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## Photodynamic stimulation causes sustained increase in intracellular calcium concentration in cells of small cell lung carcinoma

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### Abstract

Photodynamic agents, due to their selective uptake by tumor cells and photon-dependent selective activation, have immense implications for cancer treatment. The present study provided direct evidence that the photon activation of chloro-aluminum phthalocyanine sulphonate (AlPcS<sub>4</sub>) in the presence of extracellular Ca<sup>2+</sup> caused a rapid increase followed by a sustained increase in intracellular concentration of calcium ion ([Ca<sup>2+</sup>]<sub>i</sub>) in a small cell lung carcinoma (SCLC) cell line, SBC-3. The [Ca<sup>2+</sup>]<sub>i</sub> increase by photodynamic stimulation was completely inhibited by the removal of extracellular Ca<sup>2+</sup> and reintroduction of extracellular Ca<sup>2+</sup> immediately led to a rapid elevation of [Ca<sup>2+</sup>]<sub>i</sub>. However, the increase was not inhibited by application of Ni<sup>2+</sup>, nifedipine, or SK&F 96365, a receptor-mediated and voltage-dependent Ca<sup>2+</sup> entry blocker. The photosensitizer AlPcS<sub>4</sub> alone or light alone (4 min) had no effect on [Ca<sup>2+</sup>]<sub>i</sub>. Cytotoxicity examination by trypan blue exclusion test, however, suggested photodynamic stimulation-induced cell injury which was observed in both the presence and the absence of extracellular Ca<sup>2+</sup>. These results indicate that [Ca<sup>2+</sup>]<sub>i</sub> increase may not be mandatory for photodynamic stimulation-induced cell injury. Whether [Ca<sup>2+</sup>]<sub>i</sub> increase can accelerate, at least in part, cell death under the physiological condition, whether the mechanism(s) of cell death can be different in the presence and the absence of extracellular Ca<sup>2+</sup>, and whether [Ca<sup>2+</sup>]<sub>i</sub> increase can be totally unrelated to cell death await further work.

Key words: [Ca<sup>2+</sup>]<sub>i</sub>, cell injury, chloro-aluminum phthalocyanine sulphonate (AlPcS<sub>4</sub>), methacholine

### Introduction

Photodynamic therapy using photosensitizing drugs is a new cancer treatment modality that is effective for various solid tumors<sup>10,34</sup>. Photodynamic stimulation is the process which involves activation of photosensitizing drugs by photon energy. The transfer of this photon

energy to ground state generates the excited delta singlet molecular oxygen and subsequently singlet oxygen-mediated cellular responses<sup>7</sup>. Photosensitizing drugs include porphyrins, chlorins, phthalocyanins, pupurins, porphycenes<sup>9,17</sup>. In these drugs some porphyrin derivatives, especially photofrin II, have been approved for clinical usefulness<sup>10</sup>. However detailed cellular

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mechanisms of photodynamic stimulation remain to be clarified.

It has previously been found that, after short-term incubation of cells, photosensitizers localize mainly to the plasma membrane *in vitro*<sup>13)</sup>. Upon subsequent light activation, these membrane-bound drugs can cause contraction in smooth muscle cells<sup>22)</sup>, slow phasic increase of intracellular calcium concentration in fibroblasts, thymocytes, and cardiomyocytes<sup>3,13,38,39)</sup>, amylase secretion in pancreatic acinar cells<sup>19,20)</sup>, and inhibition of amylase secretion in AR4-2J cells<sup>1,21)</sup>. Photosensitizers could directly regulate phospholipase A<sub>2</sub><sup>2)</sup>, activate adenylate cyclase<sup>31)</sup>, stimulate p<sup>21</sup>-ras and MAP kinase<sup>33)</sup>, and increase the expression of immediate early response genes (fos, jun, myc, and egr-1)<sup>18)</sup>. In a recent work, it was reported that photoactivation of a photosensitizer, chloro-aluminum phthalocyanine sulphonate (AIPcS<sub>4</sub>), triggered recurrent [Ca<sup>2+</sup>]<sub>i</sub> spike generation in pancreatic acinar cells<sup>7)</sup>. Such [Ca<sup>2+</sup>]<sub>i</sub> spiking is often seen upon ligand activation of cell surface receptor coupled to phosphatidyl inositol hydrolysis. This probably reflects the periodic opening and closing of inositol trisphosphate-(IP<sub>3</sub>)-gated Ca<sup>2+</sup> channels. Therefore these agents may become a major class of drugs targeting cellular signal transduction pathways.

Small cell lung carcinoma (SCLC) is a malignant neuroendocrine cancer that expresses many proteins of neuronal phenotype<sup>28,37)</sup>. Cultured SCLC cells have been shown to express voltage-dependent potassium<sup>30)</sup> and sodium channels and to generate action potentials<sup>14)</sup>. Voltage-gated Ca<sup>2+</sup> channels have been identified using biochemical and electrophysiological criteria in virtually all SCLC cell lines<sup>6,26,28,30,35)</sup>, and depolarization-induced secretion of peptides from SCLC cells requires Ca<sup>2+</sup> influx<sup>6)</sup>. Stimulation with a G-protein activator caused various types of [Ca<sup>2+</sup>]<sub>i</sub> dynamics in a SCLC cell line, SBC-3<sup>15)</sup>. In the present study, we monitored [Ca<sup>2+</sup>]<sub>i</sub> dynamics in

SBC-3 cells after loading with AIPcS<sub>4</sub>.

## Materials and Methods

### Drugs

AIPcS<sub>4</sub> was a photosensitizer received from Dr. Z. J. Cui in Faculty of Biomedical Sciences, Beijing Agricultural University, Beijing, P. R. China; SK&F 96365 was a receptor-mediated and voltage-dependent Ca<sup>2+</sup> entry blocker obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA, U.S.A.); methacholine chloride was a nicotinic and muscarinic receptors agonist obtained from Tokyo Kasei Industry (Tokyo, Japan); atropine sulfate monohydrate was a muscarinic receptor antagonist obtained from Wako Pure Chemicals (Osaka, Japan); and O,O'-bis(2-aminoethyl) ethyleneglycol-N, N, N'-N'-tetraacetic acid (EGTA) was obtained from Dojindo Laboratories (Kumamoto, Japan).

### Cell Culture

Human SCLC cells (SBC-3) were kindly provided by Prof. M. Hoshino from School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan. The cells were grown in suspension in RPMI 1640 medium (Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA, U.S.A.), 100 IU/ml penicillin and 100 µg/ml streptomycin (Gibco BRL) in 25 cm<sup>2</sup> tissue culture flasks. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. The cells were maintained in log growth phase by splitting every 4 to 5 days.

### [Ca<sup>2+</sup>]<sub>i</sub> Measurement

The composition of 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered Ringer solution (HR) was as follows: 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.13 mM MgCl<sub>2</sub>, 1.0 mM Na<sub>2</sub>HPO<sub>4</sub>, 5.5 mM D-glucose, 10.0 mM HEPES (Dojindo Laboratories) supplemented with bovine serum albumin (2 mg/ml, Fraction V: Sigma Chemical, St. Louis, MO, U.S.A.) and enriched with Eagle's minimal essen-

tial amino acids medium (Gibco BRL) and L-glutamine (2 mM, Wako Pure Chemicals). The pH of the solution was adjusted to 7.4 with NaOH and the solution was gassed with 100% O<sub>2</sub>. Cultured cells were mechanically freed from the flasks and were washed with HR. The arrangements for perfusing cells and measuring Fura-2 fluorescence have been described previously<sup>16</sup>. In brief, the cells were loaded with Fura-2/AM (1 mM in DMSO : Dojindo Laboratories) at a final concentration of 3 μM for 40 min at 37°C with gentle shaking at every 10 min. The Fura-2-loaded cell suspension was rinsed and diluted with HR and 1 ml aliquots were transferred to several Sykus-Moore chambers with the bottom coverslip coated with Cell-Tak (Becton Dickinson Labware, Bedford, MA, U.S.A.). The cells were allowed to attach at least for 15 min before perfusion with HR at 1 ml/min. The buffer was pre-warmed in a water bath to 37°C and HR containing relevant drugs was delivered to the cell through perfusion. The Sykus-Moore chamber was positioned on the stage of an inverted microscope (TMD-2, Nikon, Tokyo, Japan). A single cell was selected in the optical field under microscope, and fluorescence intensities with excitation at 340 and 380 nm were alternatively recorded at an interval of 5 sec. [Ca<sup>2+</sup>]<sub>i</sub> was calculated from the ratio of 340 nm and 380 nm fluorescence intensity using [Ca<sup>2+</sup>]<sub>i</sub> calibration curve<sup>11</sup>.

#### Application of drugs

First, the responsiveness of the [Ca<sup>2+</sup>]<sub>i</sub> signaling in the Fura-2-loaded cells was examined by application of methacholine which simultaneously activates nicotinic and muscarinic receptors. In viable cells, this drug evoked [Ca<sup>2+</sup>]<sub>i</sub> dynamics which may be mediated by muscarinic receptor, since the rise was inhibited by atropine (Fig. 1). Then subsequent recovery of [Ca<sup>2+</sup>]<sub>i</sub> to stable baseline levels was recorded for about 5 min prior to the application of AIPcS<sub>4</sub> contained in HR, in Ca<sup>2+</sup>-deficient HR, or in

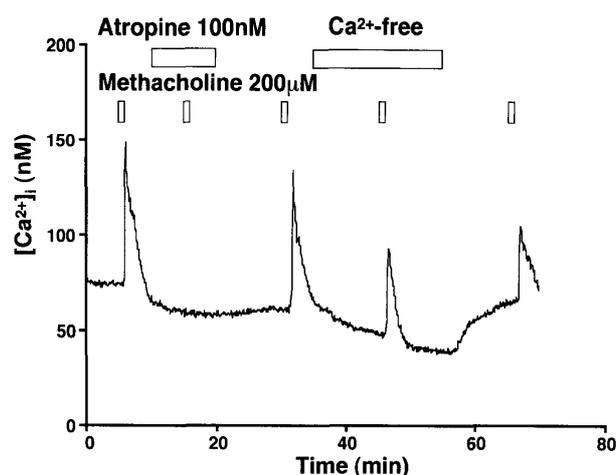


Fig. 1. An example of measurement of [Ca<sup>2+</sup>]<sub>i</sub> dynamics induced by methacholine. In all experiments (n=14) cells were continuously perfused with HR. Methacholine (200 μM) was applied as indicated by the horizontal bars and atropine or Ca<sup>2+</sup>-free buffer was superfused during the period indicated by the horizontal bars.

SK&F 96365-containing HR. The cells were superfused with the media containing 5 μM AIPcS<sub>4</sub> for 10 min, and then AIPcS<sub>4</sub> was washed out with HR for 5 min prior to the exposure to light.

#### Light illumination

Cold Light (HL 100R, Hoya-Schott, Tokyo, Japan) equipped with a focusing lens (HLL201) and a sharp-cut filter (R60, wavelength > 580 nm) was used for illuminating the photosensitizer (190 kJ/m<sup>2</sup>).

## Results

#### [Ca<sup>2+</sup>]<sub>i</sub> dynamics in SBC-3 cells

Stimulation of SBC-3 cells with 200 μM methacholine caused [Ca<sup>2+</sup>]<sub>i</sub> dynamics consisted of initial transient elevation followed by a gradual decay. Addition of 100 nM atropine completely abolished the methacholine-induced [Ca<sup>2+</sup>]<sub>i</sub> dynamics. On the other hand removal of CaCl<sub>2</sub> and the addition of EGTA (1 mM) did not affect the methacholine-induced [Ca<sup>2+</sup>]<sub>i</sub> dynamics (Fig. 1). High K<sup>+</sup> at a depolarizing concentration of 90 mM

or 10  $\mu\text{M}$  nicotine did not induce  $[\text{Ca}^{2+}]_i$  dynamics in 35 cells tested (data not shown).

*Photodynamic stimulation on  $[\text{Ca}^{2+}]_i$  dynamics in SBC-3 cells*

The effects of photodynamic stimulation on  $[\text{Ca}^{2+}]_i$  were examined as follows. After confirming methacholine-induced  $[\text{Ca}^{2+}]_i$  dynamics, the SBC-3 cells were superfused with 5  $\mu\text{M}$  AIPcS<sub>4</sub>-containing HR for 10 min. After washing out AIPcS<sub>4</sub> with HR for 5 min, the cells were exposed to 190 kJ/m<sup>2</sup> of light (>580 nm) through a fiber optic bundle, causing activation of photosensitizer molecules that remained bound to the cells. The photosensitizer AIPcS<sub>4</sub> had no effect on  $[\text{Ca}^{2+}]_i$  in the dark (ambient light <20 nW). Light illumination alone in the absence of AIPcS<sub>4</sub> also had no effect. The exposure to light caused an elevation of both 340 nm- and 380 nm-excited fluorescence intensities, inducing an alteration of the fluorescence ratio, and in turn, calculating artificial  $[\text{Ca}^{2+}]_i$  during the exposure to light. After cessation of illumination it was found that a rapid rise in  $[\text{Ca}^{2+}]_i$  was followed by a sustained increase on which small fluctuations were superimposed during the whole period of  $[\text{Ca}^{2+}]_i$

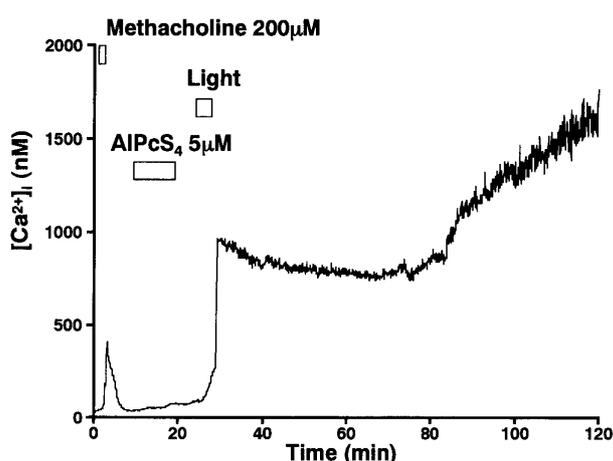


Fig. 2. An example of the effect of photodynamic stimulation on  $[\text{Ca}^{2+}]_i$  dynamics. Exposure to 190 kJ/m<sup>2</sup> of light (>580 nm) was made after AIPcS<sub>4</sub> loading (n=36). Methacholine, AIPcS<sub>4</sub>, or light illumination was applied as indicated by the horizontal bars.

measurement for up to 90 min in 36 SBC-3 cells out of 45 cells tested (Fig. 2).

*Photodynamic stimulation-induced  $[\text{Ca}^{2+}]_i$  dynamics in the absence of extracellular  $\text{Ca}^{2+}$  or in the presence of  $\text{Ca}^{2+}$  entry blockers.*

To obtain an insight into the mechanism of photodynamic stimulation that causes the sustained increase in  $[\text{Ca}^{2+}]_i$ , we examined the influence of  $\text{Ca}^{2+}$  removal from the extracellular environment or the effect of addition of various  $\text{Ca}^{2+}$  entry blockers on the  $[\text{Ca}^{2+}]_i$  response to photodynamic stimulation. The increased  $[\text{Ca}^{2+}]_i$  response to photodynamic stimulation was completely abolished by the removal of extracellular  $\text{Ca}^{2+}$ . Reintroduction of extracellular  $\text{Ca}^{2+}$  immediately led to a rapid elevation of  $[\text{Ca}^{2+}]_i$  to the plateau level on which small fluctuations were superimposed in 4 cells (Fig. 3). Addition of Ni<sup>2+</sup> (2 mM) or nifedipine (10  $\mu\text{M}$ ) did not affect the  $[\text{Ca}^{2+}]_i$  increase in response to photodynamic stimulation (n=5 and n=3, respectively, data not shown). Administration of SK&F 96365 (50  $\mu\text{M}$ ), a receptor-mediated and voltage-dependent  $\text{Ca}^{2+}$  entry blocker which could block  $\text{Ca}^{2+}$  channel currents

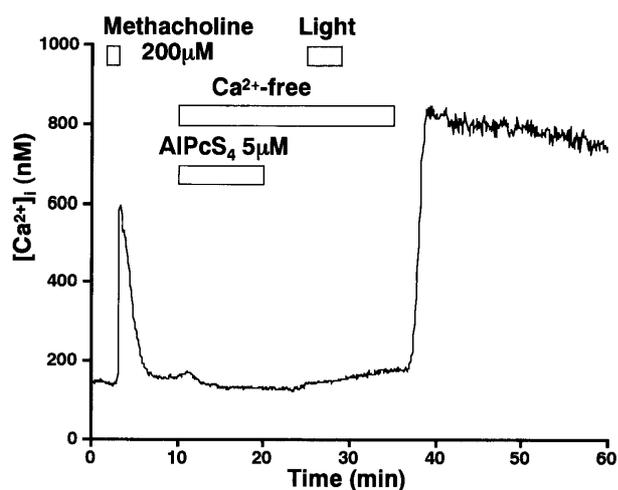


Fig. 3. An example of inhibition of  $[\text{Ca}^{2+}]_i$  increase caused by photodynamic stimulation by the removal of extracellular  $\text{Ca}^{2+}$ . (n=4). The  $\text{Ca}^{2+}$ -free buffer containing EGTA was superfused during the period indicated by the horizontal bar.

in single patch-clamped vascular smooth-muscle cells from rabbit ear artery<sup>27)</sup> and inhibit the methacholine-induced  $[Ca^{2+}]_i$  elevation in rat adrenal chromaffin cells<sup>5)</sup>, however, did not affect the response to photodynamic stimulation in 6 cells (Fig. 4). These results suggest that the photodynamic stimulation accelerates entry of extracellular  $Ca^{2+}$  which was insensitive to  $Ni^{2+}$ , nifedipine, and SK&F 96365.

#### *Cytotoxicity caused by photodynamic stimulation*

To examine cytotoxicity of photodynamic stimulation in SBC-3 cells, cell viability was examined by trypan blue exclusion test<sup>32)</sup> at 120 min after photodynamic stimulation. None of the cells tested ( $n=120$ ) could exclude the dye, indicating that photodynamic stimulation under this condition induced cell injury in SBC-3 cells. Additionally, all cells tested ( $n=123$ ) in the  $Ca^{2+}$ -free solution also showed cell injury. These results indicate that cell injury induced by photodynamic stimulation under this condition was independent of extracellular  $Ca^{2+}$ .

#### Discussion

The present study demonstrated for the first time that the photodynamic stimulation caused a rapid rise in  $[Ca^{2+}]_i$  followed by a sustained increase in SBC-3 cells in the presence of extracellular  $Ca^{2+}$ . The  $[Ca^{2+}]_i$  increase triggered by photodynamic stimulation was likely to be the result of permanent transfixation of cellular processes, leading to long-lasting  $Ca^{2+}$  entry. The photodynamic stimulation may induce oxidative stress to generate the excited delta singlet molecular oxygen<sup>7)</sup>, resulting in impairment of  $Ca^{2+}$  transport and subsequent perturbation of  $[Ca^{2+}]_i$  homeostasis, which, in turn, causes a sustained  $[Ca^{2+}]_i$  increase. The  $[Ca^{2+}]_i$  increase triggered by photodynamic stimulation was abolished in  $Ca^{2+}$ -deficient environment, but was not inhibited by SK&F 96365, a receptor-mediated and voltage-dependent  $Ca^{2+}$  entry blocker,  $Ni^{2+}$ , or nifedipine. However,

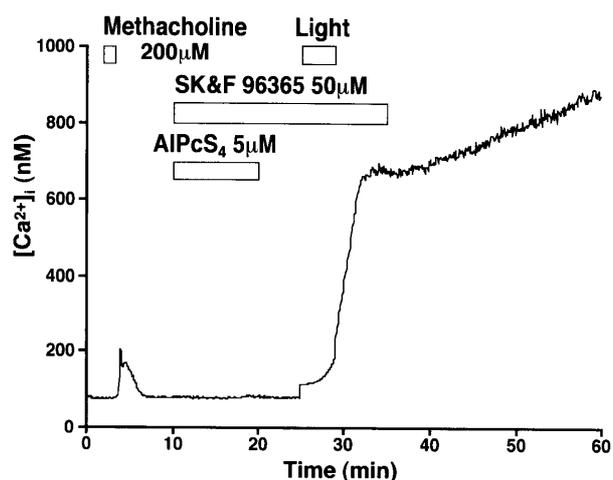


Fig. 4. An example of lack of inhibition by SK&F 96365 on  $[Ca^{2+}]_i$  increase induced by photodynamic stimulation ( $n=6$ ). SK&F 96365 was added to HR during the period indicated by the horizontal bar.

the cytotoxic results suggested that photodynamic stimulation induced cell injury regardless the presence or the absence of extracellular  $Ca^{2+}$ . These data indicate that the excited delta singlet molecular oxygen generated by photodynamic stimulation may induce membrane damage, resulting in an increase in  $Ca^{2+}$  permeability of cellular membrane, and in turn, causing rise of  $[Ca^{2+}]_i$  in the presence of extracellular  $Ca^{2+}$ . In a recent report, it was shown that illumination with ultraviolet light in the near-visible range induced a sustained nonsaturable increase in membrane conductance in various mammalian cells, to which non-selective cations current could be attributed<sup>25)</sup>. This report suggested that the light-induced non-selective cation current was caused by phototoxic or free radical-induced membrane damage, corresponding with our speculation.

The present study also demonstrated that physiological  $[Ca^{2+}]_i$  dynamics is caused by methacholine ( $200 \mu M$ ) in SBC-3 cells. The dynamics may be mediated by muscarinic receptor, a  $G_q$ -protein-coupled receptor, since the rise was inhibited by atropine.

Changes of  $[Ca^{2+}]_i$  in a physiological range within  $2\ \mu\text{M}$  can activate several cellular functions, such as secretion, contraction, enzyme activation, and cell cycle regulation<sup>4,24,36</sup>). The increase in  $[Ca^{2+}]_i$  may also be a signal for accelerating the electron transfer system. A view that mitochondrial  $Ca^{2+}$  transport regulates the free  $Ca^{2+}$  concentration of the mitochondrial matrix ( $[Ca^{2+}]_m$ ) has been proposed<sup>8,12</sup>). Hormones, neurotransmitters, and other extracellular signals which stimulate ATP-requiring processes such as secretion or muscle contraction by increased  $[Ca^{2+}]_i$  could also activate intramitochondrial oxidative metabolism and, hence, promote the replenishment of ATP<sup>8</sup>). The  $[Ca^{2+}]_i$  is maintained within a very narrow range ( $0.05\text{--}1\ \mu\text{M}$ ) by  $Ca^{2+}$  pump in the plasma membrane and  $Ca^{2+}$  stores, despite an enormous concentration gradient across the plasma membrane which tends to drive  $Ca^{2+}$  into the cell. It has been shown that an excess and sustained  $[Ca^{2+}]_i$  increase ( $>3\ \mu\text{M}$ ) elevates  $[Ca^{2+}]_m$  and brakes the electron transfer system in the mitochondrial matrix<sup>23</sup>), and activates various  $Ca^{2+}$ -dependent degradative enzymes, for example, phospholipases, proteases and endonucleases, which may contribute to cell death<sup>29</sup>).

These results indicate that, under physiological condition where extracellular  $Ca^{2+}$  is present, photodynamic stimulation of AIPcS<sub>4</sub> causes an increase in  $Ca^{2+}$  permeability of the plasma membrane, inducing extraordinary elevation of the  $[Ca^{2+}]_i$  which can be maintained within  $2\ \mu\text{M}$  by  $Ca^{2+}$  pump in the plasma membrane and  $Ca^{2+}$  stores for several tens of minutes. This may result in activation of various intracellular cascades, some of which may be, at least in part, attributable to cell injury. However, in the absence of  $Ca^{2+}$ , trypan blue exclusion test also revealed the cell injury induced by photodynamic stimulation. This result would reject the above view but whether the mechanism(s) of cell injury caused under these two different conditions is the

same or not and whether  $[Ca^{2+}]_i$  increase is not mandatory for cell death remains to be determined because trypan blue exclusion test itself cannot give any actual insights into mechanisms and time courses which may occur in the injuring cell.

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