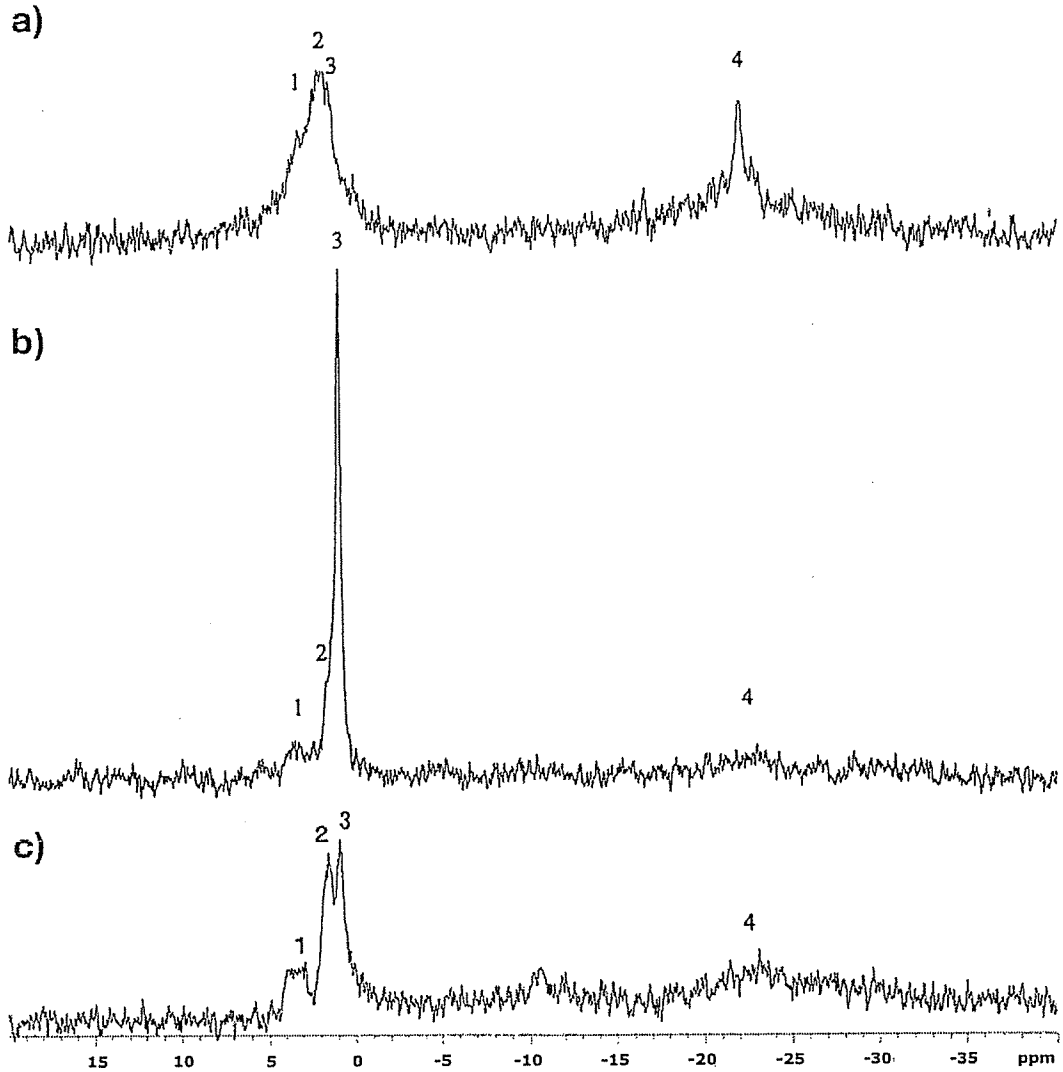

Fig. 4. The response of polyphosphate metabolism in *C. acidophila* determined by ^{31}P-NMR spectra. a: An untreated cell. b: Cell treated with Cd for 3 days. c: Cells re-incubated in Sager–Granick medium for 3 days after Cd treatment for 3 days. Signals were assigned as follows: 1, sugar monophosphate; 2, cytoplasmic inorganic phosphate; 3, vacuolar phosphate; 4, polyphosphate (non-terminal phosphate residues).

vacuolar deposits included phosphate and cadmium traces (Fig. 3b). Many researchers have reported that electron-dense deposits were from polyphosphate bodies. Polyphosphate bodies have the ability to accumulate metals and also to protect algal cells from metal toxicity [8,15].

The exact role of vacuoles in heavy-metal detoxification is not yet clear, but vacuolation could contribute to compartmentalization of toxic metals, as suggested by Davies et al. [15]. In yeasts and fungi a large proportion of the accumulated ions is located inside the vacuole in an ionic form or bound to the polyphosphates of lower molecular mass [16]. During our investigation, the peak of polyphosphate disappeared almost completely with an intense increase in the vacuolar phosphate peak (Fig. 4b) after Cd stress.

Inorganic polyphosphates are linear polymers of many orthophosphate (P_i) residues linked by high-energy phosphoanhydride bonds and are found in cells of all organisms [17]. This observation indicates that polyphosphate hydrolyzed, forming shorter-chain species or orthophosphates and combined with Cd in vacuoles. Recent studies have pointed out that the degradation of polyphosphate into orthophosphate is more important for heavy-metal tolerance than the polyphosphate level in the cells [18–20]. Cells that had recovered from the Cd stress simultaneously increased the peaks of cytoplasmic and sugar phosphate. A vacuolar phosphate peak remained (Fig. 4c) and recovered cells did not return to their former phosphate level. Under osmotic stress, a reversible precursor–product relationship existed between cytoplasmic phosphate and polyphosphate in mycelium of *Neurospora crassa* [21]. However, under Cd stress, the vacuolar phos-

phate, combined with Cd in the recovered cells, could not convert to cytoplasmic phosphate. Vacuolar phosphate–Cd must be the final state for metal detoxification.

In this study we have confirmed that the role of vacuoles and polyphosphate degradation is important in heavy-metal detoxification.

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EVALUATION OF THE CORNEA CELLS AFFECTED BY MULTI-PURPOSE SOLUTIONS FOR CONTACT-LENS

Jeongung PARK and Toshie TSUCHIYA

Division of Medical Devices, National Institute of Health Sciences, 1-18-1 kamiyoga, setagaya-ku, Tokyo 158-8501, Japan

Abstract

We studied the influence of multi-purpose solutions (MPS) on gap-junctional intercellular communication (GJIC) of cornea cells and its cell toxicity such as cell metabolism and proliferation. This study evaluates long-term safety of three types of multi-purpose solutions (MPS) in the cornea cells. Three types of MPS were investigated using scrape-loading dye transfer (SLDT) method to measure GJIC. Cell toxicity was evaluated for cell viability according to colony method and USP elution method (MEM Elution Test). In the SLDT method, one type solution was significantly showed an inhibitory effect on the GJIC in cornea cells but not the other type solutions. Comparative cytotoxicity potentials of three types of MPS also indicated similar tendency in the result of SLDT method. These results suggest that the evaluation methods are considered to be effective to confirm the safety of MPS.

Key words: multi-purpose solution, contact lense, gap-junctional intercellular communication, cytotoxicity,

1. Introduction

Recently, various multi-purpose solutions (MPS) have been introduced to the market. Lens-cleaning products and surfactants also are common component in many types of MPS [1]. These components are the most important factor in safety of cornea cells for a

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long-term use. Spanakis *et al.* reported that cultured corneal fibroblasts and myofibroblasts have functional gap junctions to maintain intercellular communication with themselves and with nonactivated keratocytes [2]. To examine gap junction dependent intercellular communication (GJIC), a fluorescent dye transfer protocol based on the scrape-loading and dye transfer (SLDT) method developed by El-fouly *et al.* [3,4,5] was used for cultured monolayers. Gap junction channels play important roles in the maintenance of the stratified structure of the corneal cells [6,7]. Gong *et al.* reported the importance of gap junctions in maintaining normal lens transparency by providing a cell-cell signaling pathway [8]. We described a method for determining the inhibitory effects of MPS on the GJIC along with its cytotoxic effects. The method provides a means for the direct exposure of cornea cells to MPS. Cytotoxicity was indicated by significant increases in the number of dead cells relative to controls. In this study, three commercial MPS containing a variety of preserving/disinfecting agents were examined. The results indicate that this method is useful for measuring the safety of a multipurpose solution for contact lenses.

2. Materials and Methods

Cell culture: Human cornea cells were cultured in DMEM (GIBCO BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO BRL) and antibiotics [penicillin (100 unit/ml)-streptomycin (100 μg/ml)]. Human cornea cells in DMEM-10% FCS medium were maintained in a humidified 5% CO_2 incubator at 37 °C. The cells were allowed to form a fully confluent monolayer.

Scrape-loading and dye transfer (SLDT) analysis: Human cornea parenchymal cells were incorporated at very high densities into the dish and allowed to form a fully confluent monolayer. The cell-monolayer was rinsed three times with phosphate-buffered saline containing Ca^{2+} and Mg^{2+} [PBS(+)] before the addition of the fluorescent dye (Lucifer yellow : MW 457.2). The cell monolayer was scraped using a surgical blade and loaded with 0.1% Lucifer yellow solution. The dye solution was left with the cells, and they were incubated at 37 °C for 5 min in a humidified atmosphere containing 5% CO_2 and 95% air. The dye solution was discarded from 35 mm^2 plastic Petri dishes, and the dishes were washed three times with PBS(+) solution to remove detached cells and background fluorescence. The distance of dye migration

was measured at room temperature under the fluorescence microscope, equipped with a type UFX-DXII and Super High Pressure Mercury Lamp Power Supply (NIKON, Japan). Measurement was carried out within 10 min after dye-loading.

MEM elution test: For measurement of cell toxicity, 5 x 10^4 cells seeded into 12 well plastic dishes. After 1 day, 0, 3.15, 6.25, 12.5, 25, 50, 100% volumes of multi-purpose solutions (MPS) in the media were applied to each well. The MPS-treated dishes were kept at 37 °C for 5 min in a humidified atmosphere containing 5% CO_2 and 95% air. After 3 days of MPS treatment, the extent of cell toxicity was measured by alamarBlue™ assay.

3. Results and Discussion

The GJIC functions of cornea cells were affected by MPS. Assessment of GJIC function was performed by SLDT assay using Lucifer yellow. Figure 1 shows fluorescent images of cells exposed to three types of MPS. The images show that Lucifer yellow diffuses through gap junctions from loaded cells to neighboring cells. The profile of percentages of dye migration in cornea cells is shown in Fig. 2. In the presence of MPS-A, dye transfer length has no significant change in comparison with the control (Fig. 2). While, the dye transfer extents of the cells treated with MPS-B and MPS-C were lower than that of the control. Decrease rate of dye transfer of MPS-B and MPS-C to the control is about 30% (Fig. 2). Therefore, these data indicated that cornea cells cultured with the MPS-B and MPS-C were induced GJIC down-regulation. These results suggest that the components of the MPS-B and MPS-C might cause the GJIC down-regulation.

Cell toxicity was evaluated for the assessment of the cell viability according to colony method and USP elution method. Figure 3 shows the estimation result of the cell toxicity performed by USP elution test. In all samples tested, the cell toxicities were higher than in saline buffer samples as the control. Thus, cell toxicities of all samples highly increased between 25% and 50% of MPS concentrations in the cell culture medium. Especially, the cell toxicity of MPS-B indicated high level in the colony method (data not shown). Based on these results, our results indicated that the conditions of MPS concentrations and MPS components are closely associated with safety of cornea cells, specifically with the expression of gap junction channels. Further investigations are required to clarify the cause of the inhibitory action on the GJIC and its *in vivo* adverse effects.

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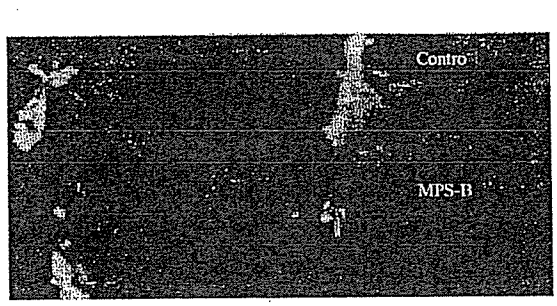

LY transfer after 2 hr incubation using various samples (20%)

Fig.1 Appearace of fluorescence dye of Lucifer yellow in human corneal cells for the estimation of the function of GJIC using Scrape loading dye transfer assay.

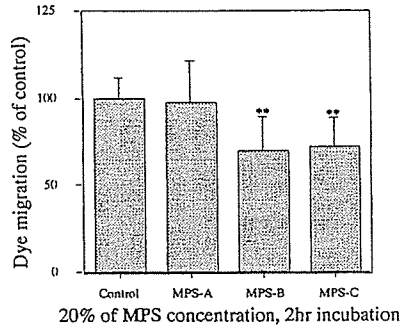

Fig.2 Determination of dye migration after scrape loading dye transfer assay using human corneal cell incubated with 20% of MPS solutions for 2 hours.

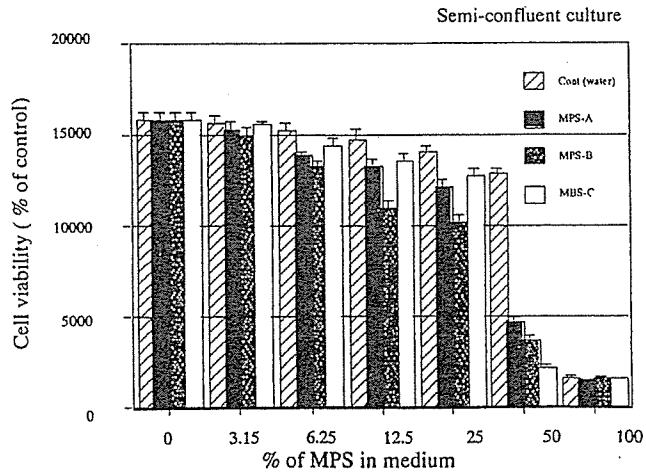

Fig.3 Cell viability of human corneal cells after exposure with various concentrations of MPS solutions using alamarBlue assay.

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Estrogens inhibit L-glutamate uptake activity of astrocytes via membrane estrogen receptor α

Kaoru Sato,* Norio Matsuki,† Yasuo Ohno* and Ken Nakazawa*

**Division of Pharmacology, National Institute of Health Sciences, Kamiyoga, Setagaya-ku, Tokyo Japan*

†Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan

Abstract

We investigated the effects of estrogen-related compounds including xenoestrogens [17β-estradiol (E2), 17α-ethynylestradiol (EE), diethylstilbestrol (DES), *p-nonylphenol* (PNP), bisphenol A (BPA) and 17α-estradiol (17α)] on L-glu uptake by cultured astrocytes via glutamate-aspartate transporter (GLAST). After 24 h treatment, E2 inhibited the L-glu uptake at 1 μM and higher concentrations. EE and DES also inhibited the L-glu uptake at 1 nM and higher concentrations. The other four compounds had no effect. The effects of E2, EE and DES were completely blocked by 10 nM of ICI182 780 (ICI). β-Estradiol 17-hemisuccinate : bovine serum albumin (E2-BSA), a membrane-impermeable conjugate of E2, also elicited the inhibition of L-glu uptake at 1 nM and higher concentrations, and the effect was blocked by ICI. 16α-Iodo-17β-estradiol (16αIE2), an estrogen receptor α (ERα) selective ligand, revealed an inhibitory effect at 10 nM, while genistein, an ERβ selective ligand, failed to reveal such an effect at this concentration. Western blot analysis showed that the predominant ER of cultured astrocytes was ERα. The colocalization of ERα with GLAST on plasma membranes was immunohistochemically detected in these cells. From these results, we concluded that estrogens down-regulate L-glu uptake activity of astrocytes via membrane ERα.

Keywords: astrocyte, L-glutamate transporter, estrogen, xenoestrogen, membrane ERα, E2-BSA.

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Abundant expression of estrogen receptors (ERs) is observed in the central nervous system (CNS), and it has been clarified that estrogens play diverse roles in regulating structures and functions of neuronal systems (Weiland 1992; Wong and Moss 1992; Wooley and McEwen 1994; Foy *et al.* 1999). Xenoestrogens, man-made non-steroidal compounds including pesticides and industrial by-products, were reported to mimic the actions of estrogens through interactions with ERs. However, recent reports have shown that estrogens have non-genomic effects through unknown mechanisms (Beyer and Raab 1998; Mermelstein *et al.* 1996; Gu *et al.* 1999). We also reported that estrogens and xenoestrogens modified mossy fiber-CA3 synapses, and thereby caused CA3-selective hypersensitivity to glutamate through non-genomic mechanisms (Sato *et al.* 2002). Thus, it is possible that xenoestrogens have more various risks than ever estimated before.

L-Glu is a major excitatory neurotransmitter in the CNS. L-Glu transporters are the only significant mechanism for the removal of L-glu from extracellular fluid and the maintenance of low and non-toxic concentrations of L-glu (Balcar and Johnston 1972; Logan and Snyder 1972; Johnston 1981). A growing body of evidence has suggested the importance of L-glu transporters in synaptic events, i.e. the time course of neuronal activation and the number of neurons activated by L-glu (Oliet *et al.* 2001; Thompson 2003). In spite of the significant importance of this system, the implication of estrogens in L-glu uptake by L-glu transporters is still

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Address correspondence and reprint requests to Kaoru Sato, Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan. E-mail: kasato@nihs.go.jp

Abbreviations used: 17α, 17α-estradiol; 16αIE2, 16α-iodo-17β-estradiol; BPA, bisphenol A; CNS, central nervous system; DES, diethylstilbestrol; DHK, dihydrokainic acid; E2, 17β-estradiol; E2-BSA, β-estradiol 17-hemisuccinate : bovine serum albumin; EE, 17α-ethynylestradiol; EMEM, Eagle's medium; ER, estrogen receptor; GFAP, glial fibrillary acidic protein; GLAST, glutamate-aspartate transporter; GLD, Glutamate dehydrogenase (NAD(*p*) +); HS, horse serum; ICI, ICI 182 780; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; L-glu, L-glutamate; PI3K, phophatidylinositol 3-kinase; PNP, *p*-nonylphenol; THA, DL-threo-β-hydroxy-aspartic acid.

unknown. Therefore, we evaluated the effects of estrogens [17β-estradiol (E2); 17α-ethynylestradiol (EE), an estrogen used for oral contraceptive pills and diethylstilbestrol (DES), a synthetic estrogen for preventing miscarriages] and xenoestrogens [*p*-nonylphenol (PNP), the degradation product of surface active agents used as a supplement of resins and bisphenol (BPA), a content of canned food, dental sealants and composites) on L-glu uptake by cultured astrocytes. Here we report novel effects of estrogens: these compounds down-regulated L-glu uptake activity of astrocytes via membrane ERα.

Materials and methods

Materials

Eagle's minimal essential medium (EMEM) was purchased from Nissui (Tokyo, Japan). Donor horse serum (HS; gelding) was from C-C Biotech Corporation (Valley Center, CA, USA). L-Glu, glutamate dehydrogenase (NAD(*p*)+) (EC 1.4.1.3) (GLD), genistein, paraformaldehyde (PFA), polyoxyethylene (10) octylphenyl ether (TritonX-100) and dimethylformamide were from Wako Pure Chemical (Osaka, Japan). β-NAD, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-methoxyphenazine methosulfate (MPMS), dihydrokainic acid (DHK) DL-threo-β-hydroxy-aspartic acid (THA), E2, EE, DES, PNP, 17α, E2-BSA, BSA, and peroxidase-conjugated anti-rabbit IgG antibody were from Sigma (St Louis, MO, USA). BPA and sodium dodecyl sulfate (SDS) were from Nacalai tesque (Kyoto, Japan). ICI was from Tocris (Ballwin, MO, USA). 16αIE2 (Hochberg and Rosner 1980) was a gift from Dr R. B. Hocheberg (Yale University, New Haven, CT, USA). Rabbit polyclonal IgG to ERα (MC-20) was from Santa Cluz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal IgG to ERβ (Ab-1) was from Oncogene Research Products (Cambridge, MA, USA). Mouse IgG to glial fibrillary acidic protein (GFAP) and enhanced chemiluminescence (ECL) kit were from Amersham Biosciences (Arlington Heights, IL, USA). The guinea pig antiserum to GLAST was from Chemicon international (Temecula, CA, USA). All second antibodies used for immunohistochemical analysis were from Molecular Probes (Eugene, OR, USA).

Cell culture

All procedures in this study were in accordance with the guidelines of the National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo, Japan. Primary cultures of astrocytes were prepared from the cerebral cortices of 3-day-old neonates of Wistar rats, as described previously (Suzuki *et al.* 2001). Briefly, dissociated cortical cells were suspended in modified EMEM containing 30 mM glucose, 2 mM glutamine, 1 mM pyruvate and 10% fetal bovine serum. They were then plated on uncoated 75 cm^2 flasks at the density of 600 000 cells/cm^2. A monolayer of type I astrocytes was obtained 12–14 days after the plating. Non-astrocytes such as microglia were detached from the flasks by shaking and were removed by changing the medium. Astrocytes in the flasks were dissociated by trypsinization and reseeded on uncoated 96 well micro titer plates or cover glasses at 20 000 cells/cm^2. When astrocytes became confluent (approximately 9–10 days after the reseeding), the medium was switched to modified EMEM containing 10% HS (gelding), followed by 7 day-incubation. In this culture, > 98% of the cells were identified as type I astrocytes by positivity for GFAP and by their flattened, polygonal appearance.

Measurement of the extracellular L-glu concentration

The extracellular L-glu concentration was measured by the colorimetric method modified by Abe *et al.* (2000). Briefly, 50 μL of culture supernatant was transferred to a 96-well micro titer plate and was mixed with 50 μL of substrate mixture consisting of 20 U/mL GLD, 2.5 mg/mL β-NAD, 0.25 mg/mL MTT, 100 μM MPMS and 0.1% (v/v) Triton X-100 in 0.2 M Tris-HCl buffer (pH 8.2). After 10 min-incubation at 37°C, the reaction was stopped by adding 100 μL of solution containing 50% (v/v) dimethylformamide and 20% (wt/vol) SDS (pH 4.7). In this reaction, MTT (yellow) is converted into MTT formazan (purple) in proportion to the L-glu concentration (Fig. 1b). The amount of MTT formazan was determined by measuring the absorbance with a microplate reader at 570 nm (test wavelength) and at 655 nm (reference wavelength).

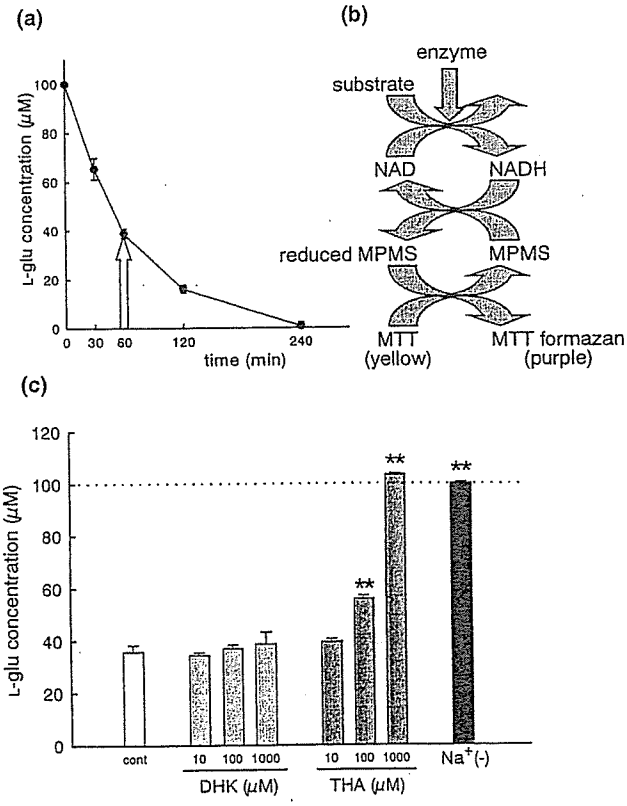


Fig. 1 The features of the extracellular L-glu clearance by cultured astrocytes. (a) The clearance of exogenously applied L-glu. Concentrations were measured by the colorimetric method using MTT. 100 μM of extracellular L-glu was totally clearanced within 4 h. (b) The principle of the colorimetric measurement of L-glu concentrations. (c) THA (1 mM) completely blocked the L-glu clearance, while DHK (1 mM) had no effect. When the extracellular Na^+ was removed, the L-glu clearance was completely blocked (**$p < 0.01$ vs. control group. $N = 4$, Tukey's test following ANOVA).

The concentration of L-glu was estimated from the standard curve, which was constructed in each assay using cell-free media containing known concentrations of L-glu.

Drug treatment

L-Glu was dissolved at 1 mM in phosphate buffered saline (PBS) and diluted to 100 μM with the culture medium. DHK and THA were freshly dissolved at 1 mM and diluted to final concentrations with the culture medium. E2, EE, DES, PNP, BPA and 17α were dissolved at 10 mM in ethanol and diluted to final concentrations with the culture medium. E2-BSA was freshly dissolved at 100 μM and diluted to final concentrations with the culture medium. ICI was dissolved at 1 mM in ethanol and coapplied with E2, EE, DES and E2-BSA at final concentrations. 16αIE2 and genistein were dissolved at 1 mM in ethanol and diluted to final concentrations with the culture medium.

Western blot analysis

Cells were washed twice with ice-cold PBS and then harvested. After intense sonication (23 kHz, 1 min × 3), the cell suspension was centrifuged at 800 g for 5 min at 4°C. An aliquot of this supernatant was removed for protein assay. Another aliquot was diluted in SDS sample buffer. Molecular mass markers and protein samples that contain equal amounts of protein were separated by electrophoresis on 10% polyacrylamide-SDS gels and transferred onto polyvinyliden difluoride membranes. The membranes were incubated with PBS containing 0.5% (v/v) Tween20 and 5% (wt/vol) skim milk for 1 h at 25°C, followed by overnight incubation at 4°C with rabbit polyclonal IgG to ERα (1 : 1000) or rabbit polyclonal IgG to ERβ (1 : 1000). The membranes were then washed for 30 min and incubated with peroxidase-conjugated anti-rabbit IgG (1 : 1000) for 1 h at 25°C. Immunoreactive proteins were visualized by the ECL kit. Specificities of the primary antibodies were confirmed by using the lysate of ovary cells in which strong expression of ERα and ERβ had already been confirmed. We also confirmed that the primary antibodies did not show any non-specific signals by using the lysate of COS7 cells that express neither ERα nor ERβ (Fig. 7a).

Immunohistochemistry

Cells were washed 3 times with PBS for 5 min and fixed with 4% PFA in 0.1 M phosphate buffer (PB) for 30 min at 4°C. After washing with Tris-buffered saline (TBS, pH 7.6), cells were treated with TBS containing 10% (wt/vol) BSA, 0.1% (v/v) TritonX-100 and 0.2% (v/v) Tween20 for 1 h at room temperature. For immunostaining of GFAP, cells were incubated overnight at 4°C with mouse IgG to GFAP diluted at 1 : 1000 in TBS containing 1% (wt/vol) BSA. After washing with TBS 3 times for 5 min, they were incubated with Alexa Fluor 488 rabbit antimouse IgG (1 : 200) for 3 h at room temperature (25°C). After washing with TBS 3 times for 5 min, fluorescent images were obtained by μ-Radiance laser scanning confocal system (Bio-Rad, Hercules, CA). For immunostaining of ERα or ERβ, cells were incubated overnight at 4°C with rabbit polyclonal IgG to ERα (1 : 500) or rabbit polyclonal IgG to ERβ (1 : 500). After washing with TBS 3 times for 5 min, cells were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1 : 200) for 3 h at room temperature. After washing with TBS 3 times for 5 min, fluorescent images were obtained by the laser scanning confocal system using a 60 × oil immersion objective. For double-immunostaining of ERα and GLAST, cells were incubated overnight at 4°C with a mixture of rabbit polyclonal IgG to ERα (1 : 500) and the guinea pig antiserum to GLAST (1 : 4000). After washing with TBS 3 times for 5 min, cells were incubated with the mixture of Alexa Fluor 488 goat anti-rabbit IgG (1 : 200) and Alexa Fluor 568 goat anti-guinea pig IgG (1 : 200) for 3 h at room temperature. After washing with TBS 3 times for 5 min, fluorescent images were obtained by the laser scanning confocal system using a 60 × oil immersion objective.

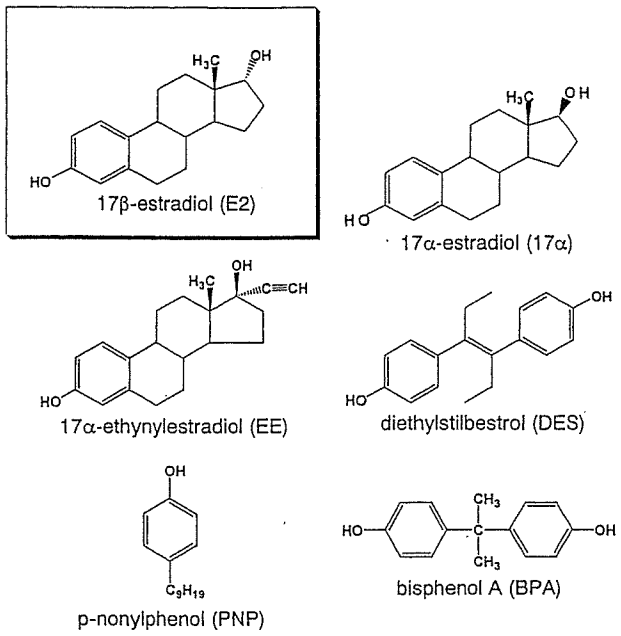
Statistical analysis

Data were obtained from 4 independent experiments (averaged values of 4 wells for each). Data are expressed as means of these data ± SEM values. Tests of variance homogeneity, normality and distribution were performed to ensure that the assumptions required for standard parametric ANOVA were satisfied. Statistical analysis was performed by one-way repeated-measure ANOVA and post hoc Tukey's test for multiple pairwise comparisons.

Results

L-Glu uptake activity of cultured astrocytes was evaluated by the clearance of exogenously applied L-glu. The principle of the measurement of L-glu concentrations is shown in Fig. 1(b). When L-glu was applied to the culture medium at 100 μM, it decreased to below half within 60 min and then to below 1 μM within 4 h (Fig. 1a). We measured the clearance of L-glu 60 min after the application as an index in following experiments. When the extracellular Na^+ was removed, the L-glu clearance was completely abolished (Fig. 1c), indicating that the extracellular L-glu was uptaken by Na^+ dependent L-glu transporter(s). To identify the predominant L-glu transporter(s) of cultured astrocytes, we co-applied THA (Balcar *et al.* 1977; an inhibitor of both of GLT-1 and GLAST) or DHK (Johnston *et al.* 1978; a selective inhibitor of GLT-1) with L-glu. THA (1 mM) completely blocked the L-glu clearance, while DHK (1 mM) had no effect, indicating that L-glu was uptaken by GLAST.

We investigated the effects of E2, EE, DES, PNP, BPA and 17α on the L-glu uptake by cultured astrocytes (Fig. 3). The chemical structures of these compounds are shown in Fig. 2. After 24 h treatment, E2 inhibited the L-glu clearance at 1 μM and higher concentrations. EE and DES also inhibited the L-glu clearance at 1 nM and higher concentrations. At 100 μM, E2, EE and DES (1 μM) inhibited the L-glu clearances by 34, 40 and 22%, respectively. The other four compounds had no effect. When ICI, a pure anti-estrogen, was co-applied at 10 nM with E2, EE and DES, it completely blocked the inhibitory effects of these compounds (Fig. 4), indicating that the effects were mediated by classical ER(s). When the extracellular Na^+ was removed or 1 mM THA was applied, the effect of E2 (1 μM) completely disappeared (Fig. 8),



Transactivity: E2≒EE≒DES>PNP>BPA>>17α

Fig. 2 The chemical structures of estrogens and xenoestrogens used in this study.

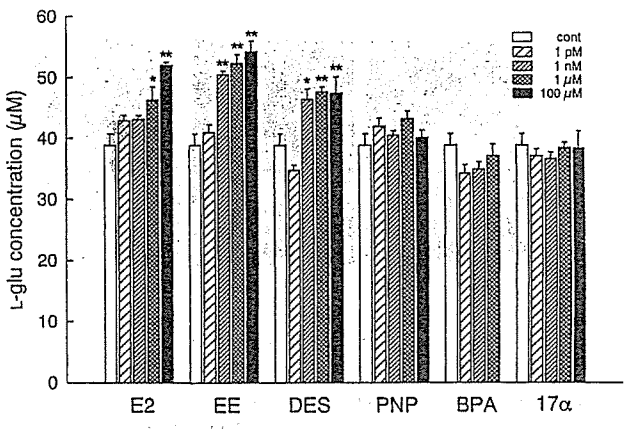


Fig. 3 The effects of estrogens and xenoestrogens on the L-glu clearance by cultured astrocytes. E2 (1–100 μM), EE (1 μM-100 μM) and DES (1 μM-100 μM) significantly inhibited L-glu clearance after 24 h treatment (*$p < 0.05$, **$p < 0.01$ vs. control of each group. $N = 4$, Tukey's test following ANOVA).

demonstrating that the inhibition of L-glu clearance was caused by down-regulation of L-glu uptake via GLAST.

Some reports have suggested that a part of classical ERs are translocated to plasma membranes and mediates acute effects of estrogens (Pappas *et al.* 1995; Razandi *et al.* 1999). Therefore, we next investigated the effect of E2-BSA, a membrane-impermeable conjugate of E2 and BSA, on the L-glu uptake by cultured astrocytes. As is shown in Fig. 5(a),

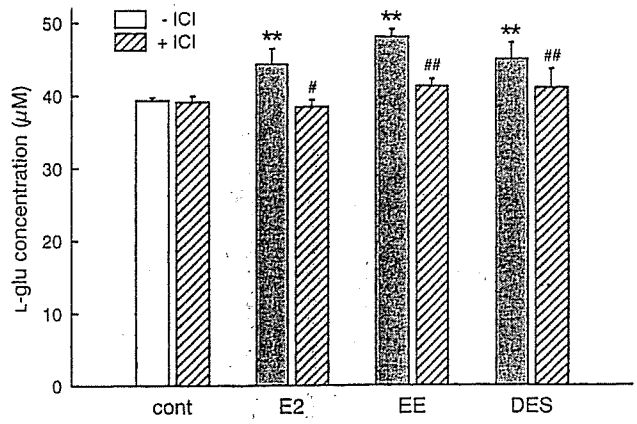


Fig. 4 The effects of ICI on the inhibitory effects of E2, EE and DES. When ICI (10 μM) was co-applied with these compounds (1 μM), the inhibitory effects were completely blocked (**$p < 0.01$ vs. control group without any treatments, ##$p < 0.01$ vs. the group exposed to E2, EE or DES, respectively. $N = 4$, Tukey's test following ANOVA).

L-glu clearance was inhibited dose-dependently by a 24 h treatment of E2-BSA. The effect was significant at 1 nM and higher concentrations, and the clearance was inhibited by 30% at 100 μM. BSA alone had no effect at the concentrations corresponding to those included in E2-BSA. ICI (10 nM) completely blocked the effect of E2-BSA (1 μM), implying that this effect was mediated by classical ERs on plasma membranes. When the extracellular Na^+ was removed or 1 mM THA was added, the effect of E2-BSA (1 μM) disappeared (Fig. 8), demonstrating that the effect of E2-BSA was caused by down-regulation of GLAST.

The results mentioned above indicate that estrogens down-regulate GLAST via classical ER(s) locating on the plasma membranes of cultured astrocytes. Therefore, we tried to identify the receptor isotype(s) mediating these effects. First, we tested the effects of 16αIE2 (Shughrue *et al.* 1999; Singh *et al.* 2000; a selective ligand for ERα) and genistein (Witkowska *et al.* 1997; a selective ligand for ERβ) on the L-glu uptake by cultured astrocytes (Fig. 6). At 10 nM, 16αIE2 revealed a significant inhibitory effect, while genistein had no effect at this concentration. When the extracellular Na^+ was removed or 1 mM THA was added, the effect of 16αIE2 (10 nM) disappeared (Fig. 8), demonstrating that the effect of 16αIE2 was caused by down-regulation of GLAST as in the case of E2 and E2-BSA. Western blot analysis of the lysate of cultured astrocytes detected a single band of ERα, while little or no signal of ERβ was observed (Fig. 7a). Thus, we compared the subcellular localization of ERα with that of GLAST immunohistochemically (Fig. 7b). Although ERα was distributed over the whole cell, the strong signals were observed on the plasma membrane and in the nucleus (left). Although weak signals of GLAST were distributed in the nucleus and the soma, much stronger signals scattered on the plasma membrane (center). When

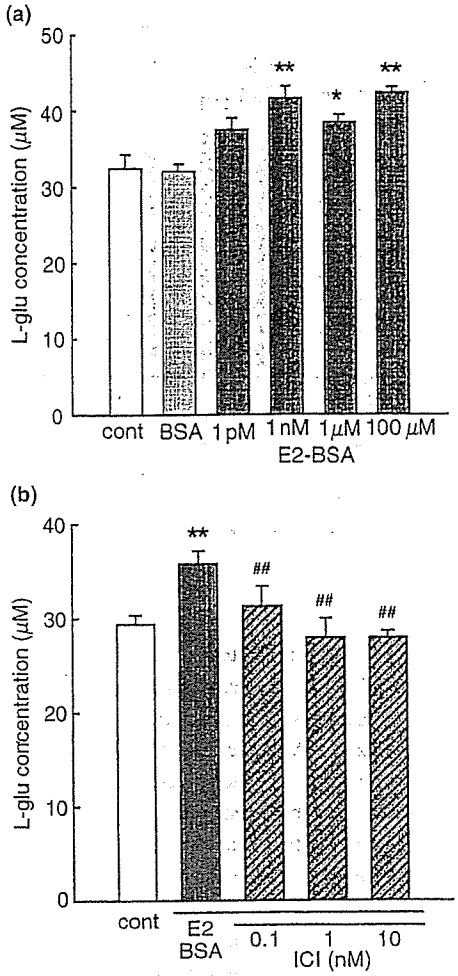


Fig. 5 The effect of E2-BSA on the L-glu clearance by cultured astrocytes. (a) E2-BSA (1 μM-100 μM) significantly inhibited the L-glu clearance after 24 h treatment (**$p < 0.01$ vs. control group. $N = 4$, Tukey's test following ANOVA). (b) ICI (10 μM) completely blocked the effect of E2-BSA (1 μM) (**$p < 0.01$ vs. control group without any treatment, ##$p < 0.01$ vs. the group exposed to E2-BSA. $N = 4$, Tukey's test following ANOVA).

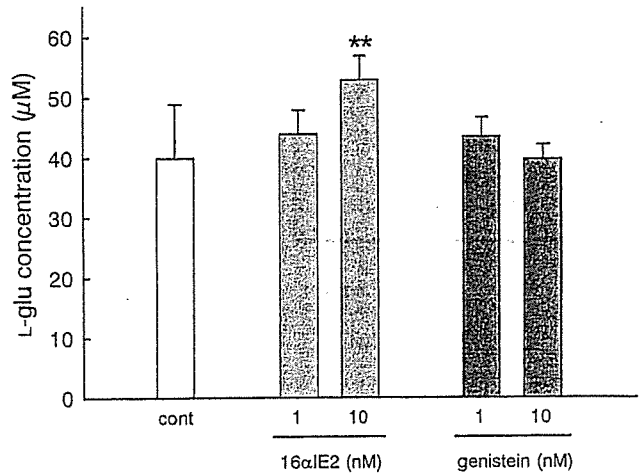


Fig. 6 The effects of 16αIE2 and genistein on the L-glu clearance by cultured astrocytes. Only 16αIE2 inhibited the L-glu clearance at 10 μM (**$p < 0.01$ vs. control group. $N = 4$, Tukey's test following ANOVA).

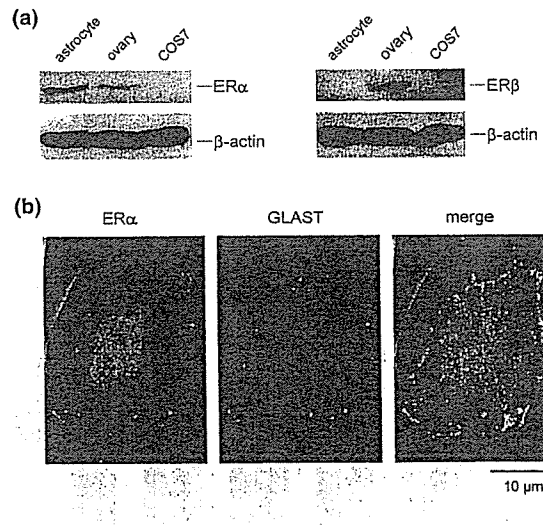


Fig. 7 Identification of ER isotype locating on plasma membranes of cultured astrocytes. (a) Western blot analysis of the lysate of cultured astrocytes showed a single band of ERα, while little or no signal of ERβ was observed. (b) Double immunostaining of ERα and GLAST. ERα was detected over the whole cell (green, left), whereas GLAST was detected on the plasma membrane and in the nucleus (red, middle). In the merged image (right), colocalization of ERα with GLAST on the plasma membrane became apparent (yellow dots).

two fluorescent images were merged, co-localization of ERα with GLAST on the plasma membrane became apparent (yellow dots, right). Taken together, these results strongly suggest that ERα is expressed on plasma membranes of cultured astrocytes and its activation causes the down-regulation of GLAST.

Discussion

We investigated the effects of estrogens and xenoestrogens on L-glu uptake activity of cultured astrocytes. Our results are summarized as follows: (1) estrogens (E2, EE and DES) inhibited the L-glu uptake of cultured astrocytes by 24 h treatment, while xenoestrogens had no effect, (2) ICI completely blocked the effects of E2, EE and DES, (3) E2-BSA inhibited the L-glu uptake by 24 h treatment and the effect was completely blocked by ICI, (4) 16αIE2 inhibited the L-glu uptake, while genistein had no effect, (5) the predominant ER of cultured astrocytes was ERα, (6) ERα was co-localized with GLAST on plasma membranes of cultured astrocytes.

L-Glu is a major excitatory neurotransmitter in the CNS. The only rapid way to remove L-glu from the extracellular fluid surrounding the receptors is by cellular uptake by L-glu

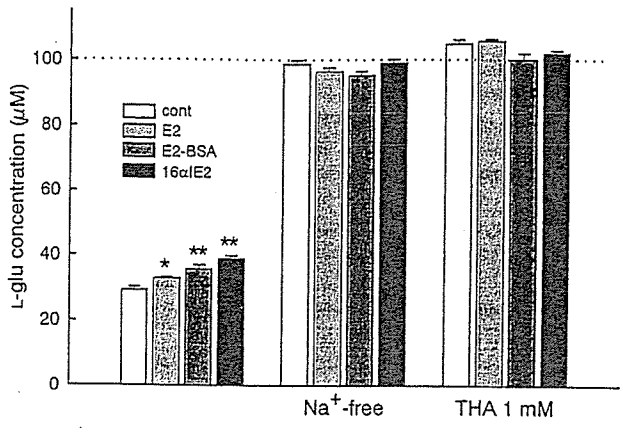


Fig. 8 E2, E2-BSA and 16αIE2 inhibited the extracellular L-glu clearance via the down-regulation of GLAST. When extracellular Na^+ was removed or 1 mM THA was added, the inhibitory effects of E2 (1 μM), E2-BSA (1 μM) and 16αIE2 (10 μM) completely disappeared (*$p < 0.05$, **$p < 0.01$ vs. control group. $N = 4$, Tukey's test following ANOVA).

transporters (Balcar and Johnston 1972; Logan and Snyder 1972; Johnston 1981). To evaluate L-glu uptake activity of cultured astrocytes, we monitored the changes in the concentration of exogenously applied L-glu by a modified colorimetric method. This assay has already been confirmed to be optimal for measuring 1–200 μM of L-glu (Abe *et al.* 2000). The precise L-glu concentration in the synaptic cleft following release is still controversial. Shikorski and Stevens 1997) reported the peak concentration of 12 μM based on the assumption that the vesicle emptying is fast and complete. On the other hand, Clements *et al.* (1992) estimated it 1.1 mM by EPSC recording technique.

Among estrogen-related compounds used in this study, estrogens (E2, EE and DES) inhibited the L-glu uptake by cultured astrocytes, while the other compounds had no effect. Because of the transactivities of these compounds (E2 ≒ EE ≒ DES > PNP > BPA) (Nishikawa *et al.* 1999), we speculated that the effects of E2, EE and DES were mediated by classical type of ER(s). This speculation was further supported by the finding that ICI, a pure inhibitor for both of ERα and ERβ, completely blocked the inhibitory effects of these compounds.

Some reports have suggested that a part of classical ERs are translocated to plasma membranes and mediate acute effects of estrogens. Razandi *et al.* (1999) reported that the transfection of CHO cells with classical ERs caused their expression in both nuclei and membranes. ERs on the plasma membranes of tumor cells were structurally similar to classical ERs (Pappas *et al.* 1995). In the present study, we tested E2-BSA, a membrane-impermeable conjugate of E2 and BSA. E2-BSA also inhibited the L-glu uptake by cultured astrocytes and its effect was completely blocked by ICI. E2-BSA is generally used for the discrimination between classical nuclear ERs and ERs locating on plasma membranes. However, Stevis *et al.* (1999) pointed out that 1/100 free E2 was present in the commercial preparations of E2-BSA. In our experiment, the inhibitory effect of E2-BSA was observed even at lower concentration than that of E2. Thus, we considered that E2-BSA itself revealed the effect through classical ER(s) locating on plasma membranes.

We tried to identify the receptor isotype(s) mediating these effects. To date, 2 isotypes of classical ERs (ERα and ERβ) have been cloned. We first investigated the effects of 16αIE2 and genistein on L-glu uptake. The affinity of 16αIE2 for ERα and ERβ are 5.03 nM and 146.7 nM, respectively, and the selectivity for ERα has been ensured below 10 nM (Shughrue *et al.* 1999; Singh *et al.* 2000). Genistein binds preferentially to ERβ in the low to mid nanomolar range (Witkowska *et al.* 1997). L-Glu uptake was significantly inhibited by 16αIE2, but not by genistein at 10 nM, suggesting that ERα mediates the inhibitory effects of estrogens. Western blot analysis showed that the predominant ER isotype of cultured astrocytes was ERα. Immunohistochemical analysis showed that ERα was co-localized with GLAST on plasma membranes. These results support the contribution of ERα to the L-glu uptake inhibition further. Although some reports have suggested the existence of membrane ERs in astrocytes (Beyer *et al.* 1999; Hosli *et al.* 2000), their isotypes are unknown. Recently, Toran-Allerand *et al.* (2002) reported that ER-X, a novel plasma membrane-associated ER, elicited rapid activation of mitogen-activated protein (MAP) kinases, ERK1 and ERK2, in organotypic neocortical cultures. Because the effects mediated through ER-X were not inhibited by anti-estrogens (Singh *et al.* 1999), we considered that ER-X did not contribute to the inhibitory effect of estrogens on L-glu uptake.

At present, several signal transduction pathways are known to down-regulate L-glu uptake via GLAST. Activation of cAMP pathway resulted in a transient decrease in GLAST mRNA (Espinoza-Rojo *et al.* 2000). Although PKC was also reported to interact with GLAST, its effect was more complicated. Pan-isoform PKC inhibition suppressed GLAST uptake activity (Bull and Barnett 2002; Lortet *et al.* 2002 ; Conradt and Stoffel 1997), while their activation decreased GLAST mRNA (Espinoza-Rojo *et al.* 2000). Cytokines were also reported to down-regulate GLAST uptake activity via NO production (Ye and Sontheimer 1996; Hu *et al.* 2000). Interestingly, estrogens have long been known as an important vasoprotective molecules that cause the rapid dilation of blood vessels (Guetta and Cannon 1996). Recent studies clarified that estrogen activated endothelial nitric oxide synthase (eNOS) through activation of PI3K via ERα locating on plasma membranes of endothelial cells and produced NO, thereby causing the rapid dilation of blood vessels (Chen *et al.* 1999; Haynes *et al.* 2000). We are currently investigating the mechanisms underlying the down regulation of GLAST via membrane ERα. We have confirmed that a 10 min-treatment of E2 or

E2-BSA elicits a significant inhibitory effect via the activation of phophatidylinositol 3-kinase (PI3K) (unpublished data). These results raise the possibility that the inhibitory effects of estrogens are mediated by the mechanisms including NO production. Taken the co-localization of ERα with GLAST into consideration, there might exist the signal transduction unit comprised of ERα, PI3K, NOS, GLAST, etc. in the vicinity of the cell surface, thereby causing the rapid down-regulation of GLAST. Whether the down regulation of GLAST was caused by the decrease in the transport activity or in the number of functional GLAST remains unknown. Although we have confirmed that total expression of GLAST is not changed by E2 or E2-BSA (unpublished data), the translocation of GLAST is currently under investigation.

To our knowledge, this is the first report showing that estrogens down regulate L-glu uptake activity of astrocytes via ERα locating on plasma membranes. A growing body of evidence has suggested that complex interplay between astrocytes and neurons modifies the formation, maintenance and efficacy of synapses (Oliet *et al.* 2001; Thompson 2003). L-Glu transporters of astrocytes regulate extracellular L-glu concentrations in the synaptic clefts, thereby terminating synaptic transmissions and preventing the neuronal excitotoxicity. Oliet *et al.* (2001) reported that the degree of the astrocytic coverage of neurons determined the diffusion of L-glu in the extracellular space and that the reduction in L-glu uptake by astrocytes affected neuronal transmitter release. Ultrastructural analysis showed that in the astrocytes that contact with neurons, ERα was translocated to the cell surface that surrounded neuronal spines (Milner *et al.* 2001). Estrogens might modulate synaptic functions via ERα on plasma membranes of astrocytes.

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2003

Effects of Multipurpose Solutions (MPS) for Hydrogel Contact Lenses on Gap-Junctional Intercellular Communication (GJIC) in Rabbit Corneal Keratocytes

Taizo Sumide, Toshie Tsuchiya

Division of Medical Devices, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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Abstract: To ensure the effects of multipurpose solutions (MPS) for hydrogel contact lenses on the cornea, the inhibitory activity of three types of MPS on corneal cells has been evaluated with the use of scrape loading and dye transfer assay (SLDT assay) and Western blotting on rabbit corneal keratocytes (RC4). In SLDT assay, MPS-A and poloxamine showed dose-dependent inhibitory activity, suggesting the inhibitory action of MPS-A and poloxamine to gap junctional intercellular communication (GJIC) in the tested cells. Moreover, after treatment with MPS-A, the GJIC was initially inhibited within 4 h, and thereafter gradually turned to an approximately 60% level of the initial value. When MPS-A was removed from the incubation media after exposure of the cells, the recovery of GJIC was time dependent and returned to approximately initial levels at 8 h. Complete recovery was established after approximately 24 h. These findings suggested that the inhibitory action of MPS-A on corneal keratocytes was reversible. This inhibition was accompanied by a decrease in the quantity of connexin-43, which is a major protein constituting the gap junctional channel of these cells, and its change in the phosphorylation state. Taken together, it was suggested that MPS-A interacts with connexin-43, inducing an inhibitory action on GJIC. © 2002 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 64B: 57–64, 2003

Keywords: multipurpose solution (MPS); gap junctional intercellular communication (GJIC); metabolic cooperation assay (MC assay); scrape loading and dye transfer (SLDT assay); connexin-43

INTRODUCTION

The use of contact lenses has gained popularity due to the comfort and convenience of lens wear. Contact lenses are medical devices that require proper care for safe use. Most complications associated with contact lens wear, such as conjunctivitis and red-eye syndrome, have been attributed to improper hygiene and noncompliance with recommended lens care procedures. Proper care of contact lenses requires the wearer to follow a precise lens care regimen, which normally includes cleaning, rinsing, and disinfection.

In general, there are two ways to disinfect hydrogel contact lenses. One is thermal disinfection, which was the first method used; the other is chemical disinfection. The advantages of thermal disinfection include short cycle times, efficacy against a variety of vegetative microorganisms, and low risk of ocular reaction when unpreserved saline is used. On the other hand, it may not be compatible with all lenses types, and repeated heating may change the physical parameters of lenses. Furthermore, denatured proteins adhering to the lenses because of heating cause an allergic reaction in some individuals. In an attempt to provide a more convenient, less destructive regimen than thermal disinfection, chemical systems were introduced. These contain antimicrobial agents that interact with microorganisms; each agent has a unique chemical structure and reactive groups that induce varying degrees of microbial damage. This system, which does not use heat, is termed cold disinfection. Multipurpose solutions (MPSs), a type of cold disinfection, were introduced recently. MPSs that can be used for cleaning, rinsing, disinfecting, and storing have become as popular as cold disinfection. The convenience of MPSs leads to high compliance. On the other hand, in contrast to other care solutions, the eye, especially the cornea, is exposed directly to MPS. Although the numbers of those who use MPSs have been increasing, there are few exact criteria to evaluate whether the MPSs have severe effects on the cornea.

Gap junctions are membrane channels that permit the transfer of ions and small molecules between neighboring cells.[1–3] These channels are composed of hexametric hemichannels, or connexons, which are attached to the con-

Correspondence to: T. Tsuchiya, Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
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TABLE I. Main Ingredients of Three Types of MPS

Chemical Disinfectants	Main Ingredients
MPS-A	Polyhexamethylenebiguanide, poloxamine
MPS-B	Polyhexamethylenebiguanide
MPS-C	Polidronium chloride

nexon in the plasma membrane of neighboring cells. Gap junctional intercellular communication (GJIC) is suggested to play an important role in cell growth, cell differentiation, and tissue homeostasis. Moreover, GJIC inhibition by chemical substrates and some materials has been recognized as being involved in tumorigenesis.[4,5] The gap junction is composed of connexin, which is a membrane protein. On the cornea, connexin-43 is expressed in epithelial cells, keratocytes, and endothelial cells.[6–9]

Previous studies have assessed three types of MPS with the use of a V79 MC assay,[10] which evaluates the inhibitory activity of the intercellular gap-junctional communication that plays important roles in tissue homeostasis with the use of the V79 cell line, as this assay is a more sensitive method to evaluate contact lens care solutions. The results indicated that one MPS had an inhibitory action on GJIC. Moreover, the surfactant, poloxamine, which is one of the ingredients, had an inhibitory action on GJIC. In the present study, the effects of MPS on gap-junctional intercellular communication were assessed by SLDT assay, which evaluates the GJIC. Moreover, an attempt was made to conduct an immunohistochemistrical method to focus on connexin-43, which is a major protein-constituting gap junctional channel of these cells, in rabbit corneal keratocytes.

MATERIALS AND METHODS

Materials

Three types of multipurpose solution (MPS-A, MPS-B, MPS-C) were investigated in this study. Their main ingredients are shown in Table I. PHMB was obtained from Avecia Co, Ltd. Poloxamine and poloxamer-407 were obtained from BASF. Lucifer Yellow was purchased from Molecular Probes, Inc.

Cell Culture

The standard medium of ME10 in this experiment was Eagle's Minimal Essential Medium (MEM) (Nissui Pharmaceutical Co., Ltd., Japan) containing 10 vol% fetal calf serum (Sanko Junyaku Co., Ltd., Japan).

Rabbit corneal keratocytes (RC4) were obtained from the RIKEN gene bank. They were thawed and cultured in ME10 medium in 25-cm^2 culture flasks (Corning) in incubators under standard conditions (37 °C, 5% CO_2, 95% relative humidity). The cells were fed every 2 days with ME10 and subcultured in 75-cm^2 culture flasks (Corning).

Scrape Loading and Dye Transfer Assay (SLDT Assay)

GJIC was assessed by scrape loading and dye transfer assay (SLDT assay).[11,12] As test solutions, appropriate concentrations of MPSs or their ingredients diluted by ME10 were used. After confluent RC4 cells were treated in 35-mm^2 dishes with test solutions, they were washed with phosphate-buffered saline containing $CaCl_2$ and $MgCl_2$ [PBS(+)]. Ten scrapes were made with a steel-blade scalpel and the cells were incubated with 0.2% Lucifer Yellow solution for 5 min under standard conditions (37 °C, 5% CO_2, 95% relative humidity). The cells were then washed with PBS(+).

The distance that Lucifer Yellow had traveled through gap junctions was observed and recorded with an inverted fluorescent microscope equipped with a camera. The distance was measured at 30 points per dish and the average value was calculated. Each value was analyzed statistically with Tukey(a) multiple-comparison test. Each experiment was performed in triplicate.

Western Blotting

First, confluent cultures of RC4 cells were treated with ME10 containing 0.065 vol% MPS-A for 30 min under standard conditions (37 °C, 5% CO_2, 95% relative humidity). Then, they were quickly washed with ice-cold PBS(+). The cells were lysed by treating with 2% SDS containing 1-mM phenylmethyl sulfonyl fluoride (PMSF), 50-mM NaF, and 100-mM Na_3VO_4. These cell lysates were centrifuged (10,000 rpm) at 4 °C for 20 min to remove insoluble material. The protein concentration of the lysates was determined with BCA assay (Pierce, IL). The proteins were separated by 8% SDS polyacrylamide gels and transferred to PVDF membrane at 120 V for 30 min. Connexin-43 was detected with the use of anti-connexin-43 monoclonal antibody (anti-CX43 Chemicon, CA), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibody and detection with ECL chemiluminescent detection reagent (Amersham Pharmacia Biotech UK Ltd., UK).[13]

Balb 3T3 Two-Steps Cell-Transformation Assay

Balb 3T3 clone A3-1-1-1 cells were provided by Dr. Kuroki, University of Tokyo. The cells were cultured in ME10 in incubators under standard conditions (37 °C, 5% CO_2, 95% relative humidity). For the cell-transformation assay, 10^4 cells were plated per 60-mm tissue-culture dish; 15 dishes were used for each point in all cell-transformation assays. After 24 h, 0.5-mg/ml 3-methylcholanthrene (MCA), a positive control, was added to culture medium, and 72 h later the cultures were washed with fresh medium and incubated in ME10 for 3 days. The cells were subsequently cultured in normal medium until the end of the culture. The medium was changed twice per week for 6 weeks. In the case of the assay for MPS, the medium was changed following a procedure similar to that described above, except there was addition of MCA. The cells were fixed and stained with 5% Giemsa solution. The types of transformed focus were determined

TABLE II. Inhibitory Potentials of MPS-A, MPS-B, and MPS-C in V79 MC Assay

Chemical Disinfectants	MC Assay[a] Result	Lowest Effective Concentration	Cytotoxicity IC50[b]
MPS-A	+	0.625 vol%	1.8 vol%
MPS-B	−	−	>10 vol%
MPS-C	−	−	>10 vol%

[a] The results of MC assay were obtained from a previous study (Reference 10). +, the inhibitory activities were observed in noncytotoxic concentrations; −, no inhibitory activities.

[b] IC50: the concentration that suppressed colony formation to 50% of the control value.

TABLE III. Inhibitory Potentials of Poloxamine, PHMB, and Poloxamer-407 in V79 MC Assay

Chemical Substrate	MC Assay[a] Result	Lowest Effective Concentration	Cytotoxicity IC50[b]
Poloxamine	+	0.125%	0.4%
PHMB	−	−	1.2 ppm
Poloxamer-407	−	−	0.4%

[a] The results of MC assay were obtained from a previous study (Reference 10). +, the inhibitory activities were observed in noncytotoxic concentrations; −, no inhibitory activities.

[b] IC50: The concentration that suppressed colony formation to 50% of the control value.

under a dissecting microscope. Foci that showed a clearly transformed phenotype were counted as described in the report of the IARC/NCI/EPA Working Group.[14] Each finding (the average number of transformed foci) was statistically analyzed with Tukey's (a) multiple-comparison test.

RESULTS AND DISCUSSION

In previous studies, three types of multipurpose solutions were investigated with the use of the cytotoxicity test and the MC assay.[10] The MC assay is a method used to clarify the inhibitory activity of gap junctional intercellular communication. The method was described previously,[15,16] with results as shown in Table II. The IC50 value of MPS-A was approximately 1.8 vol%. On the other hand, those of MPS-B and MPS-C were greater than 10 vol%. In the MC assay, MPS-A inhibited GJIC at concentrations ranging from 0.625 vol% (lowest effective inhibitory concentration) to 1.25 vol%, whereas on MPS-B and C no inhibitory activities were observed. The order of the strength of MC assay was therefore judged to be MPS-A > MPS-B and MPS-C. To analyze what caused the inhibitory activities of MPS-A, the main ingredients of MPS-A, PHMB and poloxamine, were evaluated with the use of a cytotoxicity test and an MC assay. At the same time, poloxamer-407, which also belongs to the surfactant of Pluronic acid was also evaluated. The results of the cytotoxicity test and MC assay are shown in Table III. The IC50 value of PHMB was approximately 1.2 ppm, and those of poloxamine and poloxamer 407 were approximately 0.4% each. Poloxamine inhibited GJIC at concentration ranging from 0.125% (the lowest effective inhibitory concentration) to 0.25%, while on PHMB no inhibitory activities were observed. This result suggested that the inhibitory activity observed in MPS-A was caused by poloxamine. Interestingly, no inhibitory action was observed in poloxamer-407, although poloxamer-407 has a comparable cytotoxicity to poloxamine. Because the conventional cytotoxicity test was not able to detect the difference between poloxamine and poloxamer-407, this MC assay is suitable for the screening of ingredients of MPSs.

Then an *in vitro* assay of GJIC was conducted by SLDT assay with the use of using RC4. The SLDT assay is a method to evaluate the GJIC by the distance of dye migration through the gap junction. The inhibitory action on GJIC could be detected by measuring the distance of dye (Lucifer yellow) migration through gap junctions (Figure 1). If there is an inhibitory action on GJIC, the distances of dye transfer de-

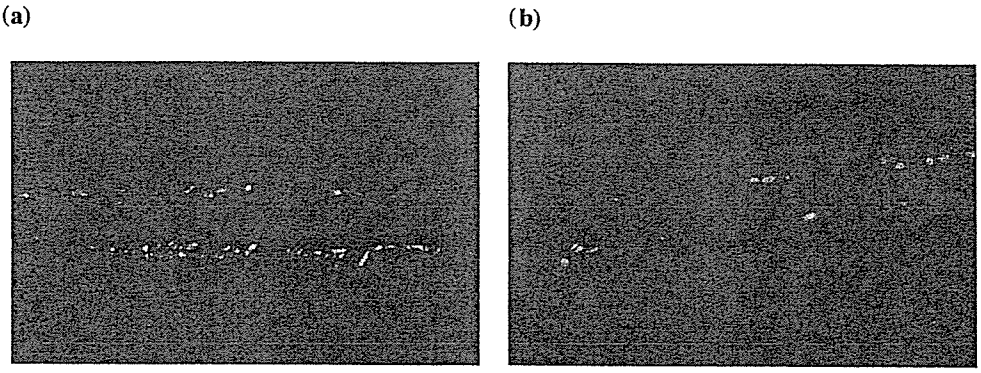

Figure 1. The image of SLDT assay. (a) Example where no inhibitory action was observed. (b) Example where inhibitory action was observed.

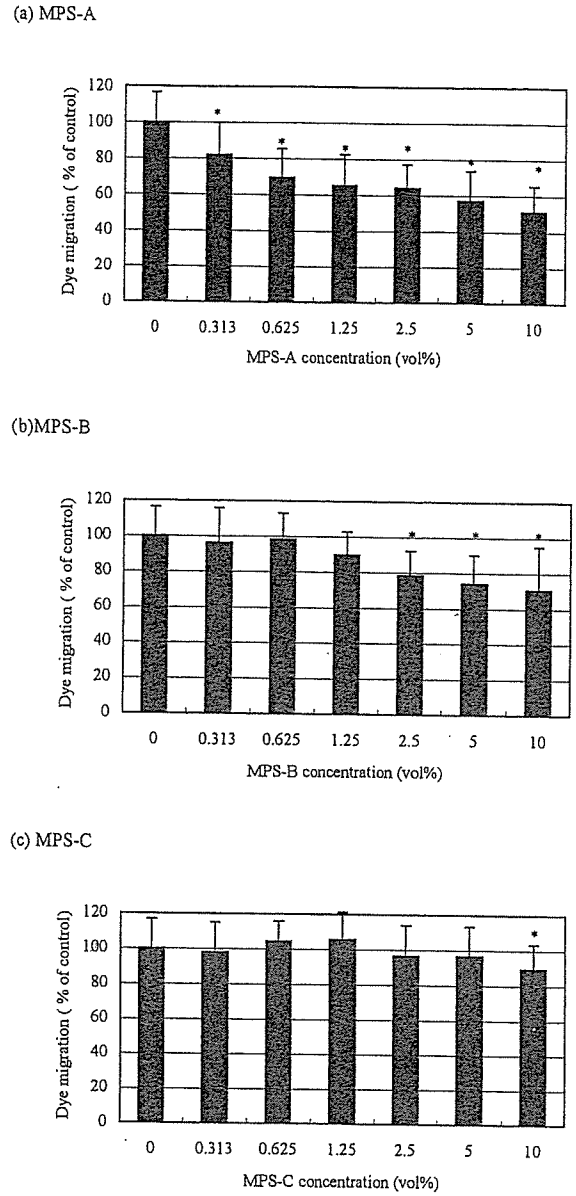

Figure 2. Dose-related inhibition of GJIC in RC4 cells treated with three types of MPS. (a) MPS-A, (b) MPS-B, (c) MPS-C. All data are expressed as the mean ± standard deviation of 30 determinations and treated statistically with Tukey's(a) multiple-comparison test. *$p < 0.01$, significant difference in comparison with control.

crease. First the concentration-dependent inhibition of MPSs were evaluated. These results are shown in Figure 2. After 24-h incubation under standard conditions, a dose-dependent inhibition was observed in all tested MPS. Especially for MPS-A, the inhibition was observed from 0.3125 vol% to a maximum at 10 vol%, revealing a 50% decrease. MPS-B produced dose-dependent inhibition from 2.5 vol% to a maximum at 10 vol%, compared with nontreatment cells [Tukey's(a) multiple-comparison test; $p < 0.01$]. There was an inhibitory action of GJIC in MPS-C at 10 vol% compared with the nontreatment cells [Tukey's(a) multiple-comparison test; $p < 0.01$]. Compared with MC assay, SLDT was more sensitive for the detection of inhibitory action on GJIC.

Second, the concentration-dependent inhibitions of poloxamine, PHMB, and poloxamer-407 were evaluated. These results are shown in Figure 3. After 24-h incubation under standard conditions, a dose-dependent inhibition was observed in poloxamine. It was observed from 0.03125% to a maximum at 1%, revealing a 50% decrease, whereas PHMB was not in its noncytotoxic concentration, compared with the nontreatment cells [Tukey's(a) multiple-comparison test; $p < 0.01$]. These results coincided with those of the MC assay, so it strongly suggesting that the inhibitory activity observed in MPS-A was caused by poloxamine. Moreover, a difference between poloxamine and poloxamer-407 in the method of

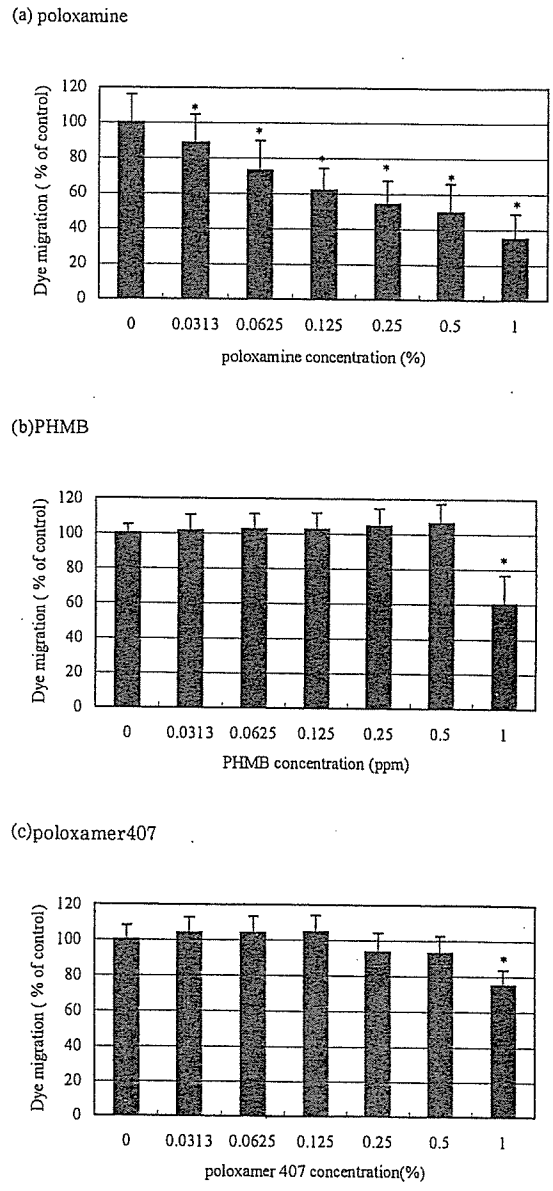

Figure 3. Dose-related inhibition of GJIC in RC4 cells treated with poloxamine, PHMB, and poloxamer-407. (a) Poloxamine, (b) PHMB, (c) poloxamer-407. All data are expressed as the mean ± standard deviation of 30 determinations and treated statistically with Tukey's(a) multiple-comparison test. *$p < 0.01$, significant difference in comparison with control.

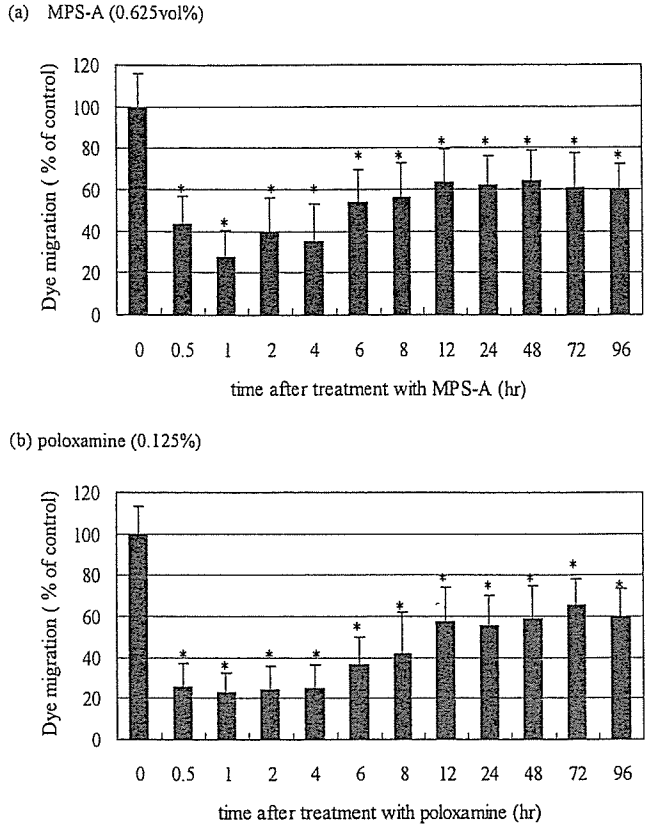

Figure 4. Time course of GJIC in RC4 cells after treatment with MPS-A and poloxamine for up to 96 h. (a) MPS-A, (b) poloxamine. All data are expressed as the mean ± standard deviation of 30 determinations and treated statistically with Tukey's(a) multiple-comparison test. *$p < 0.01$, significant difference in comparison with control.

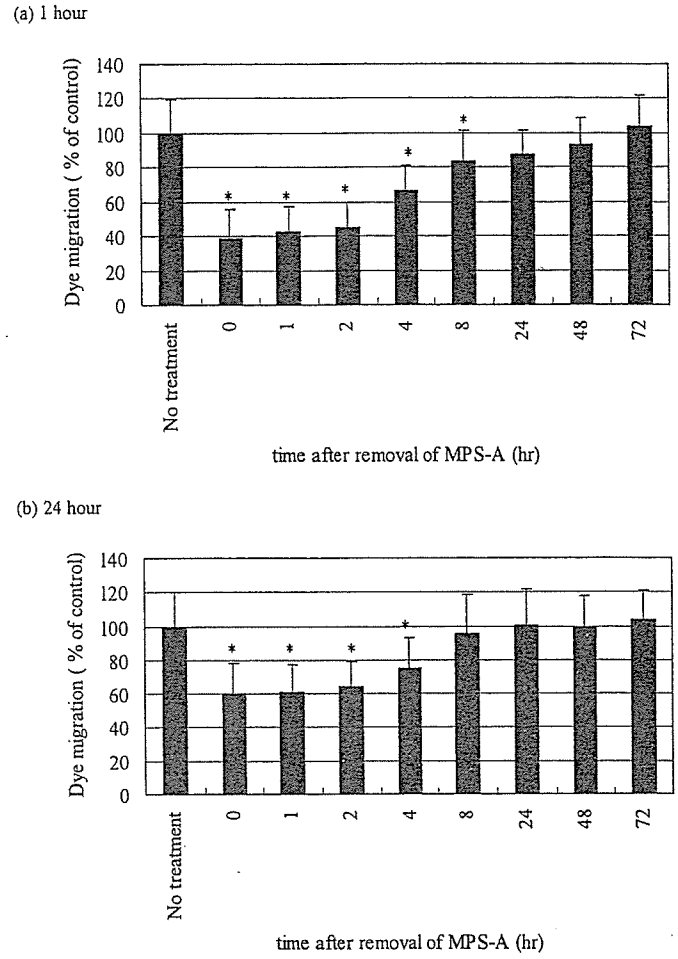

Figure 5. Time course of recovery of GJIC in RC4 cells after removal of MPS-A for up to 72 h. (a) 1-h treatment with MPS-A, (b) 24-h treatment with MPS-A. All data are expressed as the mean ± standard deviation of 30 determinations and treated statistically with Tukey's(a) multiple-comparison test. *$p < 0.01$, significant difference in comparison with no treatment of control.

influencing on GJIC was suggested. These results also coincided with those of the MC assay.

Time-dependent GJIC inhibition was assessed after RC4 cells were treated with ME10 containing 0.625 vol% MPS-A or 0.125% poloxamine (Figure 4). In this experiment, confluent cultures of RC4 cells were treated with MPS-A or poloxamine under standard conditions. After that, the distance of dye migration was measured at appropriate times. (0–96 h after treatment). In MPS-A, an apparent inhibition of approximately 40% was observed at 30 min, and then GJIC was gradually returned to 60% at 8 h, but the GJIC was not completely restored to the initial level after 8 h [Tukey's(a) multiple-comparison test; $p < 0.01$]. In poloxamine, similar inhibitory action on GJIC was observed, and GJIC was not restored completely [Tukey's(a) multiple-comparison test; $p < 0.01$].

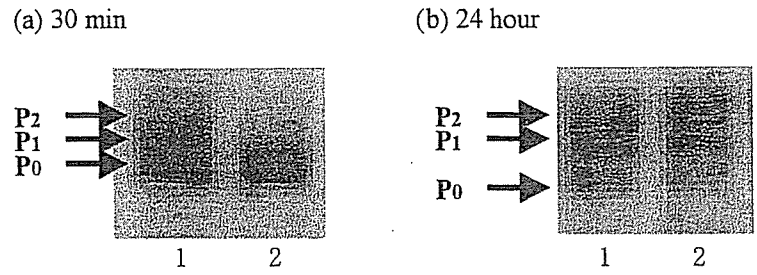

Figure 6. Western blotting of connexin-43 in RC4 cells treated with MPS-A. (a) 1-h treatment with MPS-A, (b) 24-h treatment with MPS-A. Connexin-43 is phosphorylated at least twice.[21] This results in three species[21] of connexin-43, which can be detected by Western blot (P_0, P_1, P_2).[21] P_0 is not phosphorylated, and P_1 and P_2 are phosphorylated.[21]