NMDA–Type Glutamate Receptor Is Associated with Cutaneous Barrier Homeostasis

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Glutamate receptors play an important role in the excitatory synaptic action of the central nervous system. In this study, effects of glutamate receptor agonists and antagonists on skin barrier homeostasis were studied using hairless mouse. Topical application of L-glutamic acid, L-aspartic acid (non-specific glutamate receptor agonists) and N-methyl-D-aspartate (NMDA, NMDA type receptor agonist) delayed the barrier recovery rate after barrier disruption with tape stripping. On the other hand, topical application of D-glutamic acid (non-specific antagonist of glutamate receptor), MK 801 and D-AP5, (NMDA-type receptor antagonists) accelerated the barrier repair. The non–NMDA type receptor agonist, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), did not affect the barrier recovery. Topical application of MK-801 also promoted the healing of epidermal hyperplasia induced by acetone treatment under low environmental humidity. Immediately after barrier disruption on skin organ culture, secretion of glutamic acid from skin was significantly increased. Immunohistochemistry, reverse transcription polymearse chain reaction (RT-PCR) and in situ hybridization showed an expression of NMDA-type receptor-like protein on hairless mouse epidermis. NMDA increased intercellular calcium in cultured human keratinocytes and the increase was blocked by MK 801. These results suggest that glutamate plays an important role as a signal of cutaneous barrier homeostasis and epidermal hyperplasia induced by barrier disruption. *Key words: keratinocyte/epidermis/glutamate receptor/calcium channel. J Invest Dermatol 120:1023–1029, 2003*

T he uppermost thin layer of the skin, stratum corneum, plays a crucial role as a water impermeable barrier. Acute disruption of the barrier results in an increase in epidermal DNA synthesis (Proksch *et al*, 1991) and cytokine production (Wood *et al*, 1992). Even when the damage of the barrier is relatively small, when it is repeated (Denda *et al*, 1996) or under low environmental humidity (Denda *et al*, 1998), the damage induces an obvious epidermal hyperplasia and inflammation. Moreover, various kinds of dermatoses, such as atopic dermatitis, psoriasis and contact dermatitis, are associated with barrier dysfunction (Grice, 1980).

The stratum corneum is composed of two components, i.e., protein–rich nonviable cells and intercellular lipid domains (Feingold and Elias, 2000). When the barrier function of the stratum corneum is damaged by a surfactant, organic solvent or tape stripping, the homeostatic system is accelerated and the barrier function recovers its original level (Feingold *et al*, 2000). First, exocytosis of lipid-containing granules, lamellar bodies, is accelerated and the inside lipids are secreted into the intercellular domain between the stratum granulosum and stratum corneum and forms a water impermeable membrane (Feingold *et al*, 2000).

Previous studies suggested that ion gradation in the epidermis is strongly associated with the barrier repair system (Mauro *et al*, 1998; Denda *et al*, 2000; Menon *et al*, 1994; Lee *et al*, 1994). When the barrier was damaged, gradation of calcium and magnesium in the epidermis immediately disappeared (Mauro *et al*, 1998; Denda *et al*, 2000). When the calcium concentration in the upper epidermis increases by sonophoresis, the lamellar body secretion is prevented (Menon *et al*, 1994). Lee *et al* demonstrated that Ca^{2+} channel blocker, verapamil, prevents the delay of the barrier repair induced by increased extracellular calcium concentration (Lee *et al*, 1994). These results suggest that a calcium flux into the keratinocyte perturbs the lamellar body secretion and consequently delays the barrier repair. However, the type of the channels responsible for the skin barrier homeostasis has not yet been clarified.

Recently, we demonstrated that ionotropic receptors, originally found in the nervous system, played an important role in epidermal barrier homeostasis (Denda *et al*, 2002b; Denda *et al*, 2002c). These results suggest that other ligand–gated ion channel also associates with the skin barrier function. Glutamic acid is the principal neurotransmitter with a primarily excitatory synaptic action in the central nervous system, and several different types of receptors have been reported in the nerve system (Shepherd, 1994). One group of receptors comprises the G-protein-coupled metabotropic receptors and another group the glutamate-gated ion channels, such as NMDA (N-methyl-D-aspartate)-type or AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionate)-type receptor. Previous studies demonstrated that these glutamate-gated ion channels were expressed in rat epidermal keratinocyte cell membrane and that an antagonist of the receptor

affected the colony formation of cultured keratinocytes (Genever *et al*, 1999; Morhenn *et al*, 1994). Especially, NMDA receptor passes calcium ion selectively (Hollemann *et al*, 1991) and a voltage-dependent magnesium ion block regulates the permeability of the channel (Paoletti *et al*, 1995). Moreover, we demonstrated that topical application of magnesium salts accelerated the skin barrier repair after its damage (Denda *et al*, 1999). We also reported that an external electric potential altered calcium and magnesium ion gradation in the epidermis and also affected the skin barrier homeostasis (Denda *et al*, 2002a).

From these previous studies, we hypothesized that a glutamate-gated ion channel is associated with cutaneous barrier homeostasis. In this study, we examined the effects of topical application of glutamate receptor agonists and antagonists on the skin barrier recovery rate after barrier disruption and the effects of these reagents on epidermal hyperplasia induced by barrier disruption under low environmental humidity. We also examined the effects of glutamate receptor agonists and antagonists on the calcium concentration in cultured human keratinocytes.

MATERIALS AND METHODS

Animals All experiments were performed on 7- to 10-wk-old male hairless mice (HR-1, Hoshino, Japan). All procedures of the measurement of skin barrier function, disruption of the barrier and application of test sample were carried out under anesthesia. All experiments were approved by the Animal Research Committee of the Shiseido Research Center in accordance with the National Research Council Guide (1996).

Reagents (+)-MK 801, AMPA, NMDA and D-AP5 were purchased from TOCRIS (Tocris Cookson, Inc., Ballwin, MO, USA). L-glutamic acid, L-aspartic acid and D-glutamic acid were purchased from WAKO (Osaka, Japan). Glutamate detection kit was purchased from Molecular Probes (Amplex TM Red Glutamic Acid / Glutamate Oxidase Assay Kit, Molecular Probes, Eugine OH). Normal human keratinocytes (neonatal skin) were purchased from BioWhittaker (Walkersville, MD, USA).

Cutaneous barrier function Permeability barrier function was evaluated by measurement of transepidermal water loss (TEWL) with an electric water analyzer, as described previously (Denda *et al*, 1996). Four mice were used for the evaluation of each treatment. In total, 13–14 points were measured for each treatment. The barrier recovery results are expressed as percent of recovery because of variations from day to day in the extent of barrier disruption. In each animal, the percentage of recovery was calculated by the following formula: (TEWL immediately after barrier disruption - TEWL at indicated time point)/(TEWL immediately after barrier disruption - baseline TEWL) × 100%. Topical application of each reagent was carried out immediately after barrier disruption. In each case, 100 μl of 1 mM aqueous solution was applied on 2×4 cm^2 of tape stripped flank skin and occluded with plastic membrane for 20 min. Then the membrane was removed and the treated area left to dry spontaneously.

Epidermal hyperplasia induced by barrier disruption under low humidity Animals were kept separately in 7.2 liter cages in which the relative humidity was maintained at less than 10% with dry air as described previously (Denda *et al*, 1998). Four mice were used for the evaluation of each treatment. Animals were first kept in the dry conditions for 48 h and then bilateral flank skin was treated with acetone-soaked cotton balls, as described previously (Denda *et al*, 1998). The procedure was terminated when TEWL reached 2.5–3.5 mg per cm^2 per h. Immediately after treatment, 200 μl of 1 mM test sample 8 cm^2 of back skin of mouse, and water was applied on the other side. Then the animals were again kept in the dry conditions for 48 h. After the experiments, animals were euthanaized with diethyl ether inhalation, and skin samples were taken from the treated area. One hour before the euthanization, 20 μl per g body weight bromodeoxyuridine (BrdU) 10mM solution was injected intraperitoneally. Untreated control mice were also treated with BrdU at the same time. After fixation with 4% paraformaldehyde, full thickness skin samples were embedded in paraffin, sectioned (4 μm), and processed for hematoxylin and eosin staining. For the assessment of DNA synthesis, the sections were immunostained with anti-BrdU antibodies. On each section, five areas were selected at random; the thickness of the epidermis was measured with an optical micrometer, and the mean value was calculated. In total, 16–20 sections were used for each evaluation. Measurements were carried out in an observer-blinded fashion.

Quantification of glutamate release from the skin We quantified glutamate by the method arranged from previous reports (Wood *et al*, 1996; Ashida *et al*, 2001a). Four mice were used for the experiment. After the animals were euthanized, skin samples were immediately taken from both flanks. Subcutaneous fat was removed with a scalpel and the skin samples were cut into 2×3 cm^2 exactly. The two pieces of skin from both flanks were placed, epidermis side upwards, in separate culture dishes kept in an ice water bath, and one of them was tape stripped four times. The other piece of skin was not treated. Three pieces of circle-shaped section (exactly 5 mm diameter) was cut from each skin. Each section was put in a well (12 mm diameter) separately. Then, 200 μl of chilled buffered saline solution containing 150 mM NaCl, 10 mM glucose, 25 mM HEPES, 5 mM KCl, 1.2 mM NaH$_2$PO$_4$, 1.2 mM MgCl$_2$, and 1.8 mM CaCl$_2$, adjusted to pH 7.4 with NaOH, was added to each well and incubated for 30 m at 37°C. After the incubation, Sample solution (25 μl) and 0.1 M Tris-HCl pH 7.5 (25 μl) were mixed to make 50 μl of reaction mixture supplied in the Amplex™ Red Glutamic Acid/Glutamate Oxidase Assay Kit (Molecular Probes, Inc. Eugene, OR, USA) according to the manufacturer's instruction. Briefly, L-glutamate was oxidized with glutamate oxidase to produce α-ketoglutarate, NH$_3$ and H$_2$O$_2$. L-glutamate was regenerated by transamination of α-ketoglutarate, resulting in multiple cycles of the initial reaction and a significant amplification of the H$_2$O$_2$ produced. The hydrogen peroxide reacted with 10-acetyl-3,7-dihydroxyphenoxazine in a 1:1 stoichiometry in the reaction catalyzed by horseradish peroxidase to generate the high fluorescent product, resorufin which has absorption and fluorescence emission maxima of 563 nm and 587 nm, respectively. After incubation for 5 min at 37°C the fluorescence was detected using a Fluoroskan Ascent FL Plate Reader (Thermo Labsystems, Inc., Helsinki, Finland). Excitation and emission wavelength were 548 nm and 587 nm. Standard calibrations performed with L-Glutamic acid ranged from pmol to 2 nmol under same conditions.

Localization of glutamate in mouse epidermis Glutamate detection was performed in situ on a gel mounted with Amplex™ Red Glutamic Acid/Glutamate Oxidase Assay Kit reaction mixture. The method was a modification of our previous method (Denda *et al*, 2000a). Briefly, a gel on a slide glass was made of 2% agarose. The reaction mixture of the assay kit was diluted 10 times with 0.1 M Tris buffer (pH 7.5), and 50 μl of the solution was spread over the gel. One side of the mouse flank skin surface was stripped with adhesive tape three times, and the other side was not treated as a control. After the animals were killed under anesthesia, skin sections were taken from each side and frozen immediately. The tissues were subjected to frozen sections with its 6 μm thickness. The section was put on the gel and incubated at 37°C for 5 min. The detection of fluorescence was immediately executed when the skin slice was mounted onto the reaction gel. To confirm the reproducibility, we observed five different sections from three different animals each.

Analysis of expression of NMDA receptorζ1 mRNA in epidermis and brain tissue Epidermis of the skin tissue was removed by incubation with 10 mM EDTA PBS solution at 37°C for 30 min. Mouse total RNA was obtained from epidermis using TRIzol reagent (Gibco, BRL, Grand Island, NY, USA). cDNA was produced from the RNA by random hexamer priming and reverse transcription was undergo using SuperScript II (Life Technologies), according to the manufacturer's instructions. RT-PCR was carried out using TaKaRa ExTaq (Takara shuzo, Shiga, Japan) with 2 μM sense and antisense oligonucleotide primers designed for mouse NMDA receptor subunit ζ1 (NMDARζ1) and GAPDH, respectively (Yamazaki *et al*, 1992; Meguro *et al*, 1992; Ikeda *et al*, 1992). The primer sequences were as follows: NMDAζ1 sense: CTGTTTATGGCTTCTGCGTTGA, antisense: GTTCACCTTAAATCGG-CCAAA, GAPDH sense: CCCATCACCATCTTCCAG, antisense: CCT-GCTTCACCACCTTCT. The denaturing temperature and the polymerase working temperature were 94°C and 72°C for 1 min, respectively. The annealing was carried out at 57.5°C for 1 min, respectively. All reactions were carried on 30 cycles.

Imunohistochemistry for NMDARζ1 All mouse tissues were subjected to frozen sections with 5 μm thickness. The detection for mouse NMDARs was carried on using anti NMDARζ1(C-20) polyclonal antibody (derived from goat serum) supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of glutamate (NMDA) receptorζ1 of human origin and the sequence is identical to corresponding mouse and rat sequence. The binding of the first antibody was detected by the fluorescent anti-goat secondary antibody (Alexa Fluor® 488 rabbit anti-goat IgG (H + L), Molecular Probes, Inc.). Nuclei were stained with DAPI using Vectashield with DAPI Vector Laboratories, Inc., Burlingame, CA, USA). To confirm the

reproducibility, we observed five sections from three different animals each.

***In situ* hybridization** We subcloned the PCR product of NMDA receptor subunitζ1 into pCR II TOPO (Invitrogen, San Diego, CA, USA) vector and generated the pCR II TOPO-NMDAR 1-1 vector. The NMDAR 1 digoxigenin labeled antisense and sense cRNA probe was generated by *in vitro* transcriptions by using T7 and SP6 RNA polymerase (Roche Diagnostics, Mannheim, Germany) with pCR II TOPO-NMDARζ1-1 respectively. All the mouse tissues were subjected to frozen sections with 5 μm thickness on the poly L-Lysine coated slide. We carried out the *in situ* hybridization using digoxigenin labeled cRNA probes by the method arranged from a previous report (Schaeren-Wiemers & Gerfin-Moser, 1993). The sections were fixed in 4% paraformaldehyde/PBS for 10 min at room temperature and acetylated with aqueous solution containing 0.25% acetic anhydride, 0.1 mM triethanolamine, and 0.175% HCl. The acetylated sections were washed twice with PBS. Then the sections were pre-hybridized with hybridization solution containing 50% formamide, 250 μl/ml herring sperm DNA (Roche Diagnostics, Mannheim, Germany), 500 μg/ml yeast derived total RNA (Sigma-Aldrich Japan, Tokyo, Japan) and $1 \times$ Denhart's solution (Sigma-Aldrich Japan, Tokyo, Japan) in $4 \times$ SSC ($1 \times$ SSC: 150 mM NaCl, 15 mM sodium citrate, pH 7.4) at room temperature for 2 h. Then the sections were hybridized with the hybridization solution containing the heat denatured 200 ng/ml sense or antisense digoxigenin labeled cRNA probe in a humidified chamber at 60°C overnight. Hybridized sections were washed first with 5xSSC solution at 50°C and then washed with 0.2xSSC solution twice at 50°C for 30 min. Again the sections were washed with 0.2xSSC solution at room temperature for 5 min. The sections were washed with B1 buffer (0.1 M TrisHCl, 0.15 M NaCl, pH 7.5) and then incubated with B1 buffer including 10% heat inactivated goat serum (HINGS) for 1 h at room temperature. After the removal of the solution, the sections were incubated with B1 buffer including 0.02% anti-digoxigenin-Ap, Fab fragments (Roche Diagnostics, Mannheim, Germany) and 1% HINGS at 4° C overnight. The sections were washed with B1 buffer three times, buffered with a solution (0.1 M TrisHCl pH 9.5, 0.1 M NaCl, 50 mM MgCl₂) and the staining reaction was started with a staining solution (75 mg/ml nitroblautetrazoliumchloride, 50 mg/ml, 5-bromo-4-chloro-3-indolyl-phosphate p-toluidine salt and 0.24 mg/ml levamisole in 0.1 M TrisHCl pH 9.5, 0.1 M NaCl, 50 mM MgCl₂). After 6 h incubation at room temperature, the reaction was stopped with 1 mM EDTA in TrisHCl 10 mM, pH 7.5 and the sections were rinsed, air-dried, dehydrated with ethanol, treated with xylene and mounted with Mount-Quick (Cosmobio, Tokyo, Japan).

Calcium dynamics in keratinocyte culture system Evaluation of intracellular calcium concentration ($[Ca^{2+}]i$) in cultured human keratinocytes was measured with fura-2 as described by Grynkiewicz *et al* (1985) with minor modifications (Koizumi and Inoue, 1997). Details of the method using keratinocyte was the same as previously reported (Denda *et al*, 2002a). The coverslip was mounted on a fluorescence microscope (IX70, TS Olympus, Tokyo, Japan) equipped with a 75 W xenon-lamp and band-pass filters of 340 nm and 380 nm wavelengths. Measurements were carried out at room temperature. Imaging data, recorded by a high-sensitive silicon intensifer target camera (C4742, Hamamatsu Photonics, Hamamatsu, Japan), were regulated by a Ca^{2+} analyzing system (AQUA/RATI01, Hamamatsu Photonics, Hamamatsu, Japan).

Statistics The results are expressed as the mean $\pm$ SD. Statistical differences between two groups were determined by a two-tailed Student's t-test. In the case of more than 2 groups, differences were determined by ANOVA test (Fisher's protected least significant difference).

RESULTS

Barrier recovery Figure 1 shows the effects of topical application of L, D-glutamic acid and L-aspartic acid on skin barrier recovery after tape stripping. L-glutamic acid and L-aspartic acid, both non-specific agonists of the glutamate receptor, delayed the barrier recovery and D-glutamic acid, which is a weak antagonist against the glutamate receptor, slightly but significantly accelerated the barrier recovery. Topical application of NMDA delayed the barrier recovery and the delay was blocked by MK 801, antagonist of NMDA receptor (**Fig 2A**). Topical application of MK 801 or D-AP5 (another NMDA specific antagonist) alone accelerated the barrier recovery
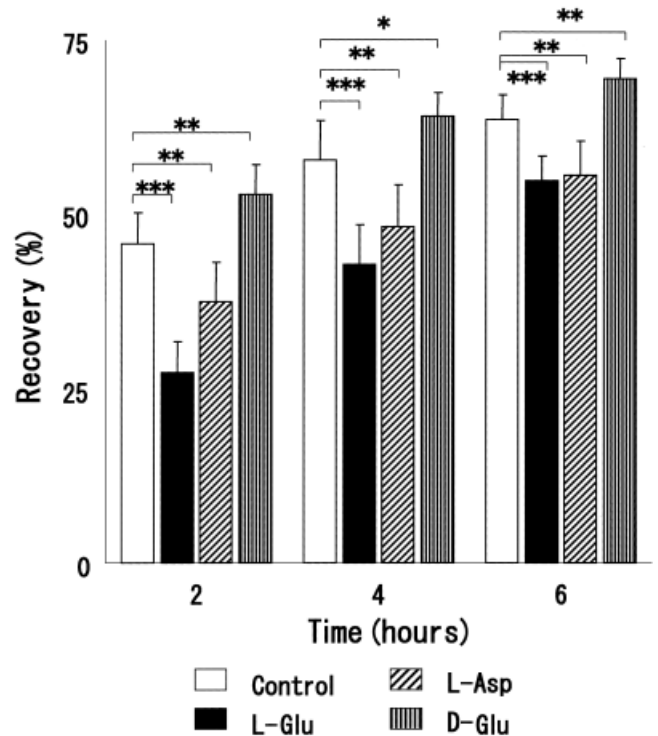

Figure 1. Topical application of non-specific glutamate receptor agonists, L-glutamic acid and L-aspartic acid, delayed the barrier recovery of the hairless mice and the non-specific antagonist of glutamate receptor, D-glutamic acid, slightly but significantly accelerated the barrier recovery at 2, 4 and 6 hours after the barrier disruption by tape stripping. Ordinate shows recovery percent of the barrier to the original level. (mean $\pm$ SD) n = 14 each. *: P < 0.05, **: P < 0.005, ***: P < 0.0005

(**Fig 2B**). Topical application of AMPA, AMPA receptor-specific agonist, did not affect the barrier recovery rate (**Fig 2A**). **Figure 2C** shows the barrier recovery rate 30 min and 24 h after tape stripping. The effects of NMDA and MK 801 were observed in the earlier stage and also one d after treatment. These results suggest that the NMDA type glutamate receptor or very similar receptor is associated with skin barrier homeostasis. The effects of NMDA receptor antagonists suggest that the presence of endogeneous glutamate increase in the epidermis after barrier disruption.

Epidermal hyperplasia Figure 3 shows the effect of topical application of NMDA and MK 801 on epidermal hyperplasia induced by acetone treatment under dry conditions. As previously demonstrated (Denda *et al*, 1998), the barrier disruption induced obvious epidermal hyperplasia under dry conditions (**Fig 3B**) compared to untreated skin (**Fig 3A**). The epidermal hyperplasia was almost completely prevented by the topical application of MK 801 (**Fig 3C**), but topical application of NMDA even worsened the hyperproliferative response (**Fig 3D**). The results of quantification of the epidermal thickness are shown in **Figure 3E**. Evaluation of epidermal proliferative response using BrdU showed the same tendency (**Fig 3F**). These results suggest that the NMDA receptor or similar receptor also plays an important role on the epidermal proliferative response induced by barrier insults.

Localization of glutamate in the epidermis Figure 4A shows the amount of glutamate released from skin organ culture with or without barrier disruption by tape stripping. The barrier disruption significantly increased the secretion of glutamate from the skin tissue within 30 min after barrier disruption. Visual
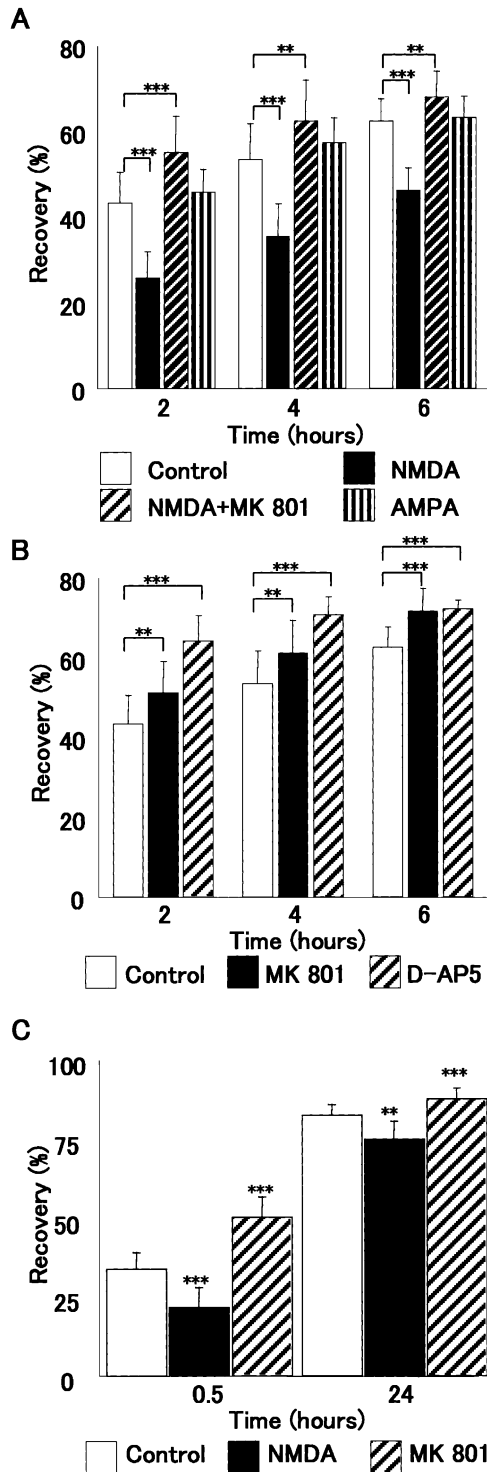
Figure 2. Topical application of NMDA receptor specific agonist, NMDA delayed the barrier recovery after tape stripping and the delay by NMDA was blocked by MK 801, specific antagonist of NMDA receptor. Topical application of AMPA, AMPA receptor specific agonist did not affect the barrier recovery rate after tape stripping (**Fig 2A**). Topical application of MK 801 or D-AP5, another NMDA receptor specific antagonist, alone accelerated the barrier recovery (**Fig 2B**). The effects of NMDA and MK 801 were observed in the earlier stage (30 min after tape stripping) and also one d after tape stripping (**Fig 2C**). Ordinate shows recovery percent of the barrier to the original level. (mean ± SD) n = 13-14 each. *: P < 0.05, **: P < 0.005, ***: P < 0.0005

images of glutamate localization with and without tape stripping are shown in **Figure 4B** and **C**. Glutamate was localized in the upper layer of the epidermis of untreated normal skin (**Fig 4B**:*
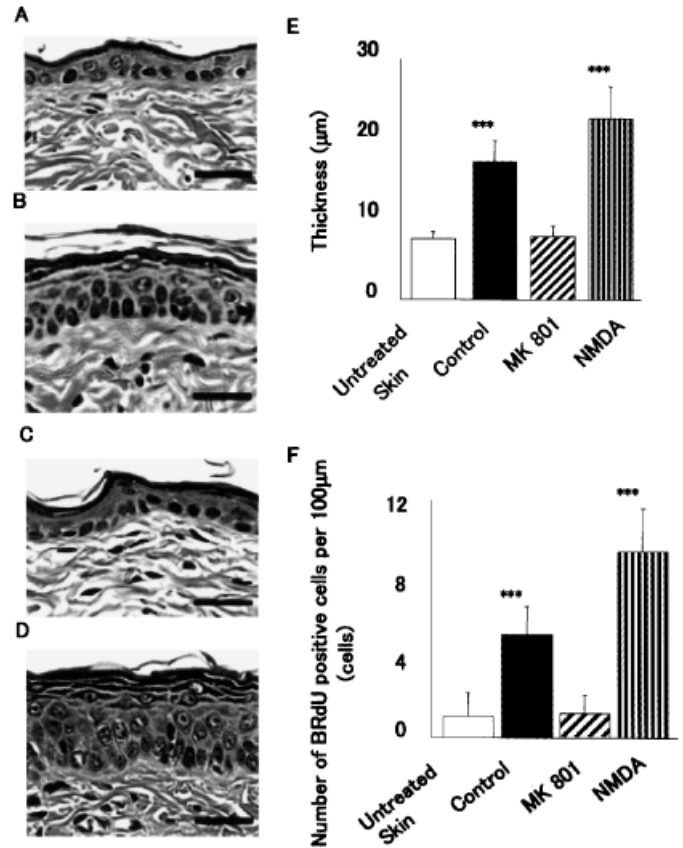

Figure 3. The effects of topical application of NMDA and MK 801 on epidermal hyperplasia induced by acetone treatment under dry conditions. As previously demonstrated (Dendá *et al*, 1998), the barrier disruption induced obvious epidermal hyperplasia under a dry condition (**Fig 3B**) compared to untreated normal skin (**Fig 3A**). The epidermal hyperplasia was almost perfectly prevented by the topical application of MK 801 (**Fig 3C**) and topical application of NMDA worsened the hyperproliferative response (**Fig 3D**). The results of quantification of the epidermal thickness are shown in **Figure 3E**. Evaluation of epidermal proliferative response using BrdU shows same tendency (**Fig 3F**). Ordinate in **Figure 3E** shows the average thickness of epidermis of each treatment. (mean ± SD) n = 16-20 each. *Bars* in **A,B,C,D** = 10 μm, ***: P < 0.0005

red color, blue color shows DAPI staining of DNA). The localization disappeared by the barrier disruption with tape stripping and some of the glutamate re-localized or re-generated in the basal layer of the epidermis (*arrows*, **Fig 4C**). With this method, we could not distinguish the stratum corneum and stratum granulosum. Rawlings *et al* (1994) previously demonstrated the low content of glutamate in the stratum corneum (Ikeda *et al*, 1992) and moreover, the red color appears only by the chemical reaction with glutamate as we described in the Methods. Thus, the result of the untreated skin likely shows the localization of glutamate in the stratum granulosum. These results suggest that glutamate in the epidermis might be associated with the signaling of the skin barrier damage.

RT-PCR assay, immunohistochemistry and *in situ* hybridization of NMDA receptor RT-PCR analysis for mouse NMDARζ1 and GAPDH on the total RNA from mouse epidermis also showed positive bands at the expected sizes (**Fig 5A**). The expected bands for ζ1 were observed on the total RNA from the mouse brain (data not shown). Immunoreactivity against anti NMDARζ1 (**Fig 5B**) was observed in the living layer of the epidermis. Blank assay without primary antibody did not show any reactivity (data not shown). *In situ* hybridization analysis of the mouse skin sections with the
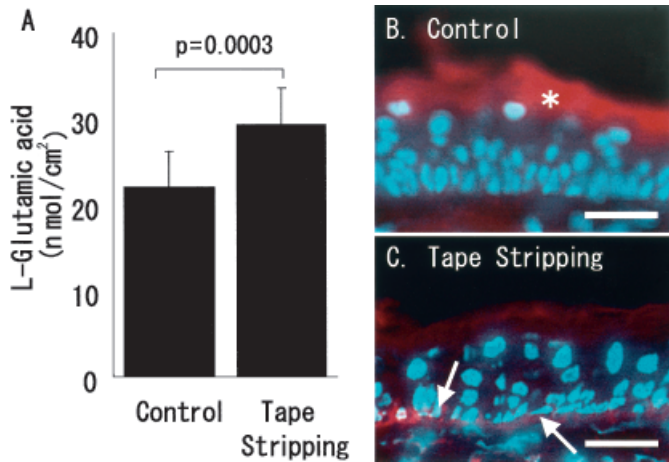
Figure 4. Glutamate was released from skin immediately after barrier disruption. The barrier disruption significantly increased the secretion of glutamate from the skin tissue within 30 min after the barrier disruption (**Fig 4A**) (mean ± SD). n = 12 each. Visual images of glutamate localization with and without tape stripping were shown in **Fig 5B** and **C**. In normal skin, glutamate was localized in the uppermost epidermis (**Fig 4B**, *Asterisk*). Barrier disruption by tape stripping released glutamate from epidermal granular layer and some of them re-localized in the epidermal basal layer (**Fig 4C**, *arrows*). *Bars in **B** and **C** = 5 μm.*
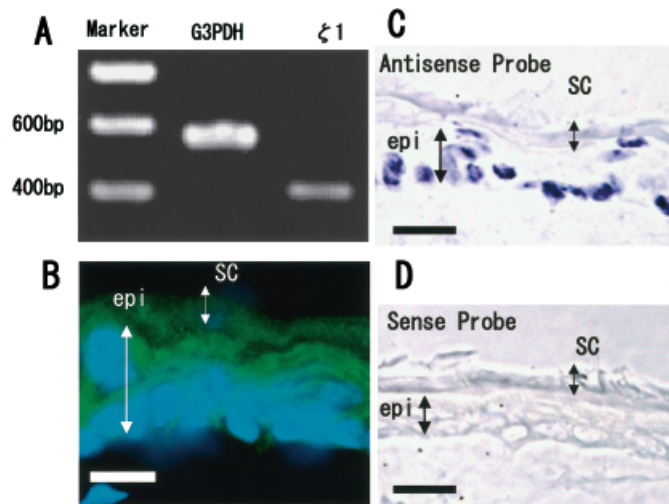

Figure 5. RT-PCR assay, immunohistochemistry and *in situ* hybridization analysis of NMDA receptor subtype, NMDARζ1. RT-PCR analysis for mouse NMDARζ1 and G3PDH on the total RNA from mouse epidermis also showed positive bands of the expected sizes respectively (**Fig 5A**). Immunoreactivity against NMDARζ1 was observed in the living layer of epidermis (**Fig 5B**, *green color*). *In situ* hybridization analysis for NMDARζ1 also showed similar localization (**Fig 5C**, antisense) and no signal was detected in an application of the corresponding sense probe (**Fig 5D**). SC; stratum corneum, epi; epidermis. *Bars in **B**, **C**, **D** = 5 μm.*

antisense probe showed NMDARζ1 mRNA expression in the epidermal cells broadly (**Fig 5C**). The analysis with sense probe did not show any staining (**Fig 5D**). These results show the existence of NMDA receptor or a very structurally similar protein in the epidermis.

Calcium concentration in the cultured keratinocytes The intracellular calcium concentration in a single cell using fura-2AM was increased when we added 50 μM NMDA (**Fig 6A**, **B** and **E**). Most of the increase was blocked by pre-incubation with 50 μM MK 801 (**Fig 6C**, **D** and **E**).
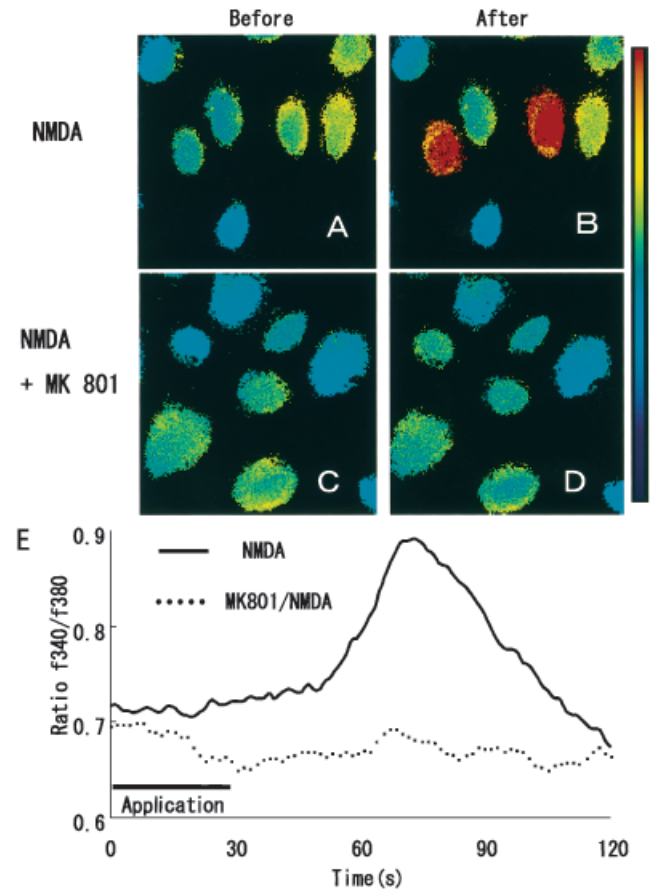

Figure 6. Effects of NMDA and MK 801 on intracellular calcium ion in cultured human keratinocytes. Application of 50 μM of NMDA increased $[Ca2+]i$ in the keratinocytes (**Fig 6A**, **B** and **E**) and the increase was blocked by pre-incubation with MK 801 (**Fig 6C**, **D** and **E**). Among 56 keratinocytes, 17 cells showed obvious increase of intracellular calcium by NMDA treatment. After pre-incubation with MK 801, only two cells among 47 keratinocytes showed the increase of intracellular calcium by NMDA treatment. Ordinate shows the ratio of relative intensity of 340 nm and 380 nm emission.

Approximately 31% of the keratinocytes showed an increase of intracellular calcium among 56 observations within 1 min after we applied NMDA. When we pre-incubated with MK 801, only 4.2% among 47 observations showed the calcium response against NMDA application. These results suggest that the NMDA receptor or a functionally very similar protein exists in the epidermis. To confirm a contamination of other cell line in the keratinocyte culture system, we carried out immuno-histochemical study with keratinocyte specific antibody (against CK14) on the culture system of this study. Almost all cells (more than 99%) showed positive immnunoreactivity against the keratinocyte specific protein antibody (data not shown).

DISCUSSION

An increase of intercellular and intracellular calcium in the keratinocyte has been suggested to perturb the exocytosis of lamellar bodies and delay skin barrier repair (Menon *et al*, 1994; Lee *et al*, 1994). In the healthy epidermis, a high concentration of calcium is observed in the uppermost epidermis and the gradation disappeared immediately after barrier disruption by tape stripping or organic solvent treatment (Mauro *et al*, 1998; Denda *et al*, 2000a). The drastic change of calcium gradation in the epidermis might be a crucial signal for the barrier repair signaling. The phase transition and fusion of intercellular granular membrane and cell

membrane is a crucial stage of the exocytosis of the lamellar body, and ions such as calcium or magnesium influence the phase of lipid bilayer structure (Denda *et al*, 1999; Denda *et al*, 2002a). Although the mechanism has not been clarified, the relative concentration of ions outside of and inside the keratinocyte cell membrane, i.e., polarity of the membrane, might be crucial for the skin barrier repair process. Zhang *et al* (2001) demonstrated that depolarization of cell membrane causes an outward movement. Increase of intracellular calcium ion might induce depolarization of the keratinocyte cell membrane, perturb inward movement of cell membrane, the exocytosis of lamellar body and delay the barrier repair.

Recently, we demonstrated that P2X purinergic receptor antagonist and GABA(A) receptor agonists accelerated the barrier repair after tape stripping (Denda *et al*, 2002b; Denda *et al*, 2002c). The former is a calcium channel and the latter is a chloride channel. These results suggest that calcium ion influx into the keratinocytes delays the barrier repair and on the other hand, chloride ion influx accelerates the barrier recovery. NMDA receptor is also a calcium channel. In neurons, chloride channels like GABA(A) receptor or glycine receptor play a role of inhibiting depolarization induced by calcium flux through other excitatory receptors such as P2X or NMDA receptor. Similar to the nerve-system, "excitation" induced by calcium influx and "inhibition" induced by chloride influx through the ligand-gated ion channel might be important for the epidermal homeostasis. The results of the present study support this hypothesis.

In this study, we first demonstrated the specific localization and dynamic of glutamate in the epidermis. In normal skin, glutamate localized in the uppermost epidermis and the localization disappeared by the barrier disruption. Interestingly, some of the glutamate re-localized in epidermal basal layer (**Fig 4C**). Barrier disruption induces epidermal DNA synthesis (Proksch *et al*, 1991) at the basal layer of the epidermis (Denda *et al*, 1998). Glutamate might play a role of signaling in the epidermal proliferative response induced by barrier disruption. However, the mechanism underlying these phenomena has not been clarified. Like the synaptic system, a transporter of glutamate might play an important role of the localization and its release from epidermis by barrier disruption. The secretion of endogenous glutamate might increase the intercellular calcium concentration through an NMDA receptor-like protein in the epidermal keratinocytes. An application of MK 801 might reduce the autocrine effect of glutamate and consequently block the delay of barrier recovery. In neurons, the glutamate-gated cation channel plays an important role of depolarization of the cell membrane induced by a cation flux. The NMDA receptor passes calcium ions through its channel, but the AMPA receptor does not (Hollemann *et al*, 1991). In this study, the antagonist and agonist of NMDA affected the skin barrier repair process, but AMPA did not affect the recovery rate. Not only an NMDA receptor, but also an AMPA receptor was expressed in epidermal keratinocytes in a previous study (Genever *et al*, 1999). Lee *et al* (1992) demonstrated that topical application of calcium chloride delayed barrier repair, but application of sodium chloride did not affect barrier recovery. These results suggest that the calcium flux through keratinocyte cell membrane is important for the skin barrier homeostasis, as we described above.

The NMDA receptor is modulated by a voltage-dependent magnesium ion block (Paoletti *et al*, 1995). We previously demonstrated that topical application of magnesium salts and a mixture of magnesium and calcium salts accelerated the skin barrier recovery (Denda *et al*, 1999). The relative ratio of magnesium and calcium ions in the epidermis might be a crucial factor in the electric potential through the epidermis (Denda *et al*, 2001a). An external electric potential altered the magnesium and calcium localization in the epidermis and also affected skin barrier recovery (Denda *et al*, 2002a). The NMDA receptor-like protein we demonstrated in this study might be associated with these phenomena we demonstrated previously.

In neurons, the polarity of cell membrane is regulated by a variety of ligand-gated ion channels such as NMDA, acetylcho-line or GABA receptor (Shepherd, 1994). Several receptors originally found in nerve cells have been reported on keratinocytes. Ndoye *et al* (1998) demonstrated that each muscrinic acetylcholine receptor subtype was localized differently in the human epidermis. Another report suggested that keratinocyte nicotinic cholinergic receptors related to calcium influx of the cell (Grando *et al*, 1996). Stoebner *et al* (1999) reported the expression of benzodiazepine receptors. Recently, we demonstrated the expression and function of the vanilloid receptor subtype 1 (VR1) in human keratinocytes (Denda *et al*, 2001b; Inoue *et al*, 2002). Ectoderm derived keratinocytes and neurons show similar expression of those receptors.

The NMDA receptor in the mouse central nervous system is basically constructed of a ζ1 subunit and ϵ subunit (Moriyoshi *et al*, 1991). Our findings suggested the expression of ζ1 in the epidermal keratinocytes. The ζ1 subunit alone has a function of glutamate-gated cation channel and a different type of ϵ subunit add variation to the channel function (Ikeda *et al*, 1992; Kutsuwada *et al*, 1992). Previous studies suggested that autocrine acetylcholine and paracrine acetylcholine both play an important role in epidermal proliferation and differentiation (Grando, 1997). They also suggested that a difference in the distribution of a variety of cholinergic receptors might be crucial for epidermal homeostasis. On the other hand, Nguyen *et al* (2001) demonstrated that nicotinic acetylcholine receptor, a calcium channel, are associated with keratinocyte differentiation. Their report is not inconsistent with this study because previous reports suggested that differentiation of epidermal keratinocytes is not always dependent on barrier homeostasis (Ekanayake-Mudiyanselage *et al*, 1998). Different types of glutamate receptors might regulate skin barrier homeostasis and other metabolic systems in the epidermis.

Recent findings suggest that an abnormality in the calcium dynamics in the epidermal keratinocytes is a crucial cause of cutaneous diseases. For example, mutation of the calcium pump of the keratinocyte is a cause of Darier's diseases and Hailey-Hailey disease (Sakuntabhai *et al*, 1999; Hu *et al*, 2000). Karvonen *et al* demonstrated that psoriatic keratinocytes have an inborn error in calcium signaling (Karvonen *et al*, 2000). Abnormal calcium localization in the epidermis has been reported on aged skin, atopic dermatitis and psoriasis patients (Forslind *et al*, 1999). Abnormalities of the barrier function in psoriasis, atopic dermatitis (Grice, 1980) and aging (Ghadially *et al*, 1995) were reported previously. Higher expression of NMDA receptor was observed in glioneuronal tumours from intractable epilepsy patients (Aronica *et al* 2001). Although the relationship between the expression of the NMDA receptors and abnormal metabolism of the cells has not been clarified, NMDA receptor might also be associated with these skin diseases.

We previously demonstrated that a psychological stress delayed the skin barrier recovery and that it was mediated by glucocorticoid (Denda *et al*, 2000b). No report has demonstrated the relationship between psychological stress and NMDA receptor expression in the epidermal keratinocytes. However, in lung epithelial cells, glucocorticoids increased mRNA of glutamine synthetase (Abcouwer *et al*, 1996). On the other hand, psychological stress also induces glutamate release in the brain (Gilad *et al*, 1990). In this study, we observed the release of glutamate by tape stripping. Although the mechanism of the glutamate secretion has not been clarified, glutamate might be another mediator between psychological factors and epidermal homeostasis.

Acceleration of barrier repair after its damage could prevent inflammatory responses induced by barrier disruption (Denda *et al*, 1997; Ashida *et al*, 2001b). The skin barrier homeostatic system of human skin is similar to that of hairless mouse (Elias *et al*, 1991). Accelerations of the barrier repair by topical application of same agents were observed in both hairless mice and human skin (Denda *et al*, 1997; Zettersten *et al*, 1997) Thus, a new strategy for improving the barrier repair may be used in human skin. This strategy could result in novel therapeutic approaches to treat the cutaneous disorders caused by barrier damage or abnormal ion dynamics in keratinocytes.

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