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理事長 中島 章 殿

研究室で撮影した本人のスナップ写真、及び発表論文のコピーを添付

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研究テーマ 初代培養網膜神経細胞を用いてグルタミン酸毒性に対するイフェンプロジルの保護作用

2．本年度の研究業績

(1) 学会・研究会等においての口頭発表 (有)・無 （学会名・内容）

ARVO (The Association for Research in Vision and Ophthalmology).

内容：Protective Effects of Vitamin B6 Against Glutamate-Induced Neurotoxicity in Cultured Retinal Neurons.

日本眼科学會総會

内容：ラット網膜初代培養神経細胞におけるグルタミン酸毒性に対するビタミンB6の保護作用

(2) 学会誌等に発表した論文 (有)予定・無 （雑誌名・論文名）

雑誌名：《Graefe's Archive for Clinical and Experimental Ophthalmology》

論文名：Protective Effects of Ifenprodil Against Glutamate-Induced Neurotoxicity in Cultured Retinal Neurons

3．今後の研究計画

アポトーシス関連因子:bcl-2のノックアウトマウスとbcl-2を過剰発現するトランスジェニックマウスより網膜神経細胞初代培養を行い、低酸素負荷あるいはグルタミン酸曝露などの条件下での生存率を対照正常マウスと比較し、bcl-2の網膜神経細胞死における役割を明らかにする。特に、NOが網膜神経細胞を障害するのに単独では障害性はなく活性酸素を必要とすることから、bcl-2がミトコンドリア等の脂質膜の活性酸素生成系への抗酸化作用を有する事より、bcl-2類縁制御遺伝子の分子機構を明らかにする上でNOおよび活性酸素との関係の解明を行いたい。

4．研究指導者の意見

現在の研究のテーマはラット初代培養網膜神経細胞を用いたグルタミン酸毒性のメカニズムの解明及びその毒性に対する保護物質の検討です。このテーマに関する研究を始めて、早くも多くの重要な発見を次々に見いだしております。その成果は世界的にも高名な英語雑誌に投稿を予定しております。また、同時に臨床面でも精力的に研究活動を行っており、京都大学同窓会学会でも共同演者として発表をしておりました。このように研究面、臨床面ともに短期間で多くの成果を上げており、今後はその発表のために数多くの学会に参加していただく予定です。申請者は日本語の勉強も熱心で、日常会話に関しては全く問題なく、近々、自身での日本語による発表も行ってもらいます。将来、母国である中国に帰国された時には、当研究室での成果あるいは日本での滞在の経験を生かし、眼科領域の研究及び臨床の発展あるいは日中友好に大きく貢献されるものと確信しております。

研究指導者氏名 本田孔士 ㊞

5．研究報告

別紙形式を参考に、報告本文4000字以上で報告して下さい（枚数自由・ワープロ使用）

タイトル・要旨等は日本語で、KEY WORDS以下は日本語或いは英語で記入して下さい。

研究成果の発表予定がある場合は発表原稿・抄録集等を添付して下さい。

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Protective Effects of Ifenprodil Against Glutamate-Induced Neurotoxicity in Cultured Retinal Neurons

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Abstract

•Purpose: To examine the effects of ifenprodil on glutamate induced neurotoxicity in the cultured retinal neurons.•Methods: Primary cultures obtained from the fetal rat retinas (gestation day 17-19) were used for the experiment. Neurotoxicity effects on retinal cultures were quantitatively assessed by the trypan blue exclusion method. The cells were exposed briefly (10 min) to excitatory amino acids (EAA, 1 mM) and then were incubated for 1 hour in an EAA-free medium. Ifenprodil (10 mM) was added during the 10-min exposure to EAA and subsequent 60-min incubation in an EAA-free medium. •Results: Ifenprodil dose-dependently prevented cell death induced by glutamate or NMDA, but did not affect that induced by kainate. The protective effects of ifenprodil against glutamate neurotoxicity were significantly reduced by spermidine, a polyamine modulatory site agonist, but not by glycine, a strychnine-insensitive glycine site agonist. •Conclusion: These results suggest that ifenprodil protected retinal neurons against glutamate neurotoxicity by selective antagonism of the polyamine modulatory site of the NMDA receptor complex..

Introduction

Recent receptor cloning studies have shown that the N-methyl-D-aspartate (NMDA) receptor is a heteromer composed of the two subunit families NMDA receptor NR1, and NR2 subunits, A-D[12]. Studies of NMDA receptor subtypes in heterologous expression systems have established that the kinetic and pharmacological properties of NMDA receptors are determined by their subunit composition. Consequently, regional and developmental changes in NMDA subunit composition have been correlated with alterations in the properties of NMDA receptors in the brain[4]. In cortical neurons, NR2B subunits were reported to predominate during embryonic and early neonatal days, whereas expression of NR2A subunits became detectable during the early postnatal period and increased through the second postnatal week[13]. These developmental changes in the subunit compositions were suggested partly by findings on the developmental alterations in the sensitivity of the NMDA receptors to ifenprodil[14,20,22]. Ifenprodil, originally considered to act as a competitive antagonist at a polyamine binding site of the NMDA receptor, has been shown to act at a distinct site which has an allosteric effect on the polyamine site and is now regarded as the NR2B subunit-selective NMDA receptor antagonist[3,8].

Developmental differences in antagonism of NMDA toxicity by ifenprodil were first indicated in an ex vivo preparation of embryonic chick retina[23,24]. However, these studies did not clarify precisely how ifenprodil interacts with NMDA receptors to rescue retinal neurons. To determine the mechanism of protective action of ifenprodil against glutamate neurotoxicity, neuronal cell

culture is required where exposure and ionic conditions can be precisely controlled and multiple drug manipulations are possible at once. According to a study using cultured hippocampal cells, it is suggested that neither polyamine nor glycine sites were linked to the neuroprotective effects of ifenprodil, but instead they were ascribed to antagonism of the NMDA receptors[17]. As we have been studying glutamate neurotoxcity in cultured retinal neurons, we decided to study the effects of ifenprodil on glutamate-induced neurotoxicity using our well-characterized culture system of embryonic retinal neurons. We report that ifenprodil protects the embryonic retinal neurons by acting as an antagonist at the polyamine modulatory site of the NMDA receptor complex.

Materials and Methods

Materials

Primary cultures were obtained from the retinas of fetal rats(16 to 19 days gestation) and were used for the experiments. Drug-induced neurotoxicity was assessed as described previously[6,7,9]. In brief, retinal tissues were dissociated mechanically and single-cell suspensions were plated on plastic coverslips (1.2 to $1.8x10^6$ cells/dish). Cultures were incubated in Eagle's minimal essential medium (EMEM, Eagle's salts, Nissui, Tokyo) supplemented with 10% heat-inactivated fetal calf serum (1 ~ 8 days after plating), or 10% heat-inactivated horse serum (1~9 days after plating) or 10% heat-inactivated horse serum (10~14 days after plating), containing 2mM glutamine, 11mM glucose (total), 24mM sodium bicarbonate, and 10mM HEPES. After a 6-day culture, nonneuronal cells were removed by the addition of 10^{-5}M cytosine arabinoside. In this study we used only those cultures maintained for 10 to 12 days invitro and

only isolated cells. Clusters of cells were excluded from the results because cells located in clusters could not be used for histologic experiments. A previous immunocytochemical study showed that these isolated cells consisted mainly of amacrine cells[7]. All animals were treated in accordance with the ARVO Resolution on the Use of Animals in Research. The Principles of laboratory animal care (NIH publication no. 85-23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, as amended, were followed, as well as specific national laws (the Japanese Law on the Protection and Care of Animals, revised 1983, and the current version of the German Law on the Protection of Animals).

Drug Application

Ifenprodil was dissolved in the incubation medium immediately before the experiments to yield final concentrations of 10^{-8} to 10^{-5} M. Mg^{++}-free Eagles's solution was used to determine NMDA-induced cytotoxicity. Cultures were exposed for 10 minutes to glutamate followed by postincubation in glutamate-free medium for more than 1 hour. The cell viability was decreased. Ifenprodil was added for 10min before, during or after the 10 min exposure to glutamate (1mM). The drug induced maximal protection when added during glutamate exposure for 10 min and to glutamate-free medium for 1 hour. Therefore, in the current study, ifenprodil and MK-801 were added during glutamate exposure for 10min and to glutamate-free medium for 1 hour.

Measurement of Neurotoxicity

The neurotoxin effects of glutamate were assessed quantitatively by the trypan blue exclusion method[7,9,10]. All experiments were performed in Eagle's solution at 37^{0}C. After the completion of drug treatment, cell cultures were stained with 1.5% trypan blue solution at room temperature for 10 minutes and then were fixed with isotonic formaldehyde solution(PH 7.0, 2^{0}C to 4^{0}C), The fixed cultures were rinsed with physiological saline and examined by Hoffman modulation microscopy at x400. More than 200 cells on each of 5 coverslips were counted randomly to determine the viability of the cell culture. We counted on the order of 10% to 20% of the total cells grown on each coverslip, because the overall density of the cultures was 50 to 100 cells / mm^2. Viability of culture was calculated as the ratio of the number of unstained cells (viable cells) to the total number of cells counted (viable cells plus nonviable cells). The significance of data was determined by Dunnett's two-tailed test.

The following drugs were used: ifenprodil was purchased from Merrill-Dow Japan (Osaka, Japan); kainate and monosodium L-glutamate from Nacalai Tesque (Kyoto, Japan); MK-801 from Research Biochemicals Co. (St. Louis, MO).

Results

Figure 1 shows a typical example of ifenprodil-induced protection against glutamate neurotoxicity. The cultured retinal neurons were exposed to glutamate (1mM) for 10min and then incubated in normal solution for 60min. The viability did not change immediately after 10 min exposure to glutamate. However, further incubation in glutamate-free media for 60 min significantly increased the number of cells stained by trypan blue which reflect non-viable cells (fig.1B). Addition of ifenprodil (10μM) to both the media with and without glutamate used for the following 60 min incubation markedly reduced the number of cells stained by trypan

blue (fig.1C). Figure 2 summarizes the dose-response relationship of the protective effects of ifenprodil against glutamate-induced neurotoxicity. Ifenprodil at concentrations of 0.01 to 10 μM restored cell viability after a brief exposure to gluatamate in a dose-dependent manner.

Figure 3 demonstrates the time dependence of the protective effects of ifenprodil against glutamate neurotoxicity in cultured retinal neurons. Ifenprodil (10μM) were added to the incubation media for 10min before, during or after the 10min exposure to glutamate(1mM). Maximal protection was obtained when ifenprodil was added during glutamate exposure (Fig. 3.c and f). Although subsequent incubation in glutamate-free media with ifenprodil (Fig. 3.c) appeared to yield slightly greater protection than such incubation without ifenprodil (Fig. 3.f), there was no significant difference. Protective effects were also noted, when ifenprodil was applied immediately before or after glutamate exposure (Fig. 3. b and d). Ifenprodil did not affect the cell death induced by glutamate, when it was added 10min before or 10min after glutamate exposure (Fig. 3 a and e). These findings indicate that the simultaneous application of ifenprodil to glutamate exposure is critical for protective action against glutamate neurotoxicity.

To determine whether the ifenprodil-induced protection against glutamate neurotoxicity was due to its action on NMDA receptors, we further examined the effects of ifenprodil or MK-801 on the neurotoxicity induced by selective agonists, NMDA or kainate. NMDA was added to Mg^{++}-free Eagle's medium for 10min then the cultures were subsequently incubated in normal media for 60min. Both ifenprodil and MK-801 prevented NMDA-induced neurotoxicity (Fig. 4), but neither agent affected cell death induced by kainate (Fig. 5). Thus, it seems likely that the protective action of ifenprodil against glutamate neurotoxicity involves modulation of the NMDA-receptor complex.

To determine the specificity of the antagonists of polyamine and glycine modulatory sites of NMDA receptors, we examined the effects of spermidine, a typical endogenous polyamine, and glycine on ifenprodil-induced protection. First, we examined the effects of spermidine and glycine on glutamate neurotoxicity, as it is known that NMDA receptors are positively modulated by polyamines and glycine. Simultaneous application of spermidine or glycine appeared to slightly enhance cell death induced by glutamate in comparison to that induced by gluatamate alone, though there was no statistically significant difference (Fig. 6A). As in Fig. 6B, the protective action of ifenprodil was markedly inhibited by the simultaneous application of spermidine but not by that of glycine, indicating that ifenprodil prevented glutamate neurotoxicity by its selective interaction with the polyamine modulatory sites of the NMDA receptor.

Discussion

Glutamate interacts with both NMDA and non-NMDA receptors. The activity of NMDA receptors is influenced by a number of endogenous substances. Such compounds include divalent cations, glycine and polyamines. Previously we demonstrated that divalent cations such as Mg^{++} and Zn^{++} reduced NMDA receptor-gated currents in cultured retinal neurons[9]. In contrast to inorganic substances, glycine and polyamines potentiate the effects of NMDA agonists. Endogenous polyamines such as spermidine selectively bind to the polyamine modulatory site linked to the NMDA receptor complex then potentiate glutamate actions on NMDA receptors. Ifenprodil is a new class of noncompetitive NMDA antagonists[19]. It has been claimed that ifenprodil blocks NMDA receptors by antagonizing the positive modulatory actions that polyamines such as spermine and spermidine have on NMDA receptor activation[2,16,18,21]. Other studies have suggested that

ifenprodil may act in vivo as an antagonist of the NMDA-coupled strychnine-insensitive glycine receptor-mediated response[15].

In our cultured retinal neurons, NMDA receptors are the predominant root of glutamate neurotoxicity, since MK-801, a selective NMDA receptor antagonist, markedly inhibited cell death induced by glutamate, as previously reported[6,7,9,10,11]. The present study demonstrates that ifenprodil significantly protected cultured retinal neurons from neurotoxicity induced not only by glutamate but by NMDA as well. Moreover, ifenprodil inhibited NMDA-induced neurotoxicity to a similar degree seen with simultaneous application of MK-801 and NMDA. However, ifenprodil failed to inhibit kainate-induced neurotoxicity. The lack of protection with ifenprodil from kainate neurotoxicity indicates the selectivity of ifenprodil for NMDA receptor subtypes among the ionotropic glutamate receptors. Therefore, it is suggested in the cultured retinal neurons that the protective effect of ifenprodil against glutamate neurotoxicity occurs by its inhibiting action against NMDA receptors.

Ifenprodil, one of the NMDA receptor antagonists, has been reported to inhibit neurotoxicity induced by glutamate and NMDA on hippocampal cultures[5]. However, there have been some controversies present as to the mechanism of its protection through NMDA receptors. Shalaby and his associates reported that protective action of ifenprodil on cultured hippocampal cells against glutamate neurotoxicity took place by inhibition of the NMDA receptor but not by interacting with the polyamine or glycine sites in contrast to those reporting the inhibitory site present with the polyamine site[17]. Previously Tamura et al[19] demonstrated in rat cultured cortical neurons that ifenprodil protects against glutamate-induced neurotoxicity by selective inhibition of the polyamine site of the NMDA receptors. Interpretation of the discrepancy between these studies is difficult, inasmuch as different brain regions were used. There may be differences between the cerebral cortex and

hippocampus in the function of NMDA receptors and the mechanisms underlying glutamate-induced cell death. As we have been studying glutamate neurotoxicity in the cultured retinal neurons[6,7,9,10,11], further study was undertaken to address this issue using our well characterized cultured retinal neurons.

Ifenprodil has been shown to inhibit NMDA receptors by inhibiting the positive modulatory polyamine sites[1], or blocking strychnine-insensitive glycine sites[15]. In order to elucidate which sites of NMDA receptors, i.e. the polyamine and/or glycine modulatory sites, were involved in the neuroprotective actions of ifenprodil on retinal neurons, we studied the effects of simultaneous applications of either spermidine, a typical polyamine, or glycine on ifenprodil-induced protection against glutamate-induced neurotoxicity. Spermidine significantly reversed the ifenprodil-induced protection against glutamate. By contrast, the simultaneous application of spermidine with glutamate appeared to enhance the cell loss induced by glutamate (1mM), although the difference was not significant. The cell viability did not change by the application of spermidine alone. Ifenprodil was initially considered a competitive antagonist at the polyamine binding site of the NMDA receptors, but recent studies on cortical neurons suggest that ifenprodil and polyamines bind to distinct sites and that there is an allosteric interaction between the ifenprodil and polyamines binding sites[8]. However, the neuroprotective effects of ifenprodil appeared not to be caused by the interaction with the strychnine-insensitive glycine binding site of the NMDA receptor, since exogenous glycine did not reverse the blockade of glutamate neurotoxicity. The simultaneous application of glycine with glutamate did not affect glutamate neurotoxicity, although there appeared to be a slight tendency for the potentiation of cell loss when spermidine was added with glutamate. There results suggest that ifenprodil protected retinal neurons against glutamate

neurotoxicity by its inhibitory action on the polyamine modulatory site of the NMDA receptor.

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Legends for Figures

Figure 1: Photomicrographs showing the effect of chronic application of ifenprodil on glutamate-induced neurotoxicity. All cultures were photographed after trypan blue staining followed by formaldehyde solution fixation using Hoffman modulation microscopy. Cells stained with trypan blue dye were regarded as nonviable. (A) Control (nontreated cells). Cells were stained without application of glutamate. (B) Cells were treated with glutamate (1mM), followed by a 1-hour incubation with glutamate-free medium. Marked cell death occurred. (C) Ifenprodil was applied during glutamate incubation for 10 minutes and glutamate-free incubation for 1 hour. Cell death was reduced markedly.

Figure 2. The dose response relationship of the protective action of ifenprodil against glutamate-induced neurotoxicity. Application of ifenprodil showed protective effects against glutamate(1mM) neurotoxicity in a dose-dependent manner at concentrations between10^{-8} M and 10^{-5} (** $P<0.01$ versus the black column).

Figure 3. Time dependence of the protective effect of ifenprodil against glutamate-induced neurotoxicity. (A) Methods of drug

application. Cells were treated with 1mM glutamate, followed by 1 hour postincubation in glutamate-free medium. Time dependence of the protective effects of ifenprodil against glutamate cytotoxicity. Closed and open columns represent the periods of exposure to glutamate (1mM) and ifenprodil(10^{-5} M). In (c) and (f), ifenprodil was added during glutamate exposure with subsequent incubation in glutamate-free media with ifenprodil (f) or without ifenprodil (c). Ifenprodil was applied immediately before (b) or after glutamate exposure(d). Ifenprodil was added 10 min before (a) or 10 min after glutamate exposure(e). (B) Potentiation of ifenprodil against glutamate-induced neurotoxicity. Ifenprodil inhibited glutamate-induced cell death (** $P< 0.01$ versus the black column) and ifenprodil (10^{-5}M) showed a maximal protective effect against glutamate neurotoxicity when applied as in (f).

Figure 4. Effect of ifenprodil and MK-801 against NMDA-induced neurotoxicity. NMDA (1mM) was added to Mg^{++}-free Eagle's medium, then ifenprodil or MK-801 were applied with NMDA for 10-min and added to NMDA-free medium for 1 hour. In this treatment, ifenprodil (10^{-5}M) or MK-801 inhibited NMDA-induced cell death(** $P<0.01$versus the black column).

Figure 5. The effects of ifenprodil and MK-801 against kainate-induced neurotoxicity. When ifenprodil(10^{-5}M) or MK-801(10^{-5}M) was administered during kainate exposure(10 minutes) and subsequent incubation in kainate-free medium for 1 hour, neither agent showed significant protection against kainate-induced neurotoxicity.

Figure 6. Effects of spermidine and glycine on ifenprodil-induced neuroprotection. (A) Spermidine (10^{-4}M) and glycine (10^{-4}M) in the

culture with and without glutamate (1mM) neurotoxicity. Spermidine did not show any effect when added in the presence or absence of glutamate ($p > 0.05$). (B) Ifenprodil was administered during glutamate (1mM) exposure (10minutes) and during glutamate-free incubation (1 hour). Protective effects of ifenprodil against glutamate neurotoxicity were inhibited by simultaneous application with spermidine but not with glycine.