 µg/ml TPKE were significantly lower than that of the sperm treated with 0.5% DMSO as the co-solvent ($p < 0.01$, Fig. 9). Hence, restrictions in the respiratory activity decreased the fluorescent intensity in sea urchin sperm stained with TPKE.

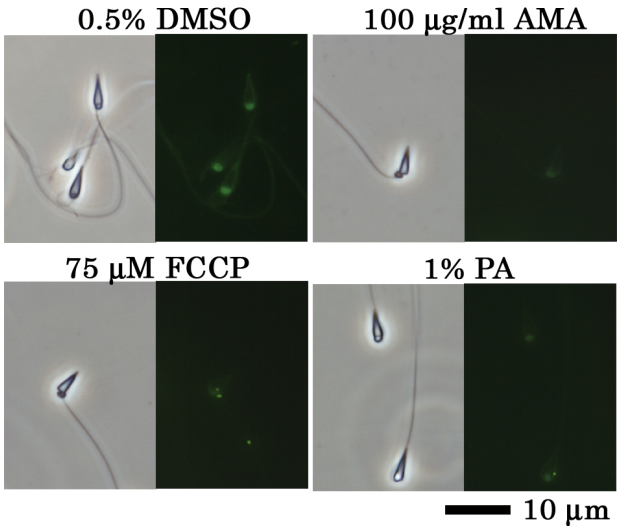

Fig. 8. Fluorescent images of sea urchin sperm stained with TPKE after the treatments. These sperm were stained with 1.0 µg/ml TPKE in the presence of 0.5% DMSO, 100 µg/ml AMA, and 75 µM FCCP. 1% PA indicates the sperm stained after fixation in 1% paraformaldehyde.

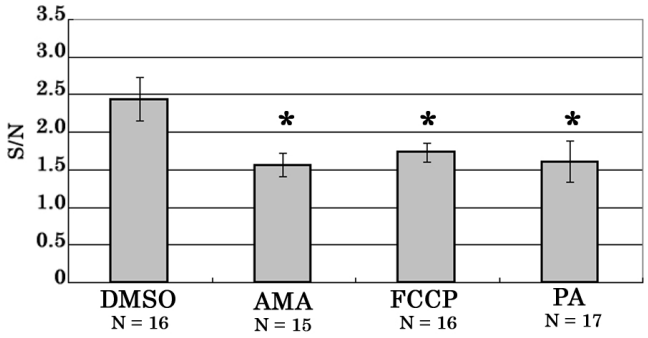

Fig. 9. The fluorescent intensity in the sperm midpiece stained with TPKE in the presence of reagents. The mean of S/N values are represented. These sperm were stained with 1.0 µM TPOX in the presence of 0.5% DMSO, 100 µg/ml AMA, and 75 µM FCCP without fixation, * indicates statistical significance ($p < 0.01$ in sperms stained in the presence of 0.5% DMSO).

The three dioxetanes

TPKE is derived from TPOX, in which the chemiluminescence is induced by bases such as NaOH. We attempted to detect the fluorescent and chemiluminescent signals of the sperms of the sea urchin *A. crassispina* by microscopy after staining with TPOX and two other dioxetanes—TROX and TPOX-Ad. These dioxetanes and TPKE were used at a concentration of 1.0 µg/ml in ASW (pH 8.0); this concentration and pH were determined condition to use TPKE in sperm of *P. depressus*.

All the four substrates showed no chemiluminescence in the sperm under the microscope. TPKE showed a clear fluorescent signal at the midpiece of the sperm of the sea urchin *A. crassispina*, like as the case of *P. depressus* (Fig. 10). The S/N value of the sperm in this image is 2.48. In the case of the three dioxetanes, namely, TPOX, TROX, and TPOX-Ad, the midpiece showed green (S/N = 1.89), faint green (S/N = 1.58), and blue (S/N= 1.40) fluorescence, respectively. However, these three fluorescent signals were weak. The highest fluorescence was observed with TPKE staining.

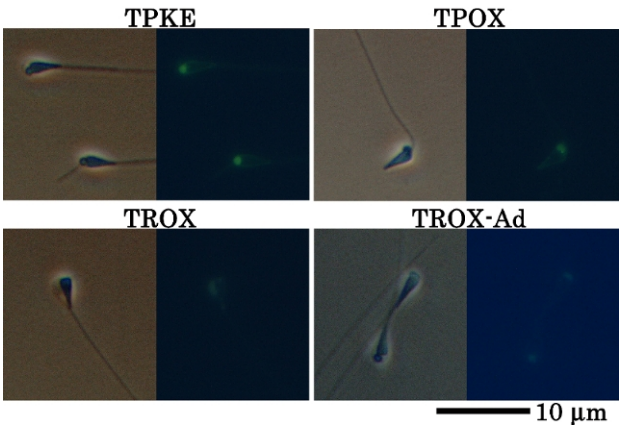

Fig. 10. *A. crassispina* sperms stained with TPKE and 3 dioxetanes. *A. crassispina* sperm were stained with 1.0 µg/ml TPKE and 3 dioxetanes in ASW (pH 8.0). These typical images were obtained after each wash with fresh ASW.

Discussion

The sea urchin sperm is a single cell with a simple structure that is detectable using appropriate fluorescent probes. TPKE is a fluorescent substrate produced by base-induced chemiluminescent TPOX decomposition [1]. Sea urchin sperm stained with TPKE emitted a fluorescent signal from the region corresponding to the midpiece, which is composed of a single mitochondrion. During staining, TPKE concentration was adjusted in the range of 1.0–5.0 µg/ml in ASW (pH 8.0) to acquire clear images; the fluorescent intensity, as denoted by the S/N values, was higher in this range of TPKE concentration. At the lower TPKE concentrations, no or faint fluorescent signals were

revealed. Background fluorescent intensity increased with higher TPKE concentrations.

Higher S/N value is more desirable for observation. In this study, sperm suspensions were washed by centrifugation (3000 *g*, for 10 min at 4°C) with fresh ASW twice after staining. Further rounds of washing with fresh ASW may be required to minimize background fluorescent intensity. Another disadvantage of TPKE staining was that real-time detection of the fluorescent signal was not possible. All fluorescent images in this study were acquired after an exposure time of 10 sec. Detection systems with a greater sensitivity may be able to overcome this problem.

TPKE is produced by the base-induced chemiluminescent decomposition of TPOX [1]. TPOX chemiluminescence is induced by bases such as NaOH. The fluorescence efficiency of TPOX was estimated in an NaOH solution. The S/N fluorescence intensity values in ASW with pH values of 8.0 and 9.0 were higher than in the case of ASW with pH values of 6.0 and 7.0. Although evidence for the pH dependency of TPKE fluorescence is insufficient, the use of basic ASW helped obtain clear images. The elevation of TPKE fluorescence is probably dependent on the pH of ASW.

From another viewpoint, acidic ASW restricts the respiratory activity in sea urchin sperm [7–9]. AMA or FCCP, which are an inhibitor and an uncoupler of the mitochondrial respiratory chain [10], are also effective for mitochondrial disruption in sea urchin sperm [6, 11, 12]. Under these conditions, faint or no fluorescence was observed with TPKE. Moreover, faint or no fluorescence was observed even after fixation. Consequently, the TPKE signal of sea urchin sperm decreases under conditions inducing low respiratory activity.

Sea urchin spawn, fertilize, and live in sea water. The respiration, intracellular pH, and motility of sea urchin sperm increase with elevation in the sea water pH[7,13]. Motility of sperm in sea water is initiated by extracellular Na^+/intracellular H^+ exchange [4,14]. Simultaneously, the oxygen consumption increases owing to the production of adenosine diphosphate (ADP) by adenosine triphosphate (ATP) hydrolysis in the flagella [12]. Before the initiation of flagellar motion, the respiratory activity of sea urchin remains in state 4, the idle state [12, 15]. Hence, clear images of the TPKE signal were obtained by mitochondrial activation of sea urchin sperm.

The fluorescence of several mitochondrial fluorescent probes are dependent on the membrane potentials across the mitochondria of living cells; these probes include rhodamine 123: methyl *o*-(6-amino-3′-imino-3H-xanthen-9-yl) benzoate monohydrochloride [16], JC-1: 5,5′,6,6′-tetrachloro- 1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide [17], and so on. TPKE fluorescence may indicate mitochondria with intact respiratory activity, and it may depend on the potentials across mitochondrial membrane.

TPKE showed fluorescence in the mitochondrion of living sea urchin sperm; however, three dioxetanes—TPOX, TROX, and TPOX-Ad— showed no or faint fluorescence. The faint fluorescence may be derived from the corresponding ketoesters that contaminated or decomposed spontaneously. Further research on these ketoesters is required to clarify how stronger fluorescence can be induced and to subsequently develop fluorescent probes.

Acknowledgments

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