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Effects of a New Excitotoxic Amino Acid, Dysiherbaine, on Cultured Müller Cells

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Purpose: To determine the pharmacological response when dysiherbaine acts on cultured Müller cells, considering the glutamate receptor functions. Dysiherbaine is a new excitotoxic amino acid, which was recently isolated from the liquid extract of a certain sponge.

Methods: Retinas of adult rabbits were used to culture the Müller cells. Intracellular calcium ion concentration ($[Ca^{2+}]_i$) analysis was done by fluorophotometry with the calcium indicator, acetoxymethyl ester fura-2 (Fura-2 AM).

Results: A transient increase of $[Ca^{2+}]_i$ was observed following the administration of dysiherbaine (2.5 μM–2.5 mM) to Müller cells, but no $[Ca^{2+}]_i$ increase was observed in the extracellular calcium-free solution. This increase was blocked by the non-N-methyl-D-aspartate (NMDA) glutamate receptor antagonist, 6-cyano-7 nitroquinoxaline-2,3-dione (CNQX). A dysiherbaine-induced increase in $[Ca^{2+}]_i$ following preincubation of the NMDA glutamate receptor antagonist, (5R,10S)-(+)-5-methyl-10,11-dyhydro-5H-dibenzo [a,d] cyclohepten-5, 10-imine hydrogen maleate (MK 801) was seen in the same number of Müller cells with and without the antagonist.

Conclusions: Dysiherbaine appears to act primarily as a non-NMDA glutamate receptor agonist, having a secondary action as a NMDA glutamate receptor agonist. **Jpn J Ophthalmol 2002;46:153–159** © 2002 Japanese Ophthalmological Society

Key Words: Cultured Müller cells, dysiherbaine, glutamate receptor, intracellular calcium concentration.

Introduction

In 1997, Dr. Ryuichi Sakai and his colleagues,[1] who were collecting sea creatures and examining their excitotoxicity, found that the liquid extract of a certain sponge, *Dysidea herbacea*, commonly seen in the Micronesian Islands area, induced spasm in rats. The intraperitoneal administration of this extract at lower concentrations induced characteristic spasms similar to those of domoic acid and caused death after spasms at higher concentrations in rats. This extract was presumed to be a selective agonist of non-N-methyl-D-aspartate (NMDA) glutamate receptors based on the results of radio-ligand binding assays using synaptic membranes from the brain of rats. Thereby, a new excitotoxic glutamate receptor agonist, dysiherbaine, was discovered. This is a novel diamino dicarboxylic acid, which consists of a *cis*-fused hexahydrofuro[3,2-*b*]pyran ring replaced by a 3-[2-aminopropanoic acid] side chain. The molecular formula was determined to be $C_{12}H_{20}N_2O_7$.[1]

Müller cells are specific glial cells of the retina, which have now been proven to have multipotential functions. It has been shown that these cells are involved in the regulation of ionic concentration, the absorption and metabolism of released neurotransmitters, and the constituents of the b-wave of electroretinographs.[2,3]

Although the roles of Müller cells are not yet fully established, the cells are important for neuroglial, glioneuronal, or glioglial signalling.[4] It has been considered that glutamate receptors are present only in neurons. However, Puro reported, in 1993, that a NMDA glutamate receptor was also present in

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Müller cells.[5] Then, in 1994, Wakakura reported that a non-NMDA glutamate receptor was also present in Müller cells.[6] Schwartz and Keirstead reported in 1993 and in 1997, respectively, the presence of a metabolism control NMDA glutamate receptor.[7,8] Thus, it has been determined that Müller cells express glutamate receptors.

To clarify the characteristics of the glutamate receptors in Müller cells, we investigated the cellular response of cultured Müller cells to a newly found glutamate agonist, dysiherbaine.

Materials and Methods

Culture of Müller Cells

Culture of Müller cells was performed as reported previously.[9] In brief, the retinas of adult white rabbits weighing 2–4 kg were used to prepare the Müller cell culture. The eyes were removed after anesthetizing the rabbits with 25 mg/kg sodium pentobarbital (Nembutal®) given intravenously. After removal of the eyes, the animal were sacrificed with intravenous injection of an excessive dose of Nembutal®. The retinal tissue pieces were prepared as follows. The scleras at a distance of 2 mm from the cornea were dissected circumferentially and then the anterior chamber and lens were removed together. The vitreous bodies were removed from the posterior pole of the eyeball, and the retinas were removed from the optic discs. The retinal tissue pieces from which myelinated nerve fibers were removed were placed in serum-free Glasgow minimum essential medium (GMEM; Gibco BRL, Rockville, MD, USA) and were dissected into about 0.25 mm^2 pieces. The dissected tissue pieces were centrifuged at 179 *g* for 5 minutes, suspended in GMEM containing fetal calf serum, and incubated in 5% CO_2/95% air at 37°C for about 1 week. The culture solution was collected and centrifuged again; the pelleted tissue was resuspended in fresh GMEM containing 10% fetal calf serum. The confluent culture of Müller cells was collected on glass coverslips 2–3 weeks later. The cultured Müller cells were treated with 0.25% trypsin and cultured in a culture dish having a silicon wall (Flexperm disc, Toyobo, Osaka) with fresh culture solution. The cells were used after a lapse of 1–4 weeks.

Intracellular Calcium Ion Concentration Analysis

An Argus 100/CA (Hamamatsu Photonics, Hamamatsu) was used to analyze the intracellular calcium ion concentration ($[Ca^{2+}]_i$).[10] Calcium ions were labeled with a fluorescence calcium ion indicator, acetoxymethyl ester fura-2 (Fura-2 AM), which combined with free calcium ions to emit fluorescence. The fluorescence intensity of the labeled ions was measured at two different excitation wavelengths, 340 and 380 nm, and the 340/380 ratio was automatically calculated by the analyzer. The change in this ratio reflects the change of $[Ca^{2+}]_i$.[11]

Müller cells in a culture dish were first washed lightly with Hanks solution (NaCl 13.7 mM, KCl 0.5 mM, $Na_2HPO_4 \cdot H_2O$ 0.03 mM, KH_2PO_4 0.5 mM, HEPES 20 mM, $NaHCO_3$ 10 mM, $CaCl_2$ 1.26 mM, $MgSO_4 \cdot 7H_2O$ 0.8 mM, glucose 5.6 mM) and placed in 5 μM Fura-2 AM at 37°C for about 45 minutes.

Fura-2 AM was washed from the cells with Hanks solution, and 25 μL of the Hanks solution was dropped into the culture dish, which was placed on the stage of an inverted epi-fluorescence microscope (Olympus IMT-2; Olympus, Tokyo).

The fluorescence of labeled cells was captured using an intensified charge-coupled camera (C2400-08H; Hamamatsu Photonics) mounted on the instrument mentioned above. From this image, the fluorescence intensity 340/380 ratio was calculated using $[Ca^{2+}]_i$ software (Intracellular calcium ion concentration analyzing software U3390-02, version 3.7; Hamamatsu Photonics), and image analysis was performed. Cultured cells were treated by dysiherbaine or α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) at various concentrations (2.5 μM - 2.5 mM). The agonist, 25 μL in Hanks solution, was dripped on each coverslip. To determine the source of calcium activity, the agonist was administered to the cells in calcium-free Hanks solution containing the calcium chelating agent, 5 mM ethylene glycol bis (β-aminoethylether)-N,N,N1,N1-tetraacetic acid (EGTA). Cultured cells were treated by dysiherbaine or AMPA with or without preincubation with one of following receptor antagonists, the non-NMDA glutamate receptor antagonist; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) at 50 μM, (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo [a,d] cycrohepten-5,10-imine hydrogen maleate (MK 801) at 50 μM, and the calcium channel-blocking agent diltiazem hydrochloride, at 100 μM. Dysiherbaine was also administered in glycine (1 mM) containing magnesium-free Hanks solution to activate the NMDA receptor.

Measurements were performed for 40-second periods separated by 4-second intervals. Each experiment was repeated five or more times. Cells with more than a 120% increase in the 340/380 ratio were defined as positively responding cells. The rate of cells with a positive response was calculated from observation of at least 100 cells.

Pharmaceutical Agents

Dysiherbaine was supplied by Dr. Ryuichi Sakai, Kitasato University School of Fishery. The structural formula of dysiherbaine is shown in Figure 1. The glutamate receptor agonist (AMPA) and glutamate receptor antagonists (CNQX and MK 801) were purchased from RBI (Natick, MA, USA). Fura-2 AM and the calcium chelating agent (EGTA) were purchased from Dojin Kagaku (Kumamoto). Diltiazem hydrochloride, the antagonist selective to L type calcium channel, was purchased from RBI.

Results

Phase contrast micrography of cultured Müller cells is shown in Figure 2. The Müller cell is thick and has a core in the center of the cell, and the cytoplasm partly overlaps other cells. Calibration solution, instead of Müller cells, made of physiological saline with Fura-2 AM suspended in paraffin oil, was analyzed using the Argus100/CA. The calibration curve calculated for $[Ca^{2+}]$, was similar to that of Kawasaki et al.[10]

An example of the effect of 250 μM dysiherbaine on cultured Müller cells is shown in Figure 3. More than half the cells showed a significant increase in fluorescence intensity ratio and an increase in $[Ca^{2+}]_i$ within 4 seconds. The fluorescence intensity ratio increased following administration of 2.5 μM to 2.5 mM dysiherbaine and the response was concentration-dependent (Figure 4). Regarding the effect of AMPA on the cultured Müller cells measured as a control, the number of AMPA responding cells exceeded that of dysiherbaine at every concentration except at 2.5 μM and also was concentration-dependent (Figure 4).

Thus, the responses of cultured Müller cells to both agents were quite similar. Figure 5 shows an example of the effect of 250 μM dysiherbaine in calcium-free EGTA containing Hanks solution. There was no positive response with 250 μM dysiherbaine in calcium-free EGTA containing Hanks solution.

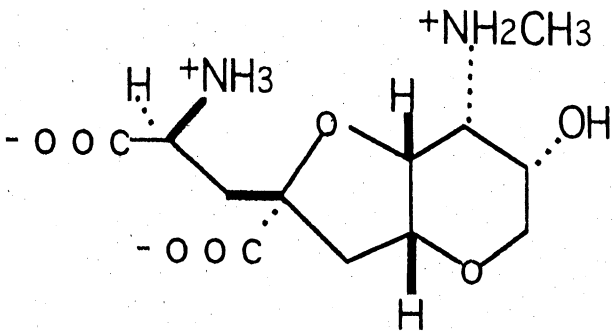

Figure 1. Structural formula of dysiherbaine.

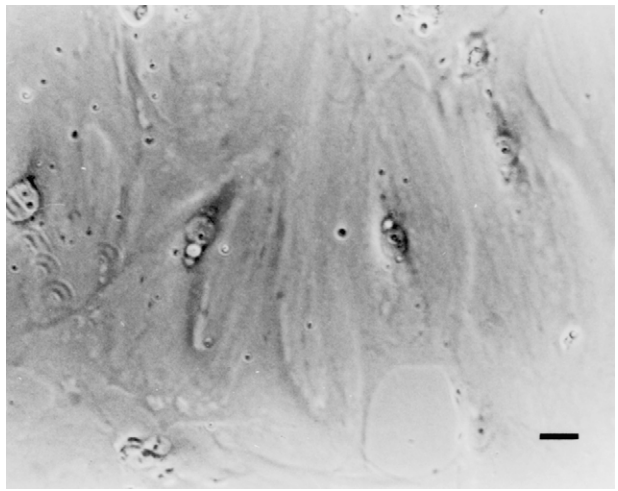

Figure 2. Phase-contrast micrography of cultured Müller cells. Bar = 50 μm.

In addition, the response of dysiherbaine was not blocked following administration of 100 μM of the selection L-type voltage-dependent calcium channel blocker agonist, diltiazem hydrochloride.

To determine whether the $[Ca^{2+}]_i$ increased through the glutamate receptor or not, dysiherbaine was reacted with cultured Müller cells following administration of the glutamate receptor antagonist. Figure 6 shows an example of the response of dysiherbaine following administration of CNQX. Only 5% of the total cell number (n = 120) showed an increase in fluorescence intensity ratio after administration of CNQX, proving that the action of dysiherbaine was apparently blocked by CNQX.

The number of responding cells following preincubation with 50 μM MK 801 was not significantly different from that without MK 801. The same result was obtained regarding the number of responding cells following administration of AMPA (Figure 7). However, the dysiherbaine-induced fluorescence intensity ratio without preincubation of MK 801 was always higher than that following preincubation with MK 801 (Figure 8). There was no difference for the AMPA-induced intensity ratio either with or without administration of MK 801.

Following perincubation of the cells with CNQX, dysiherbaine administration at 250 μM induced an increase in the fluorescence intensity ratio in 25% of the cells in the magnesium-free extracellular solution.

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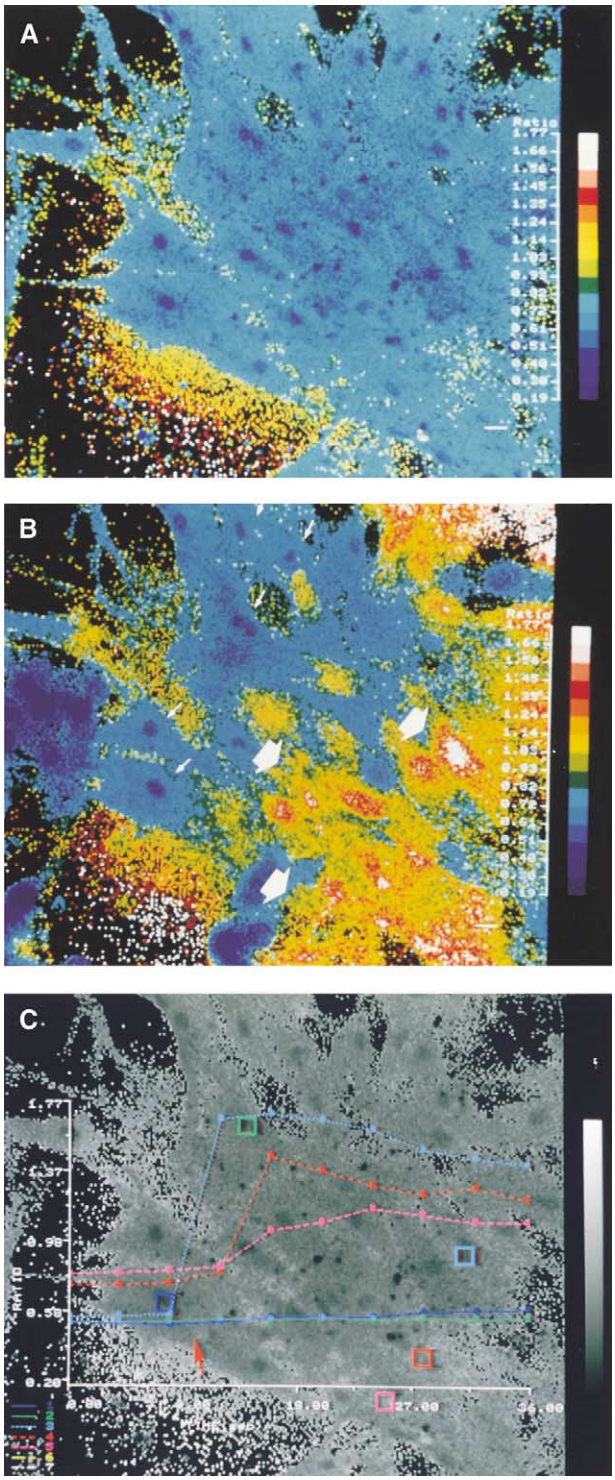

Figure 3. (**A**) Distribution of $[Ca^{2+}]_i$ in cultured Müller cells before dysiherbaine administration, demonstrated by pseudocolor coding. Bar = 50 μm. (**B**) Distribution of concentration of calcium ions in cultured Müller cells after dysiherbaine administration, demonstrated by pseudocolor coding. Cells with prominent increases in $[Ca^{2+}]_i$ are shown by large arrows and those without change, small arrows. Bar = 50 μm. (**C**) Temporal change of ratio at each cell indicated by square is illustrated. Red arrow represents time when 0.25 mM dysiherbaine is added. Diagram is superimposed on a cell image where analysis was carried out. Temporal change in cells indicated by squares of five different colors is shown by lines with same colors in the diagram. Increase in $[Ca^{2+}]_i$ is observed in some cultured Müller cells.

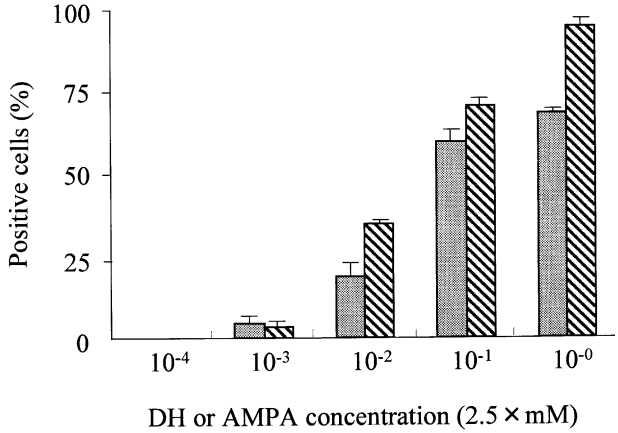

Figure 4. Comparison of percentage of cells showing an increase in $[Ca^{2+}]_i$ in cultured Müller cells following administration of either dysiherbaine (DH) or α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA). Mean ± standard error □: DH, ▨: AMPA.

Discussion

Several excitatory amino acid receptor agonists have been identified. For example, kainic acid was extracted and isolated from an insecticidal substance contained in *Digeniea-simplex*, *Rhodomelaceae*, *Flage*, and domoic acid from the seaweed, *domoi*. These agents have helped in determining the pharmacological response of glutamate receptors.

Dysiherbaine, used in the present study, was extracted from a sponge. We carried out this study with the expectation that a pharmacological action different from that of the agonists already known might be found.

The retinas of adult white rabbits are said to be vascularized, with the vessels being found on the surface of the retina. Because genuine Müller cells that do not contain astrocytes or oligodendrocytes can be obtained when cultured after removing myelinated nerve fibers, the retinas of rabbits are considered to be very suitable for culturing Müller cells.[12] Müller cells cultured by this method preserve the in vivo conditions of cell polarity even in the secondary or later cultures. It has also been confirmed, by the pos-

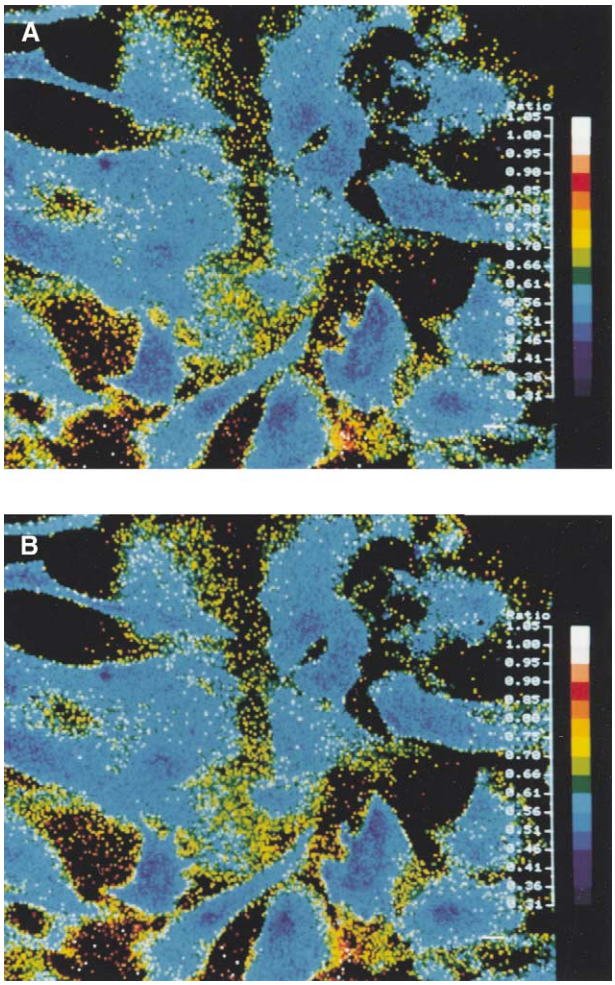

Figure 5. (**A**) Distribution of $[Ca^{2+}]_i$ in cultured Müller cells in calcium-free Hanks solution before dysiherbaine administration, demonstrated by pseudocolor coding. Bar = 50 μm. (**B**) Cultured Müller cells after 250 μM dysiherbaine administration, demonstrated by pseudocolor coding. Exposure did not express any change in distribution. Compare Figure 5B with Figure 5A. Bar = 50 μm.

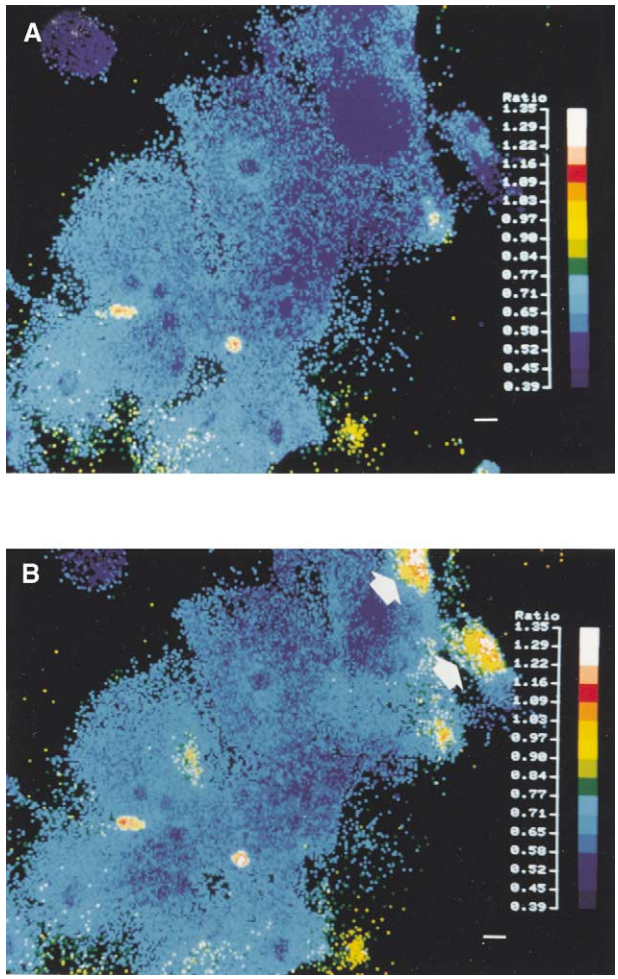

Figure 6. (**A**) Distribution of $[Ca^{2+}]_i$ in cultured Müller cells exposed to 50 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) before dysiherbaine administration, demonstrated by pseudocolor coding. Bar = 50 μm. (**B**) Distribution of $[Ca^{2+}]_i$ in cultured Müller cells exposed to 50 μM CNQX after 250 μM dysiherbaine administration, demonstrated by pseudocolor coding. Only two cells (arrows) showed increase in distribution. Bar = 50 μm.

itivity of a specific glial marker, carbonic anhydrase C, that the form and antigenicity of Müller cells are well preserved during the culture procedure.[9]

In the present study, the effect of dysiherbaine on cultured Müller cells was evaluated by measuring the increase in $[Ca^{2+}]_i$, which was detected by the change in the 340/380 ratio using the Argus 100CA. The measurement of fluorescence intensity ratio using Fura-2 AM as an indicator for changes in $[Ca^{2+}]_i$ has already been established as the method to determine response-related pharmacological changes. Using this methodology, dysiherbaine proved to have an effect on Müller cells through an increase in $[Ca^{2+}]_i$. In calcium-free solutions, there was no increase in $[Ca^{2+}]_i$, which suggests that the dysiherbaine-induced increase of $[Ca^{2+}]_i$, was mostly a result of influx from extracellular space. However, the involvement of a secondary increase in intracellular calcium was not clarified in this study.

Although the presence of L type calcium channels in Müller cells has been reported,[6,13] the effect of dysiherbaine was not altered after administration of diltiazem hydrochloride. Thus the dysiherbaine-induced increase in $[Ca^{2+}]_i$ was not the result of dysiherbaine directly stimulating and opening the L-type calcium channel.

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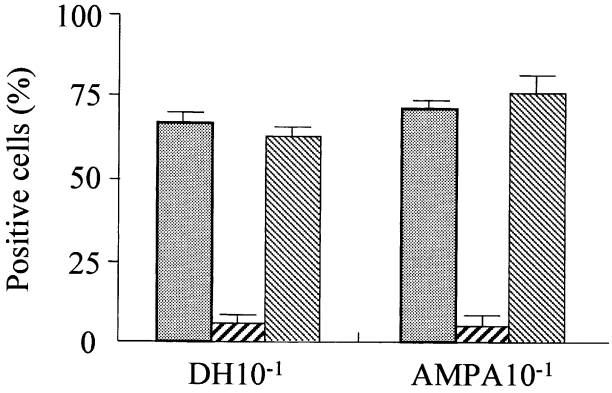

Figure 7. Comparison of percentage of cells showing an increase in $[Ca^{2+}]_i$ in cultured Müller cells following 250 μM dysiherbaine or 250 μm (AMPA) α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) administration with or without an antagonist. Concentration of each antagonist used, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) or (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cycrohepten-5,10-imine hydrogen maleate (MK801), is 50 μM. Mean ± standard error. □: Without an antagonist, ▨: With CNQX, ▧: With MK 801.

Because the effect of dysiherbaine on Müller cells following preincubation with CNQX was apparently blocked, dysiherbaine acts mainly through non-NMDA glutamate receptors. Concerning the effect of dysiherbaine on Müller cells following preincubation with MK 801, the number of responding cells was similar to that seen without the antagonist. However, when the response of each cell was examined, the increase of the fluorescence intensity ratio was apparently blocked. This finding can be interpreted as follows. The NMDA glutamate receptors maintain a high level of permeability to calcium ions but are not expressed in normal nerve transmissions because of a blockage by magnesium ions in the extracellular solution.

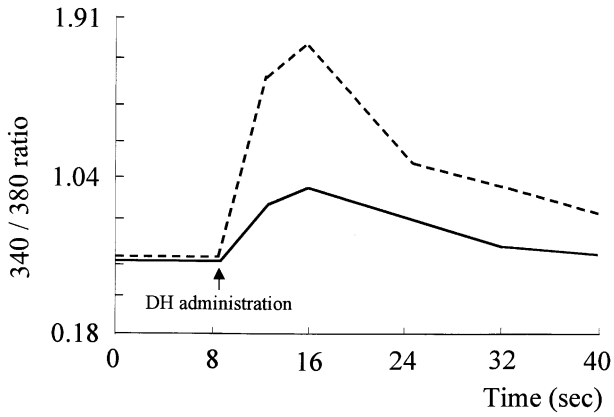

Figure 8. Comparison of temporal change in ratio following 250 μM dysiherbaine administration with and without (solid line) exposure (5R,10S)-(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cycrohepten-5,10-imine hydrogen maleate.

If depolarization does occur on the cell membrane due to some unknown factors and blockage by magnesium ion is eliminated, then non-NMDA glutamate receptors should be free to function. For example, when the non-NMDA glutamate receptor interacts with its agonist, the receptor is activated and $[Ca^{2+}]_i$ increases immediately. Consequently, the cell membrane is depolarized and the blockage by magnesium of the NMDA glutamate receptor is relieved.

If the NMDA receptor agonist is present, $[Ca^{2+}]_i$ should be expected to continue to increase via further receptor activation. Thus it could be considered that, with these processes, dysiherbaine can act as both a non-NMDA and a NMDA glutamate receptor agonist. The NMDA glutamate receptor does not work unless the non-NMDA glutamate receptor functions to cause depolarization. This is why blockage of the non-NMDA glutamate receptor did not induce an increase of $[Ca^{2+}]_i$. Also, this is why the rate of increase in the fluorescence intensity ratio did not change before and after preincubation with MK 801. A selective non-NMDA agonist such as AMPA does not react to NMDA glutamate receptors.

About 25% of the cells showed an increase in fluorescence intensity ratio in the extracellular magnesium-free solution after preincubation with the non-NMDA glutamate receptor antagonist, CNQX. This result supports our hypothesis that the non-NMDA receptor is the primary reactive site, and the NMDA receptor is the secondary functioning receptor.

Based on the aforementioned hypothesis, the increasing curve of the fluorescence intensity ratio could show bimodality, however, it was not shown in the present experiment. Because activation of both receptors occurred in a very short time (15 msec in an experiment which used bipolar cells[14]), our measurements were conducted every 4 seconds. However, this timing appeared to be too long to detect modality.

Dysiherbaine did not block the NMDA glutamate receptor antagonist, [^{3}H]CGS-19755, in the radioligand binding assay, which is used in brains of rats, indicating that dysiherbaine is an agonist of non-NMDA glutamate receptors. Our study also showed clearly that dysiherbaine acts by combining with the non-NMDA glutamate receptors on Müller cells. The possibility that dysiherbaine acts also on the

NMDA glutamate receptor seems contradictory to this hypothesis. The difference in the agents and the cells used in the experiments may be the cause of this contradiction; the agents were [^{3}H]CGS-19755 and MK 801, and the cells were Müller cells and cerebral cells.[5] Only the presence of NMDA on Müller cells was identified by a patch-clamp method,[5] but both NMDA and non-NMDA glutamate receptors have been identified on Müller cells.

In the central nervous system, it is considered that an increase in extracellular glutamate concentration during ischemia causes death of the neurons, but can be reduced by blocking the NMDA glutamate receptor.[15] The same phenomenon seems to occur in the retina.[16,17] The Müller cells that have both NMDA and non-NMDA receptors might act to reduce neuronal death. Analyzing the effect of new agonists such as dysiherbaine on Müller cells not only helps to define the pharmacological characteristics but also becomes one of the clues that can be used to determine the function of the Müller cells and their relationship with the retinal neurons.

Furthermore, it also can lead to finding the cause and prevention of the death of the retinal neurons.

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