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## Photodynamic action inhibits compound 48/80-induced exocytosis in rat peritoneal mast cells

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### Abstract

Photostimulation of sulfonated aluminum phthalocyanine (SALPC)-loaded mast cells (20,000 lux, 2 min) itself caused neither exocytosis nor  $[Ca^{2+}]_i$  increase in isolated rat peritoneal mast cells. This result is incompatible with that reported in other cell types such as pancreatic acinar cells. Stimulation with 50  $\mu$ g/ml compound 48/80, a direct G-protein activator, induced massive exocytosis which was easily detectable under conventional microscope. The fluorescent granules stained with sulforhodamine B were found to be numerous on the perimetry of mast cells, confirming occurrence of exocytosis. The stimulation also increased  $[Ca^{2+}]_i$  and cell volume before initiation of exocytosis. Pretreatment of the cells with photodynamic action with 5  $\mu$ M SALPC inhibited the compound 48/80-induced exocytosis, but the  $[Ca^{2+}]_i$  increase and the increase of cell volume were unaffected.  $NaN_3$  at 0.5 mM could relieve the photodynamic action-induced inhibition of exocytosis. These results indicate that, unlikely to other secretory or contractile cells, photodynamic action with SALPC does not directly affect exocytotic machinery but modulates some functional proteins involved in signal transduction process which may be posterior to G-protein activation in mast cells. Singlet oxygen may be involved in the photodynamic action-induced modulation. A possible target protein can be a protein in the cell membrane which binds with a protein of a granular membrane during the course of exocytosis.

**Key words :** confocal microscopy, exocytosis, mast cell, rat, SALPC

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## Introduction

Photodynamic action produces singlet oxygen by transferring photon energy from a photosensitizer to ground state molecular oxygen. The singlet oxygen can affect various cellular functions<sup>5</sup>. The photodynamic action is adopted for some clinical purposes such as photodynamic therapy against carcinoma and sterilization of the blood<sup>4</sup>. However, the mechanisms by which various cellular responses are modified are not fully understood<sup>15,17</sup>. It has been reported that photodynamic action modulates contraction of smooth muscle cells and secretory responses of exocrine cells<sup>5</sup>. An assumption is made that these modulatory actions can be induced by direct stimulation or inhibition by singlet oxygen of certain factor(s) of intracellular signal transduction process. Illumination of gadolinium porphyrin-like macrocycle B (PLMGdB) - or sulfonated aluminum phthalocyanine (SALPC)-preloaded pancreatic acinar cells causes oscillatory changes in cytosolic  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ )<sup>3,4</sup>. The oscillatory changes in  $[Ca^{2+}]_i$  are analogous to those produced by stimulation with a physiological secretagogue, cholecystokinin (CCK), at a low concentration or with carbachol<sup>3</sup>. Although the oscillatory  $[Ca^{2+}]_i$  changes terminate after the withdrawal of the stimulant, those produced by photodynamic action last for more than several tens of minutes even after the cessation of illumination. As a phospholipase C (PLC) inhibitor, U 73122, or an antagonist of  $IP_3$  receptor, 2-aminoethoxydiphenylborate (2-APB), inhibits photodynamic action-induced  $[Ca^{2+}]_i$  changes, it has been presumed that singlet oxygen can activate PLC or the processes prior to PLC activation<sup>4</sup>.

To further investigate the effect of photosensitizer on exocytotic processes in secretory cells, the present experiments were conducted

using rat peritoneal mast cells. Exocytosis is easily detectable in mast cells under conventional microscope as mast cells possess relatively large secretory granules<sup>8</sup>. Qualitative experiments were carried out to investigate photodynamic modification of exocytosis by directly observing exocytosis with conventional microscope and with confocal microscope by use of sulforhodamine B. The latter is one of exocytotic probes which binds with intragranular matrix protein after exocytosis and produces fluorescence. In addition,  $[Ca^{2+}]_i$  was also monitored with indo-1.

## Materials and Methods

### Cell isolation

Mast cells were prepared by peritoneal lavage from i.p. pentobarbitone-anesthetized or decapitated male Sprague-Dawley rats (250-300 g body weight) as previously reported<sup>8</sup>. Freshly isolated cells were resuspended in HEPES-buffered solution (HS), the composition of which was as follows (mM) : NaCl, 137; KCl, 4.7;  $CaCl_2$ , 2.5;  $MgCl_2$ , 1.13; D-glucose, 5.5; and HEPES, 10. The solution was supplemented with 1 mg/ml bovine serum albumin (Sigma Chemical, St. Louis, MO, USA). pH was adjusted to 7.4 with NaOH and the solution was gassed with 100%  $O_2$ . Experiments were done at room temperature (25-30°C).

### Loading of SALPC

A membrane localized photosensitizer, SALPC (Porphyrin Products, Salt Lake City, USA) was used. Coverglass-attached mast cells were perfused with HS containing 5  $\mu$ M SALPC for 7 min. The SALPC-loaded cells were illuminated (>580 nm, 20,000 lux) for 2 min with Cold Light (HL100R, Hoya-Schott, Japan). All these manipulations were carried out under the dark.

### Detection of exocytosis with sulforhodamine B

After the SALPC-loaded cells were illu-

minated, the cells were perfused with HS containing 50  $\mu\text{g/ml}$  compound 48/80 (Sigma), a stimulant of exocytosis in mast cell. The microscopic photographs were taken before and approximately 5 min after the stimulation with compound 48/80. Exocytosis was also observed under the confocal microscope with 20% w/v sulforhodamine B (Cosmo Bio Co. Ltd., Tokyo, Japan), an exocytotic probe which binds with intragranular matrix protein after exocytosis and produces fluorescence, with G excitation (543 nm) and the fluorescence was detected at 580 nm<sup>16,25)</sup> approximately 5 min after the stimulation with compound 48/80. At least five separate areas were chosen at random in each preparation.

#### *Measurements of $[\text{Ca}^{2+}]_i$ dynamics*

The  $[\text{Ca}^{2+}]_i$  imaging was performed as reported previously<sup>8)</sup>. Aliquots of the cell suspension were incubated with 5  $\mu\text{M}$  indo-1 AM for 40 min at 37°C. The indo-1-loaded cells were rinsed once and resuspended in fresh HS. A coverglass coated with Cell-Tak (Collaborative Research, Bedford, MA, USA) was placed to the bottom of Sykus-Moore chamber (volume, 2 ml) and an aliquot of the cell suspension was transferred into the chamber. For the experiments, the chamber was set up on the stage of an inverted microscope (TMD-300; Nikon, Tokyo, Japan) and the cells were perfused with HS at a rate of 1 ml/min with a Perista minipump (Atto, Tokyo, Japan). The  $[\text{Ca}^{2+}]_i$  was measured, displayed and analyzed with a UV laser scanning confocal microscope (RCM 8000; Nikon, Tokyo, Japan) with an excitation UV beam (351 nm) produced by an argon ion laser. The emitted fluorescence was guided through a x40 water-immersion objective (NCF Fluor N.A. 1.15) to a pinhole diaphragm, separated at 440 nm with a dichroic mirror; intensities  $>440$  nm and  $<440$  nm were detected with two separate photomultipliers. The image acquisition inter-

val (time resolution) was either 10 sec before the compound 48/80 stimulation or 5 sec after the stimulation. The  $[\text{Ca}^{2+}]_i$  signals were obtained by ratiometry and displayed with false color.

## Results

### *Exocytosis induced by compound 48/80 and photodynamic action*

Compound 48/80, a direct activator of G-protein, induced massive exocytosis in mast cells (Fig. 1). Unstimulated mast cells have smooth cell perimeter (Fig. 1A) and, during exocytosis, the released granules attached to the cell perimeter and were also scattered around the cells (Fig. 1B). Cell swelling and exocytosis were easily detectable under the light microscope.

Light illumination ( $>580$  nm, 20,000 lux) for 2 min to SALPC-loaded cells inhibited the compound 48/80-induced exocytosis most likely at a certain stage of exocytotic process (Fig. 1C, D). Cell swelling remained to occur. Administration of SALPC alone, light illumination alone, and SALPC plus light without stimulation unaffected the cells (data not shown). Stimulation of SALPC-loaded cells without light illumination or of SALPC-unloaded cells with light illumination induced exocytosis which is similar to that observed in control cells (data not shown). Pretreatment of SALPC-loaded cells with a singlet oxygen scavenger,  $\text{NaN}_3$  (0.5 mM), partially relieved the light-induced inhibition of exocytosis (Fig. 1E, F).

Photodynamic action on exocytosis was also examined with sulforhodamine B, a fluorescent exocytotic marker, under a laser scanning confocal microscope (Fig. 2). Extrusion of secretory granules by exocytosis was clearly visible (Fig. 2A). It was also confirmed with sulforhodamine B that photodynamic action inhibited the compound 48/80-induced exocy-

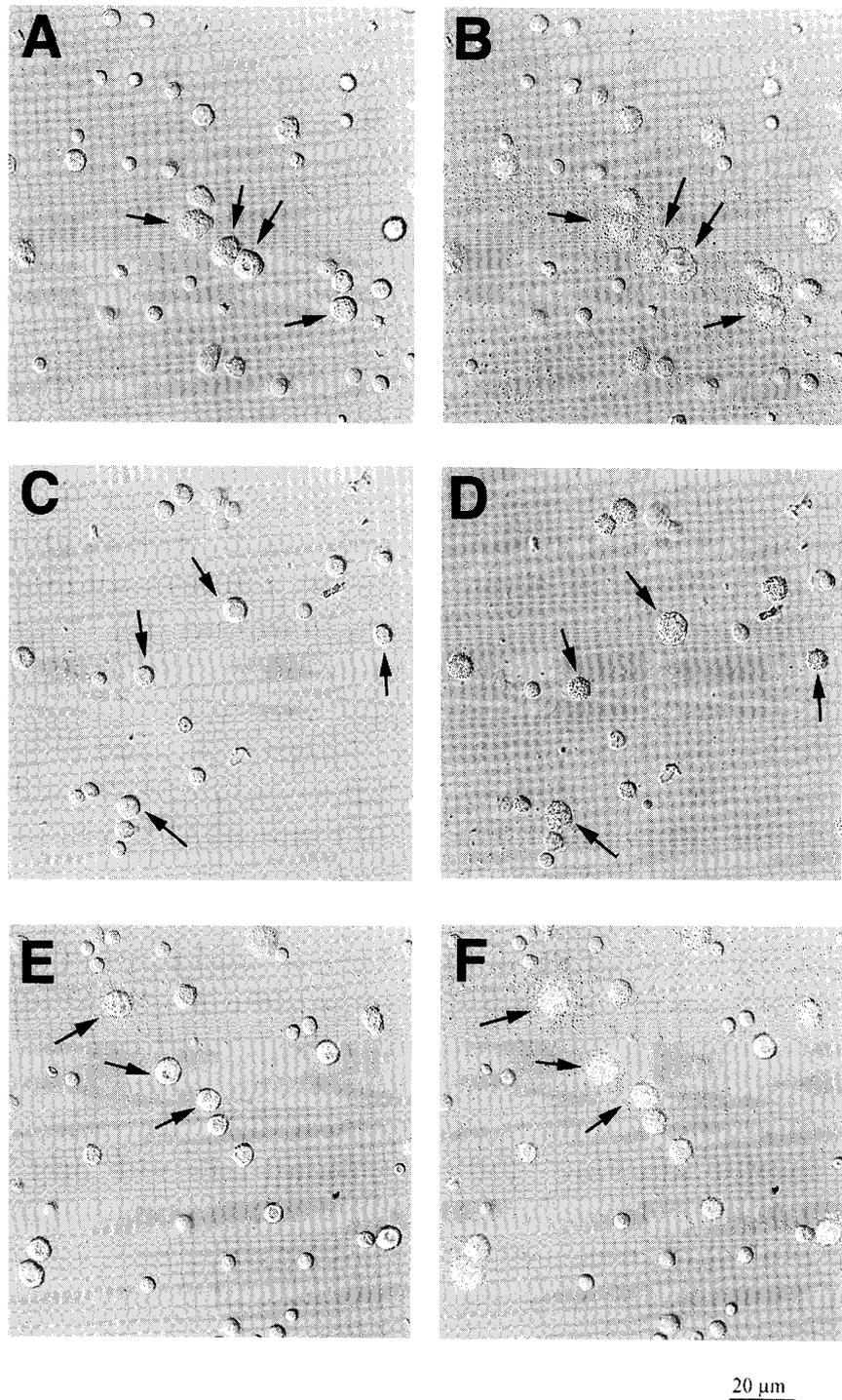


Fig. 1 . A : microscopy of unstimulated and SALPC-unloaded rat peritoneal mast cells. B : SALPC-unloaded mast cells stimulated with compound 48/80 (50  $\mu\text{g}/\text{ml}$ ). C : unstimulated and SALPC (5  $\mu\text{M}$ )-loaded mast cells before photodynamic action. D : compound 48/80-stimulated and SALPC-loaded mast cells after photodynamic action. E : unstimulated,  $\text{NaN}_3$  (0.5 mM)-pretreated, and SALPC-loaded mast cells before photodynamic action. F : compound 48/80-stimulated,  $\text{NaN}_3$ -pretreated and SALPC-loaded cells after photodynamic action. Arrows denote mast cells. For stimulated cells photographs were taken approximately 5 min after the stimulation with compound 48/80 as described in materials and methods. Each is a typical example of 6 -10 separate experiments done under each condition.

tosis (Fig. 2B). Some extent of fluorescence was detected in the cell or in the extruded granules after the photodynamic treatment. This indicates again that the extrusion of the granules seemed to be inhibited at a certain stage of exocytotic process. This inhibition was relieved by pretreatment with  $\text{NaN}_3$  (Fig. 2C).

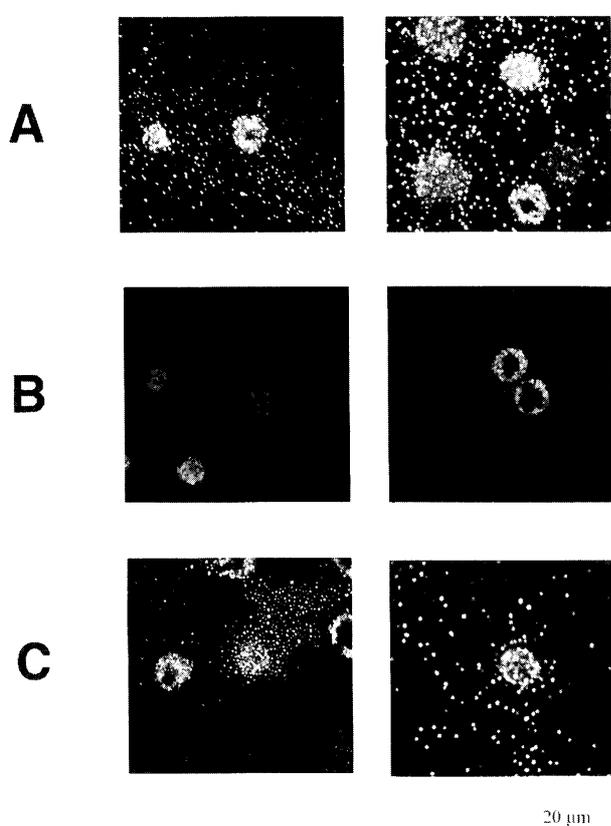


Fig. 2. A: fluorescent images of compound 48/80-stimulated mast cell observed under confocal microscope with sulforhodamine B. B: fluorescent images of sulforhodamine B in compound 48/80-stimulated and SALPC-loaded cells after photodynamic action. C: fluorescent images of sulforhodamine B in compound 48/80-stimulated,  $\text{NaN}_3$ -treated, and SALPC-loaded cells after photodynamic action. The photographs were taken approximately 5 min after the stimulation with compound 48/80 as described in materials and methods. Two examples of 6-10 separate experiments are shown for each condition.

#### Photodynamic action on the compound 48/80-induced $[\text{Ca}^{2+}]_i$ dynamics

It has been documented that the  $[\text{Ca}^{2+}]_i$  increases during exocytosis in mast cells stimulated with compound 48/80 and extracellular  $\text{Ca}^{2+}$  is relevant for this increase<sup>8)</sup>. As photodynamic action inhibited exocytosis, possible effects of photodynamic action on  $[\text{Ca}^{2+}]_i$  dynamics were examined by  $\text{Ca}^{2+}$  imaging with indo-1. Stimulation with compound 48/80 caused an increase in  $[\text{Ca}^{2+}]_i$  and invagination of cytosolic fluorescent image followed, which indicates granule extrusion (Fig. 3A). As reported previously<sup>8)</sup>, the  $[\text{Ca}^{2+}]_i$  decreased gradually, showing biphasic changes in  $[\text{Ca}^{2+}]_i$ . In the SALPC-loaded cells, illumination of the cells did not show significant effects on the compound 48/80-induced  $[\text{Ca}^{2+}]_i$  dynamics (Fig. 3C). The  $[\text{Ca}^{2+}]_i$  increased even after photodynamic action as observed in unloaded cells (Fig. 3B, D).

#### Discussion

In the present experiments, it was found that, in isolated rat peritoneal mast cells, photodynamic action by SALPC, a membrane-localized photosensitizer, caused 1) inhibition of the compound 48/80-induced exocytosis, 2)  $\text{NaN}_3$ , a singlet oxygen scavenger, relieved the inhibition, 3) the compound 48/80-induced  $[\text{Ca}^{2+}]_i$  dynamics was unaffected by photodynamic action, and 4) photodynamic action itself could not induce  $[\text{Ca}^{2+}]_i$  increase. These findings are incompatible with those reported in isolated pancreatic acinar cells. In pancreatic acinar cells, photodynamic action itself could cause oscillatory  $[\text{Ca}^{2+}]_i$  increase even in the absence of physiological stimuli<sup>3,4)</sup>. While the release of amylase is stimulated by photodynamic action in pancreatic acinar cells<sup>12,13,14)</sup>, inhibition of the release has been reported in AR 4-2 J cells<sup>1,14)</sup>, a cell-line of pancreatic acinar cell. In smooth muscle, pho-

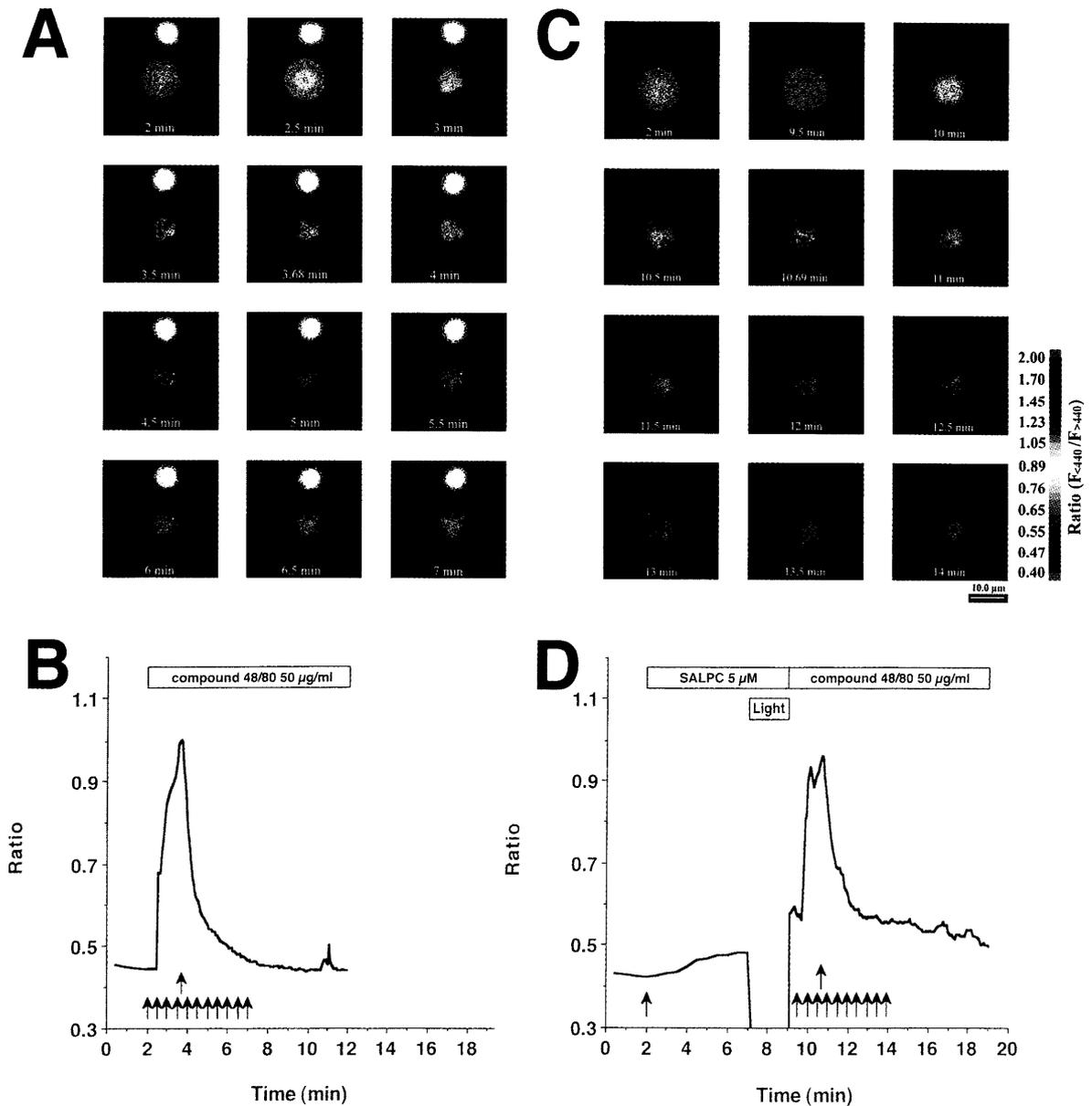


Fig. 3. A: false color images of  $[Ca^{2+}]_i$  dynamics in SALPC-unloaded mast cell. The cell was stimulated with compound 48/80. A typical example of 8 separate experiments was shown. Images displayed were selected from the time indicated by arrows in B. B: time course of  $[Ca^{2+}]_i$  dynamics in SALPC-unloaded mast cell shown in Fig. 3 A. C: false color images of  $[Ca^{2+}]_i$  dynamics in SALPC-loaded and stimulated cells after photodynamic action. A typical example of 10 separate experiments was displayed. Images displayed were selected from the time indicated by arrows in D. D: time course of  $[Ca^{2+}]_i$  dynamics in SALPC-loaded and stimulated cells after photodynamic action shown in Fig. 3C.

photodynamic action triggers contraction<sup>11)</sup>. Furthermore, photodynamic action-induced  $[Ca^{2+}]_i$  increase has been reported in fibroblast cells, thymocytes, and cardiomyocytes<sup>2,10,26,27)</sup>.

Thus photodynamic action can induce cellular responses which are similar to physiological ones in some cells but it also causes nonphysiological responses in other cells. For example, receptors for some cytokines, epider-

mal growth factor (EGF), and transforming growth factor alpha (TGF- $\alpha$ ) are deactivated by photodynamic action by pheophorbide<sup>7,18)</sup>. Recently, desensitization of  $\alpha$ -adrenergic receptor was reported in hepatocyte<sup>6)</sup>. These effects are believed to be caused by interaction of some cellular functional proteins with singlet oxygen produced by photodynamic stimulation of photosensitizers localized in the cell membrane. It has been presumed that the SALPC- or PLMGdB-induced oscillatory  $[Ca^{2+}]_i$  change is due to permanent activation of a certain functional protein that is involved in intracellular signal transduction system and is prior to PLC activation as photodynamic action-induced oscillatory  $[Ca^{2+}]_i$  changes are inhibited by an antagonist of IP<sub>3</sub> receptor, 2-APB, and by a specific PLC inhibitor, U 73122<sup>4)</sup>. Possible sites of action can be G-proteins and receptors. Deactivation or desensitization of receptors is considered to be a result of singlet oxygen-induced crosslinking between each receptor or the crosslinking with some domain(s) within the receptor molecule, hindering allosteric changes in receptor proteins<sup>6, 9, 20, 21, 22, 24)</sup>. As singlet oxygen is highly reactive (94.1 kJ/mol)<sup>19)</sup>, a membrane-localized photosensitizer can permanently alter the structure of the functional proteins in the membrane by interacting with amino acids residue of the protein<sup>23)</sup>. In fact, the  $[Ca^{2+}]_i$  changes in SALPC- or PLMGdB-loaded pancreatic acinar cells last even after the cessation of light illumination, being different from the changes caused by physiological stimuli. This long term effect may be related to photodynamic action-induced cell damage or cell death as long-lasting increase in  $[Ca^{2+}]_i$  is toxic to the cells. This mechanism can partly be involved in photodynamic therapy or photosterilization.

Since the site of action is presumably dependent on localization and distribution of

relevant protein domains, photodynamic action-induced cellular responses can be diverse. In addition, the concentration and duration of illumination can also affect the responses. In the current study, as photodynamic action by SALPC was found to inhibit exocytosis which was induced by direct stimulation of G-protein, processes including and posterior to G-protein of signal transduction system is likely to be affected. Singlet oxygen may be involved in this effect as its scavenger, NaN<sub>3</sub>, relieved the inhibition. Being different from pancreatic acinar cells, PLC, IP<sub>3</sub> production, and Ca<sup>2+</sup> entry process would not be affected because the compound 48/80-induced  $[Ca^{2+}]_i$  dynamics was normal after photodynamic action. In mast cell, cell swelling occurs just before exocytosis<sup>8)</sup>. This is believed to be caused by water entry to the cell according to transmembrane ionic movements. As cell swelling occurred as normal even after photodynamic action, the ionic mechanism may not be influenced. Based upon these findings, it is assumed that membrane-localized functional protein(s) can be target(s) of photodynamic action in mast cell. Possible protein can be a protein which is involved in interaction between granular membrane and cell membrane. Further study remains to be carried out to clarify relevant functional protein(s).

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