A caged sperm-activating peptide that has a photocleavable protecting group on the backbone amide

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Abstract **A backbone-caged sperm-activating peptide (caged speract) that has a 2-nitrobenzyl group at a backbone amide and a vastly reduced affinity for its receptor (IC_{50} = 950 nM) was synthesized. UV irradiation of caged speract photocleaves the 2-nitrobenzyl group ($\tau_{1/2}$ = 26 μs), restoring its affinity (IC_{50} = 0.67 nM) and ability to increase sperm intracellular pH and Ca^{2+}, as intact speract. Backbone caging of the biological activity was more efficient than side chain caging, which adds a nitrobenzyl group on the peptide side chain. The backbone caging strategy described can be used as a general procedure to cage biologically active peptides, which have no side chain for introduction of a caging group.**

Key words: Caged peptide; Speract; Backbone amide; Sperm

1. Introduction

Caged peptides and proteins, whose activities are masked by the introduction of photocleavable groups, have recently been recognized as a useful tool to elucidate various biological phenomena with a high spatial and temporal resolution, even in living cells. This is because active molecules can be generated at a desired time and position in a sample specimen by carrying out a photochemical reaction using pulsed and focused UV light irradiation, respectively [1,2]. Photocleavable groups have been introduced on the side chains [3–8], the N-terminal amino group [9] and C-terminal carboxyl group [10] of biologically active peptides. To provide a caging effect, the photocleavable groups need to be incorporated exactly into the active site of the target peptide. However, it is not always easy to find a site of incorporation, because an active site residue is not always at a position where it is possible to incorporate the photocleavable group. To overcome this limitation, we report here on the synthesis of caged peptides that have a 2-nitrobenzyl group, a photocleavable group, on the backbone amide of a polypeptide main chain. By using this method, we have synthesized a potent caged sperm-activating peptide (SAP or speract). SAPs are diffusible components of the echinoderm egg jelly coat [11] that show various biological effects on homologous spermatozoa [12]. Speract (or SAP-1) Gly-Phe-Asp-Leu-Asn-Gly-Gly-Gly-Val-Gly from a sea urchin was the first SAP to be purified and structurally identified [13]. A speract binds to its receptor on the sperm flagella, transiently activating a guanylate cyclase and inducing changes in intracellular pH (pH_i) and Ca^{2+} ($[Ca^{2+}]_i$) [12]. The development of caged speracts will offer a useful tool to investigate underlying events.

2. Materials and methods

2.1. Reagents and materials

Fmoc L-amino acids, resins and reagents used for the peptide synthesis were obtained from Shimadzu (Kyoto, Japan). Fmoc-caged serine and lysine were obtained from the Peptide Institute (Osaka, Japan). Fmoc amino acid fluorides were prepared according to the reported method [14]. 2′,7′-*bis*-(2-Carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) and fluo-3-AM were obtained from Molecular Probes (Eugene, OR, USA). 6-(Fluorescein-5-(and-6)-carboxamido)hexanoyl speract (F-speract) was prepared as described in a previous paper [15]. Sperm was obtained as previously described from *Strongylocentrotus purpuratus* [15].

2.2. N-α-Fmoc-N-α-2′-nitrobenzyl-glycine

A solution of 2-nitrobenzaldehyde (33.2 g, 0.22 mol) in 100 ml of methanol was added to glycine (15.02 g, 0.2 mol) in 50 ml of methanol and 50 ml of 2 N NaOH. After 1 h stirring at room temperature, $NaBH_4$ (6.0 g, 0.63 mol) was added to the solution at 0°C. After 2 h, the solution was evaporated and washed with diethyl ether. Then the pH of the aqueous phase was adjusted to about 5 with HCl and washed with diethyl ether. Thereafter, the aqueous phase was evaporated to give crude *N*-2′-nitrobenzyl-glycine. Recrystallization from water gave 12.3 g of the pure compound. A solution of 1.5 equivalent of fluorenylmethyloxy carbonyl chloride (3.89 g, 15 mmol) in 20 ml of acetone was added to *N*-2′-nitrobenzyl-glycine (2.24 g, 10 mmol) in 30 ml of 10% $NaHCO_3$, 70 ml of water and 80 ml of acetone. The solution was stirred overnight at room temperature. After evaporation of the acetone, the suspension was washed three times with ether. The pH was adjusted to about 4 with HCl. Ethyl acetate was added to the solution and the organic phase was separated, washed with water and dried over sodium sulfate. Re-precipitation with hexane gave 4.04 g (yield 94%) of the target compound as white solid. Fast atom bombardment mass spectrometry calculated for $C_{24}H_{20}N_2O_6$: 432.43; $[M^-]$ *m/z* found: 432.09.

2.3. Peptide synthesis

Peptides were synthesized using an automated peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan) by Fmoc chemistry. Fmoc amino acids were activated with benzotriazolyloxy-*tris*-pyrrolidino-phosphonium hexafluorophosphate (one equivalent), hydroxybenzotriazole (one equivalent) and *N*-methylmorpholin (1.5 equivalents) in dimethylformamide. The serine next to the *N*-2′-nitrobenzyl glycine was

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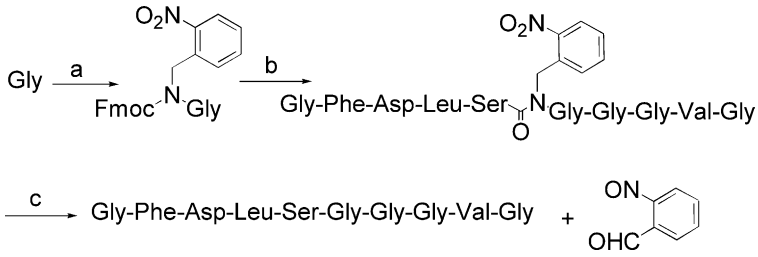

Fig. 1. Scheme of synthesis and photolysis of backbone-caged speract. a: (i) Nitrobenzaldehyde, $NaBH_4$; (ii) Fmoc-Cl. b: Solid phase peptide synthesis. c: UV irradiation.

activated with diisopropyl carbodiimide (10 equivalents) and *N*-methylmorpholin (1.5 equivalents) in dimethylformamide. A 10-fold excess of the activated amino acid was added to the resin. The target peptides were purified by reversed phase high-performance liquid chromatography (HPLC) and identified by time-of-flight mass spectrometry (TOF-MS; MALDI TOF mass spectrometer, Kratos, Manchester, UK). Molecular weight of the purified peptides agreed with the calculated values within the acceptable errors; e.g. [Ser^5, NB-Gly^5] speract: TOF-MS calculated for $C_{44}H_{60}N_{11}O_{16}$: 999.91; $[M+H^+]$ *m/z* found: 1003, $[M-NB+H^+]$ *m/z* found: 867. The fraction containing the desired peptide was lyophilized.

2.4. Study of the binding of speract to the receptor

F-speract (0.5 nM) was mixed with various concentrations of speract analogs in a glass tube (2 ml of sea water) and incubated with spermatozoa for 1 h. Since the bound F-speract emits little fluorescence due to quenching, the fraction of free F-speract can be determined by measuring the total fluorescence intensity of each sample without any mechanical separation of the bound and free ligands [15]. The fluorescence intensity was recorded on a spectrofluorometer (Aminco SLM 8000, SLM Instruments, IL, USA). The UV photolysis of the caged peptide was carried out at $\lambda = 254$ nm light with an UV crosslinker (Stratalinker, Stratagene, IL, USA).

2.5. Laser flash photolysis

A solution of the sample in a quartz cell was irradiated with the third harmonic light pulse of a Nd:YAG laser (SureLite II, Continuum, Santa Clara, CA, USA; pulse width, 8 ns; energy, 38 mJ; wavelength, 355 nm). The transient absorption during the laser irradiation was measured using a photomultiplier tube and monochromatic light of 420 nm from a 150 W xenon lamp. The photocurrent was fed into an oscilloscope (TDS340AP, Sony-Tektronics, Tokyo, Japan) and the data were transferred to a computer. All the peptide was prepared as a 1 mM solution in *N-tris*(hydroxymethyl) methyl-2-aminoethanesulfonic acid (2.5 mM, pH 7.0) containing 40% acetonitrile.

2.6. Sperm pH_i and $[Ca^{2+}]_i$ measurements

Dry sperm was suspended in 5–10-fold of LC7SW (low Ca^{2+} pH 7.0 sea water), and incubated with 20 μM of BCECF-AM or 25 μM of fluo-3-AM plus 0.4% Pluronic F-127 at 14°C for 3 h. The indicator-loaded sperm was washed with LC7SW by centrifugation ($1000 \times g$ for 7 min), re-suspended in the original volume of LC7SW and kept on ice until used. Photolysis of the caged speract was achieved using an UV flash lamp system (JML-C2, Rapp Opto Electronic, Hamburg, Germany) with an UV bandpass filter (270–400 nm) connected to the spectrofluorometer.

3. Results

3.1. Synthesis of backbone-caged peptide

Acylation of *N*-2′-nitrobenzyl glycine with the use of phosphonium reagents was unsuccessful due to the *N*-alkyl group, but acylation using acyl fluorides or carbodiimides did succeed. Therefore, the serine next to the *N*-2′-nitrobenzyl glycine was activated with the use of diisopropyl carbodiimide. The 2-nitrobenzyl group on the backbone amide, i.e. the backbone cage, was stable against treatment with base (30% piperidine in dimethylformamide) and acid (85% trifluoroacetic acid). The backbone-caged speract, [Ser^5, NB-Gly^6] speract, was obtained as the single dominant product, without significant by-products (Fig. 1).

3.2. Receptor binding and photolysis study

The biological activity was examined by the inhibition of binding of the F-speract to the receptor (Fig. 2). [Ser^5] speract inhibited the specific binding of F-speract to the speract receptor very effectively ($IC_{50} = 0.67$ nM), but the introduction of the backbone cage resulted in significant loss of inhibitory activity ($IC_{50} = 950$ nM). UV irradiation of the backbone-caged speract produced the intact peptide, [Ser^5] speract, which was confirmed by mass spectrometry and HPLC. The UV irradiation also resulted in the recovery of the inhibitory activity to the same level as that of the intact [Ser^5] speract (the inset of Fig. 2). The kinetics of photolysis were examined measuring the transient absorption of the intermediate (Fig. 3). The half-life of the decrease of the photolysis intermediate, i.e. the production rate of the intact peptide, was calculated to be 26.0 μs.

3.3. Sperm pH_i and $[Ca^{2+}]_i$ measurements

The binding of speract to the receptor transiently activates a guanylate cyclase and triggers the signaling pathways.

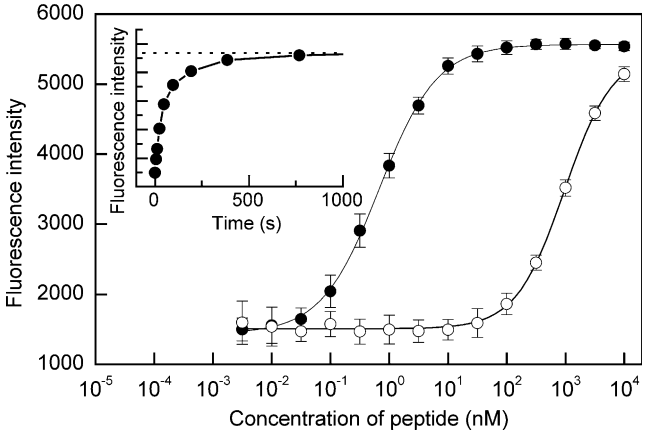

Fig. 2. Competitive binding activity of the caged and decaged speracts. Spermatozoa were incubated in sea water for 1 h with 0.5 nM of F-speract and various concentrations of [Ser^5, NB-Gly^6] speract (caged form), or [Ser^5] speract (decaged form). The fluorescence intensity ($\lambda_{ex} = 494$ nm, $\lambda_{em} = 524$ nm, the free fraction of F-speract) was plotted against the concentration of the caged speract (open circles) and the decaged speract (closed circles). Each point is the mean of four measurements (±S.D.). The inset shows the recovery of the activity of the caged speract (1 μM) as a function of UV irradiation time. The dotted line indicates the activity for the intact peptide (1 μM).

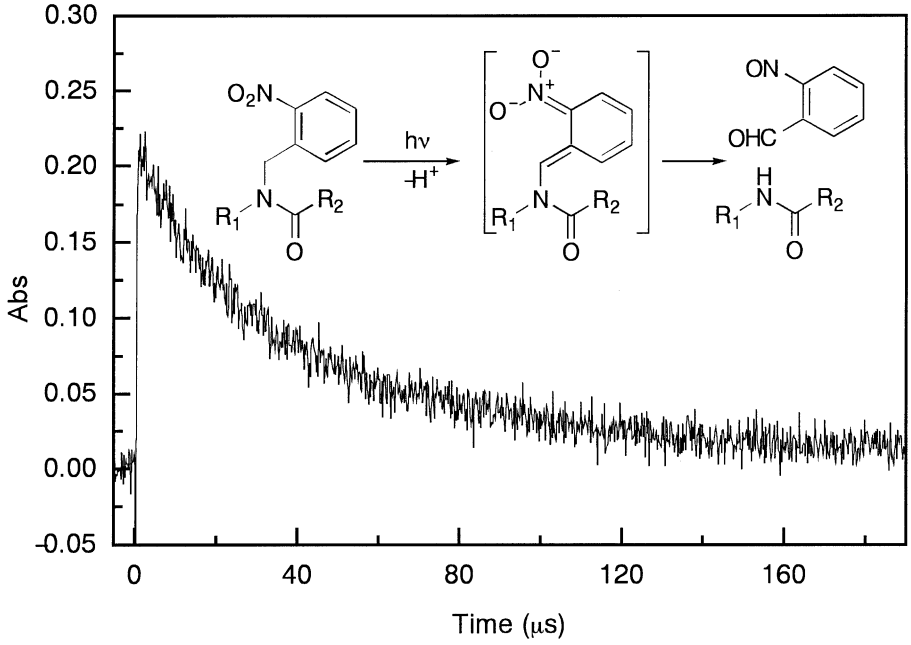

Fig. 3. Changes in absorption of [Ser^5, NB-Gly^6] speract caused by a pulse of UV light. The average of results from eight experiments is shown. The inset illustrates the reaction scheme of photolysis, where R_1 and R_2 represent the peptide fragments, Gly-Gly-Gly-Val-Gly and Gly-Phe-Asp-Leu-Ser, respectively.

Noted effects are increases in pH_i [16] and $[Ca^{2+}]_i$ [17]. As shown in Fig. 4, an UV flash evoked a significant increase in both sperm pH_i and $[Ca^{2+}]_i$ only in the presence of the backbone-caged speracts. The UV flash by itself did not produce any deleterious effect on sperm as judged by its capacity to respond to decaged speracts.

3.4. Side chain-caged peptide

We also synthesized side chain-caged speracts (Table 1). Introduction of a caged residue at the second, sixth and seventh positions resulted in a decrease in the inhibitory activity, but the analog substituted at the sixth position was excluded as a caged precursor since the intact form was inactive. The half-life of the decrease of the photolysis intermediate for the serine-caged speracts was as long as that for the backbone-caged peptide, but the lysine-caged and tyrosine-caged speracts showed slower rates.

4. Discussion

One of the indispensable properties for useful caged compounds pointed out by Kaplan and Somlyo [1] is that the caged compound precursor should be biologically inactive or, at least, some orders of magnitude less active than the photolysis product, since caged compounds are added prior to photolysis. The backbone cage was shown to exert a pronounced effect on the specific competitive binding activity of the peptide to the receptor. The IC_{50} of the caged form was about three orders of magnitude higher than that of the decaged form. Two peptides among the side chain-caged speracts, the [Tyr(NB)^2] and [Ser(NB)^7] speracts, could be used as caged speracts, but the difference in the activity between the caged and decaged forms was less than two orders of magnitude (Table 1). In this respect, the backbone-caged speract is much more useful than the side chain-caged speracts.

Another necessary property for useful caged compounds is that the photolysis reaction should be sufficiently fast [1]. The photolytic process is thought to proceed through a mechanistic pathway that includes (i) photoinduced oxygen transfer from the nitro group to the benzylic carbon position, and the formation of the *aci*-nitro compound, and (ii) hemiacetal decomposition and furnishment of the free amide [18]. Pulsed

Table 1
Inhibition of specific binding of F-speracts to the receptor (IC_{50}) and kinetics ($k_{1/2}$) during the photolysis of the caged peptides

Caged peptide	IC_{50} (nM)	$k_{1/2}$ (μs)	Intact peptide	IC_{50} (nM)
			speract	0.66
Backbone-caged speract				
[Ser^5, NB-Gly^6] speract	950	26.0	[Ser^5] speract	0.67
Side chain-caged speracts				
[Tyr(NB)^2] speract	179	123.7	[Tyr^2] speract	2.78
[Lys(NBoc)^3] speract	0.74	1299.6	[Lys^3] speract	n.d.
[Ser(NB)^5] speract	7.22	34.4	[Ser^5] speract	0.67
[Ser(NB)^6] speract	4900	36.9	[Ser^6] speract	1062
[Ser(NB)^7] speract	71.8	25.0	[Ser^7] speract	1.05
[Ser(NB)^8] speract	0.59	26.1	[Ser^8] speract	n.d.
[Ser(NB)^{10}] speract	0.64	10.9	[Ser^{10}] speract	0.76

n.d.: not determined.

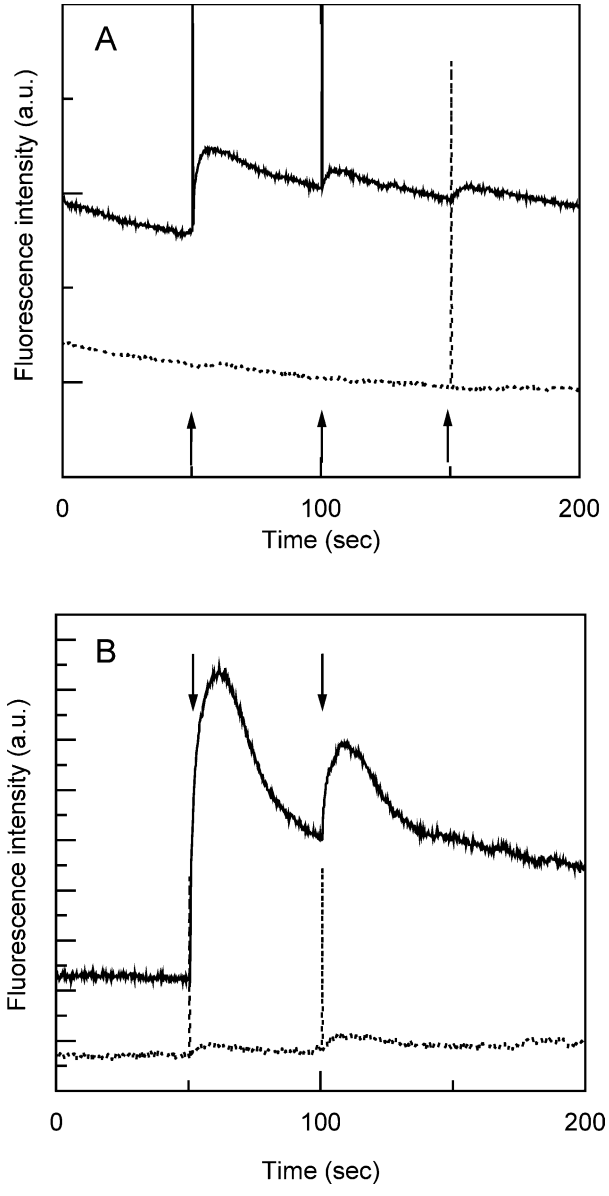

Fig. 4. Sperm responses to UV flashes in the presence or absence of caged speracts. Time courses of sperm pH_i (A) and sperm $[Ca^{2+}]_i$ (B) were measured fluorometrically using BCECF ($\lambda_{ex} = 500$ nm, $\lambda_{em} = 525$ nm)- and fluo-3 ($\lambda_{ex} = 505$ nm, $\lambda_{em} = 525$ nm) -loaded sperm. Upward changes of the fluorescence intensity indicate an increase in pH_i and $[Ca^{2+}]_i$, respectively. The sperm suspension was irradiated using an UV flash lamp, as indicated by the arrows. Continuous lines show sperm responses in the presence of 1 μM of [Ser^5, NB-Gly^6] speracts, and dotted lines indicate control experiments in the absence of [Ser^5, NB-Gly^6] speracts. The large noise at the UV flash was due to scattering.

UV light causes a rapid increase and a single exponential decay in the absorption at 420 nm, which can be ascribed to the formation of the *aci*-nitro intermediate. The half-life of the intermediate during photolysis of the [Ser^5, NB-Gly^6] speract was calculated to be 26.0 μs, indicating that the time resolution achieved is as fast as that of the side chain-caged speracts and faster than that allowed by stopped-flow techniques [19].

The pH_i and $[Ca^{2+}]_i$ measurements in live sperm shown in Fig. 4 indicate that the backbone-caged speract can be used for in vivo experiments without any problems that may arise from the side-products of photolysis or photodamage to the cell. A second flash induced a smaller response, probably owing to the saturation or desensitization of the receptor. These results clearly indicate that the backbone-caged peptide is a useful tool to study speract response. Time-resolved measurements of pH_i, $[Ca^{2+}]_i$ and motility using this caged speract will contribute to our understanding of sperm physiology.

The advantage of the backbone-caged peptide is that this strategy provides various sites for the introduction of a photocleavable group, whereas the side chain-caged peptide has an obvious limitation in the variety of amino acid residues for the introduction of a photocleavable group. A native speract has no site for the introduction of a photocleavable group by using an Fmoc derivative of caged Tyr, Lys, Ser or Cys that we have prepared in our laboratory. We therefore chose, judging from the previous results of the structure–activity relationship [11,20], the side chain residues for substitution to make the caged speract.

To the best of our knowledge, and among the caged peptides prepared by us, the backbone caged peptides show the greatest caging effect. The glycine at the sixth position in speracts is considered to be essential for the activity of this peptide because only this position is conserved among all speract isoforms (>50) purified from various species of sea urchin [11]. In fact, in our experiment, the [Ser^6] speract shows remarkably low competitive binding activity (Table 1). In addition, Nomura and Isaka synthesized various speract analogs with substitutions and deletions, and showed that the activity of the [Pro^6] speract is very low, suggesting that the amide bond $5=6$ is important for receptor binding [20]. Taking all information into account, we introduced the 2-nitrobenzyl group at the amide bond between the fifth and sixth positions. Studies using *N*-substituted analogs of biologically active peptides show that the conformational freedom of the substituted residue is highly restricted and that analogs have the potential of assuming a *cis* peptide bond [21,22]. Therefore, one possible reason for the decrease in the binding activity of the backbone-caged peptide is that the conformational constraints introduced via the 2-nitrobenzyl group are unfavorable for binding to the receptor. Another possibility is that the blockage of intermolecular hydrogen bonding sites by the *N* substitution restricts protein–protein interactions. Such intermolecular hydrogen bonding is found to be very important for the tight binding of peptide antigens in class I MHC molecules [23].

In conclusion, we have developed a simple method for the synthesis of backbone-caged peptides that satisfy the requirements for useful caged compounds. This method is applicable to any peptide sequence, whereas for the synthesis of side chain-caged peptides, substitution of the native amino acid sequence, which may alter important properties such as selectivity for receptor subtypes, is sometimes necessary, as exemplified by the present study.

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