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Regulatory mechanisms and physiological implications of the calcium oscillation in the astrocytes

（アストロサイトにおけるカルシウム振動の調節機構とその脳機能に果たす役割に関する研究）

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Kobe University Graduate School of Science and Technology

January 2004
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要旨

アストロサイトを含むグリア細胞は神経系を構成する主要な細胞集団であり、神経細胞と同じ前駆細胞から発生するが、分化に伴い電気生理学的に非興奮性となる。アストロサイトは白質と灰白質の両方に存在する大型のグリア細胞で、哺乳動物中枢神経系における最大の細胞集団である。その性質は脳部位、発生段階、病態などによって多様であるが、共通の性質として glial fibrillary acidic protein をマーカーとして発現する点が挙げられる。

アストロサイトの主要な役割は神経組織の構造と環境の維持であると長く考えられてきた。実際アストロサイトは神経細胞間の電気的絶縁状態、イオン組成、栄養物質、成長因子、神経伝達物質などを適切な状態に維持するために有効な細胞機能を持つ。一方、神経系の主要な機能である神経伝達を介した情報処理にアストロサイトは直接関与してはいないと広く信じられてきた。しかし、従来の電気生理学的手法にかえ、神経活動を測定する手段としてカルシウムイメージングが一般的に用いられるようになった 1990 年代以降、この認識に変化が生じつつある。すなわち様々な神経伝達物質に対して培養アストロサイトが顕著なカルシウム応答を示すことが示され、アストロサイトが神経活動に直接参加している可能性が示唆された。さらに近年の脳組織を用いた生理学的検討により、アストロサイトが神経活動に呼応してカルシウム応答を示し、これがさらに周囲の神経細胞に影響を与えることを示す事実が蓄積しつつある。
る。

しかし、現在のところアストロサイトのカルシウム応答が神経機能に果たす役割について確定した知見は存在しない。これを確立する上で障害となっているのがアストロサイトの多様性である。カルシウム応答の薬理学的性質（どのような刺激に対して応答を示すか）とパターン（一過性、振動性など）について、これまでの報告は一貫した結果を示しておらず、このことがアストロサイトの機能を解明する上で事態を複雑にしている。脳部位、病態、培養条件（細胞密度、培養液の組成）などがアストロサイトの性質に影響を与えた結果、多様性が生じたと予想される。しかし、こういった点に関して適切に比較検討した例は乏しく、様々な環境要因がアストロサイトに与える影響を整理することが必要だと考えられる。アストロサイトにおけるカルシウム応答の多様性について環境要因の影響という視点から実態解明することを本研究は目的とした。脳内環境に応じてアストロサイトの神経伝達物質に対するカルシウム応答がどのように変化するか明らかになれば、アストロサイトが神経系の情報処理に果たす役割を解明する上での第一歩となることが期待される。

本研究ではまずアストロサイトのカルシウム応答に多様性が生じる機構を明らかにし、次にこれに基づいてアストロサイトが脳機能に果たす役割を解明しようと試みた。第 1 章において培養アストロサイトのカルシウム応答パターンを定量的に解析する方法を開発し、第 2 章ではこれを用いて脳内の液性因子がアストロサイトのカルシウム応答パターンに与える影響を評価した。アストロサイトは相互に接着しているため、
カルシウムイメージングの際に個々の細胞を識別することが困難である。そのためカルシウム蛍光色素 Fura2 とは独立した蛍光特性を持つ色素で細胞核を染色し、これを指標に細胞の位置情報を決定する方法を開発した。これにより核近傍領域のカルシウム応答を解析し、細胞体のカルシウム応答パターンを個別に定量化して、統計的に解析することが可能となった。次にこの手法を用いて培養アストロサイトのグルタミン酸刺激などに対するカルシウム応答パターンが脳内の液性因子によりどのような影響を受けるか系統的検討した。その結果、成長因子（Epidermal growth factor と Basic fibroblast growth factor）と炎症サイトカインなど（Interleukin-1β, Tumor necrosis factor-α及び Lipopolysaccharide）が培養アストロサイトのカルシウム振動を二重に制御することが明らかとなった。すなわち無血清培養液を用いて培養したアストロサイトは、成長因子処理により振動性のカルシウム応答を示すようになるのに対し、サイトカインはこの成長因子の効果を抑制した。さらに脳スライス標本を用いてアストロサイトを刺激した際の神経細胞のカルシウム応答を検討したところ、神経細胞においてもカルシウム振動が見られ、その周期性は培養アストロサイトのものと類似していた。このことは、アストロサイトのカルシウム振動が脳組織内で神経細胞の活動を誘導することを示唆している。

このような結果を踏まえ、第 3 章ではアストロサイトが自身のカルシウム応答パターンを調節するメカニズムを解明することを目的とし、これら液性因子がアストロサイトの細胞内情報伝達経路に与える影響を検討した。刺激に用いたグルタミン酸はア
ストロサイトのGタンパク質共役型受容体を活性化し、inositol-1, 4, 5 trisphosphate (IP₃)産生を促進し、細胞内カルシウムストアーからのIP₃依存的なカルシウム放出を誘導する。そのため、細胞内カルシウムストアーと細胞内IP₃産生に対する液性因子の影響をイメージングにより検討した。その結果、アストロサイトのカルシウム振動はMAPキナーゼ経路を介したカルシウムストアーのサイズ制御により調節されていることが明らかになった。成長因子はMAPキナーゼ経路を活性化し、カルシウムストアーソのサイズを増加させるが、MAPキナーゼ経路の阻害剤、及び炎症性サイトカインはMAPキナーゼ経路とカルシウムストアーソの增加を同時に抑制した。

また、近年、いくつかの細胞種において細胞内IP₃濃度の振動がカルシウム振動の基本的メカニズムであることが示されている。本研究ではphospholipase Cδ1に由来するPleckstrin homology domainとgreen fluorescence proteinおよびその変体の融合タンパク質を用いたIP₃のイメージング法をアストロサイトに適用し、カルシウム振動におけるIP₃の動態を検討した。その結果、グルタミン酸単独刺激によるIP₃上昇はこの方法の検出感度以下であったが、カルシウムイオンフォアにより強制的な細胞内カルシウム上昇を誘起した条件下でグルタミン酸は検出範囲内のIP₃上昇を引き起こすことが明らかになった。このことはグルタミン酸単独刺激によるIP₃上昇は低いレベルであるが、強制的なカルシウム上昇に伴い高いレベルになることを示している。また、これらの性質はカルシウム振動を誘導する成長因子処理に影響を受けなかった。すなわち、アストロサイトにおいてグルタミン酸によるIP₃産生は受容体と細胞内カ
ルシウムによる二重制御を受けているが、この性質はカルシウム振動の発現とは相関していないかった。今後、より高い検出感度での検討が必要であるが、カルシウム依存的なIP₃産生がアストロサイトにおいては必ずしもカルシウム振動発現のメカニズムとして利用されていないことを本研究結果は示唆している。

以上の結果よりアストロサイトのグルタミン酸に対するカルシウム応答は、脳内に存在する液性因子により応答パターンの調節を受けることが明らかになった。また、この調節は主にMAPキナーゼ経路を介したカルシウムストアのサイズ変化によるものであった。これらの事実は従来の研究結果を再評価する上で重要な示唆を与える。

これまでの培養アストロサイトを用いたカルシウム応答研究の多くは、細胞が様々な細胞密度で調整され、血清を含む培養液中で維持されていた。今回、影響が明らかになった成長因子とサイトカインはいずれも血清中に存在すると同時に、アストロサイト自身が産生することが知られている。このため、従来の研究結果は異なった液性因子影響下におけるアストロサイトの性質を反映している可能性が高い。今後、液性因子の影響を考慮して実験結果を再検討する必要があると考えられる。

また、この液性因子の影響はアストロサイトが脳機能に果たす役割を考える上で興味深い示唆を与える。脳内の液性因子濃度は神経系の発生、記憶形成などの機能発現、病態といった状況に応じて顕著な変化を示すことが知られている。これらの因子が神経細胞に直接影響を与え、その活動を調節することは十分考えられる。しかし、今回の研究結果から、液性因子がアストロサイトを介して二次的に神経活動に影響を与える
る経路の存在が予想された。すなわち、液性因子濃度に反映される脳内の環境をアストロサイトが感知し、自身のカルシウム応答パターンを変化させることにより、神経活動が調節されるという経路である。成長因子は神経発生、記憶形成などにおいて濃度が高まるのに対し、炎症性サイトカインは各種の病態、老化に伴って産生が高まる。この傾向は、アストロサイトのカルシウム振動が神経機能の亢進に働くという考えを支持している。今後、アストロサイトの液性因子受容体、MAPキナーゼ経路、カルシウムストアのサイズ等に変異を加えたモデル動物を作成し、これを解析することにより、上記のモデルを検証することが可能であると考えられる。神経系の情報処理に関しては不明な点が多いが、アストロサイトの関与を明確に定義し、これを加えた形で検討することができれば、より実際の脳に近い形で理解することが可能となる。アストロサイトは神経系最大の細胞集団であり、この関与が解明されれば、脳研究が大きく進歩すると期待される。
ABSTRACT

Glial cells are major cellular components of the nervous system, and non-excitable cells generated from the identical progenitor cells for neurons. Astrocytes are macro-glia localizing both in the white and gray matter, and compose the largest cellular population in the mammalian central nervous system (CNS). Although astrocytes commonly express glial fibrillary acidic protein, they show diverse properties in various regions of the brain, and in development and pathological processes.

In the long history of neuroscience, the major roles of astrocytes have been considered to maintain the structure and the environment of the CNS. Actually, astrocytes possess effective cellular functions to insulate neurons and to control extracellular concentrations of ions, nutrients, growth factors and neurotransmitters. On the other hand, while it had been believed that astrocytes were not involved in the major function of the CNS, namely information processing via neuronal activities, the situation has changed since the 1990’s, when calcium imaging was established as a means to measure neural activities, in addition to electrophysiological techniques. The astrocytic calcium responses to neurotransmitters have been proposed as having direct involvement in astrocytes in neuronal activities. Furthermore, various lines of evidence showing that neuronal activities induce calcium increase in the astrocytes and the responding astrocytes influence neighboring
neurons, have been accumulating from studies using brain tissue preparations.

The involvement of astrocytes in neuronal activities via calcium responses is, however, still far from being established. Previous studies have provided contradictory results in regards to both the pharmacological properties and the patterns (transient or oscillatory) of the calcium responses, and as such it is difficult to have widely accepted conclusions. The discrepancy in results is assumed to derive from the differences of the sources (brain regions) and the conditions (cell density and the composition of culture media) in preparing astrocytes or the tissues. In fact, few reports are worth comparison of the experimental conditions for the calcium imaging of astrocytes. In order to understand the roles of astrocytes in brain function further, it is inevitable to survey the effects of various environmental factors on the astrocytic calcium responses. The purpose of the present study is to reveal the variability of calcium dynamics in astrocytes, from the point of view of the effects of brain environment on astrocytes. I believe this project will provide data, which will be valuable to understand the role astrocytes play in information processing in the CNS.

In the present study, the mechanism underlying the variety of the astrocytic calcium responses has been investigated, and I have sought the elucidation of the roles of astrocytes in brain functions. In the first chapter, a novel method for the statistical analysis of cultured astrocyte calcium response patterns was established, then in the second chapter, the effects of soluble factors in the brain, including growth factors and pro-inflammatory
cytokines were examined. When cultured astrocytes come in contact with each other closely, it is not easy to distinguish individual cells in data obtained by calcium imaging, therefore the areas of individual cell bodies were determined by staining nuclei using a fluorescent dye, which does not influence the fluorescence of the calcium indicator, Fura2. Analyzing the fluorescent changes in the areas around the nuclei, enabled me to quantify the calcium responses of the cell bodies for statistical analysis. Using this method, the patterns of the astrocytic calcium responses to glutamate was shown to be under dual regulation by growth factors (epidermal growth factor and basic fibroblast growth factor) and pro-inflammatory cytokines (interleukin-1β, tumor necrosis factor-α and lipopolysaccharide). The growth factors promoted oscillatory calcium responses in the astrocytes cultured in a defined medium. In contrast, the pro-inflammatory cytokines suppressed the effect of the growth factors. Since similar growth factor-induced promotion of calcium oscillation was observed in the astrocytic calcium responses to ATP and a direct inositol-1, 4, 5 trisphosphate (IP₃) receptor sensitizer, thimerosal, as well as to glutamate, I assumed that the growth factors altered the cellular calcium dynamics mediated by IP₃. In addition, the calcium oscillation of the cultured astrocytes possessed similar properties to those of neurons in a tissue culture preparation, indicating that the calcium oscillation of astrocytes could promote neuronal activities in vivo.

In the third chapter, in order to elucidate the mechanisms regulating the calcium
response patterns, the effects of the soluble factors on signal transduction pathways in astrocytes were examined. Glutamate is known to activate G-protein coupled receptors of astrocytes, and leads to the production of IP₃ and the release of calcium from intracellular calcium stores. The effects of soluble factors on the cellular IP₃ and the calcium store sizes were hence examined, and I found that the alteration of calcium store size via mitogen-activated protein (MAP) kinase cascade was shown to be essential for the regulation of oscillatory calcium responses in astrocytes. The growth factors were found to increase the store size, while an inhibitor for MAP kinase cascade, U0126, and the pro-inflammatory cytokines suppressed the effects of the growth factors.

In addition to the already reported mechanisms for calcium oscillation, the oscillation of cellular IP₃ has been suggested recently. In the present study, IP₃ imaging methods using fusion proteins of green fluorescent protein variants and pleckstrin homology domain from rat phospholipase C-δ1, were applied to cultured astrocytes to investigate cellular calcium and IP₃ dynamics. As a result, I found that IP₃ production induced by glutamate was below the sensitivity of these methods; however, the simultaneous stimulation of glutamate and calcium ionophore induced detectable IP₃ production. The stimulation using calcium ionophore alone was not effective for the detectable IP₃ production, either. These results indicate that glutamate induces low levels of IP₃ production, and a massive calcium increase raises the glutamate-induced IP₃ production to a high level. Since this
tendency was not affected by the growth factors, the synergistic regulation of IP3 production by glutamate and calcium did not correlate with the calcium oscillation in astrocytes.

Although the calcium dependency of the IP3 production is assumed to be a mechanism for calcium oscillation, I believe it was not the case in astrocytes.

The conclusion of the present study is that the patterns of astrocytic calcium responses to neurotransmitters are regulated by soluble factors in the brain, and the present regulation is mediated by the alteration of the calcium store size via MAP kinase cascade. This evidence provides important suggestion for the re-evaluation of previous studies that employed culture media containing serum and done under various cell densities. Since both the growth factors and the pro-inflammatory cytokines could have been present in serum, and also been produced by astrocytes by themselves, the previous studies could be under reflection of the properties of the astrocytes under the various effects of soluble factors.

In addition, the effects of soluble factors described in the present study provide important data on the roles astrocytes play in brain functions. The concentration of the soluble factors in the brain is known to change in development, memory formation and pathological processes. Although there is the possibility that these factors directly affect neurons, the present results provide another model, in which soluble factors affect neuronal activities via astrocytes. Astrocytes would detect the brain environment, which is reflected in the concentration of soluble factors, and tune the neuronal activities to appropriate levels.
by altering their own calcium response patterns. The growth factors increase in development and memory formation, in contrast, the production of pro-inflammatory cytokines is induced in pathological processes and aging. These facts support the notion that astrocytic calcium oscillation promotes neuronal activities. Analysis of genetically modified animals, in which the receptors for the soluble factors, MAP kinase cascade and the regulation of calcium store size are impaired in the astrocyte specific manner, would be useful means to evaluate the present model. There are still many unrevealed features in the information processing in the CNS, however when the participation of astrocytes, the largest population in the CNS, is elucidated, I believe progress would be achieved in research on the brain.
ABBREVIATIONS

ADM; astrocyte defined medium

AMPA; $\alpha$-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BSS; basal salt solution

bFGF; basic fibroblast growth factor

CHPG; (RS)-2-chloro-5-hydroxyphenylglycine

CNS; central nervous system

DHPG; (S)-3,5-dihydroxyphenylglycine

DHK; dihydrokinate

DMEM; Dulbecco’s Modified Eagle’s medium

EGF; epidermal growth factor

egr-1; early growth response gene-1

ERK; extracellular signal-regulated kinase

FCS; fetal calf serum

FRET; fluorescence resonance energy transfer

GFAP; glial fibrillary acidic protein

GFP; green fluorescent protein

IICR; IP$_3$-induced calcium release
IP$_3$; inositol-1,4,5 trisphosphate

IL-1β; interleukin-1β

LPS; lipopolysaccharide

mAChR; muscarinic acetylcholine receptor

MAP kinase; mitogen-activated protein kinase

NBQX; 6-Nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione

NMDA; N-methyl-D-aspartate, NMDA

PHD; pleckstrin homology domain

PIP$_2$; phosphatidyl inositol-4,5 diphosphate

PLC; phospholipase C

PKC; protein kinase C

mGluR; metabotropic glutamate receptor

tACPD; (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid

SRE; serum response element

TNFα; tumor necrosis factor-α

UV; ultraviolet
GENERAL INTRODUCTION

Classical view of astrocytes in brain function

The mammalian central nervous system (CNS) consists of neurons and glia, both of which are generated from identical progenitor cells (Gage, 2000; Price and Williams, 2001; Temple, 2001). Neurons have excitable membrane properties, while glia are non-excitable. Astrocytes belong to glia, and are localized both in the white and gray matter, and express a marker, glial fibrillary acidic protein. In the gray matter, astrocytes are the largest glial cell population, and fill the space among neuronal cell bodies and processes, synapses and blood vessels (Zonta et al., 2003). The known classical functions of astrocytes are to maintain the structure of the CNS, and to control the extracellular concentrations of ions, nutrients, growth factors and neurotransmitters (Duffy and MacVicar, 1999; Forsyth et al., 1996; Forsyth, 1996; Oderfeld-Nowak and Bacia, 1994; Schousboe, 2003). The followings are the well-established roles of astrocytes concerning neuronal activity. In order to maintain neuronal activity independency, astrocytes wrap neurons to the extent that the neurons are insulated and the extra-synaptic interactions are interfered. This, as well as the absorption of extracellular potassium, which is released from firing neurons, prevents the depolarization of the neighboring neurons. Furthermore, the astrocytic transporters rapidly absorb the synaptically released neurotransmitters, including glutamate and GABA, and contribute to
the spatial and temporal specificity of synaptic transmission. The functions of the astrocyte transporters are well represented in Figure 0-1, which shows the synaptic currents at parallel fiber synapses of Purkinje neurons in knock-out mice of the glutamate transporter (GLAST), that is expressed in Bergman glia (Marcaggi et al., 2003). The excitatory post-synaptic currents induced by single stimulations are not affected, but the restorations of the responses to repetitive stimulations are severely prolonged in the knock-out mice. These results indicate that in the absence of astrocytic glutamate transporters, glutamate leaked from the synaptic cleft and spread to the neighboring naive synapses. As described above, the classical functions of astrocytes are important for maintaining the proper and effective activities of the neuronal network.

**Novel functions of astrocytes revealed by calcium imaging**

The major function of the CNS is information processing via neuronal activities. When electrophysiology was the sole means to measure neural activities, the non-excitable astrocytes were considered not to be involved in the information processing. However, the recent establishment of calcium imaging has helped change the situation. A series of compounds, which are released to the extracellular space during neuronal activities, including glutamate, monoamine, neuropeptides and lipid metabolites have been shown to induce calcium responses in cultured astrocytes (Verkhratsky and Kettenmann, 1996). These facts suggest that astrocytes show calcium increases in response to neuronal activities in
vivo, and evidence supporting this model has been accumulating by way of calcium imaging studies using tissue slice preparations (Haydon, 2001; Pasti et al., 1997; Porter and McCarthy, 1996). Furthermore, there have been a number of studies showing that the responding astrocytes influence the activities of the neighboring neurons (Carmignoto, 2000; Parri et al., 2001; Pasti et al., 1997). As astrocytes come in close contact with synapses (Ventura and Harris, 1999), the synaptically-released neurotransmitters leak out of the synaptic cleft and interact with the receptors of astrocytes, as well as the transporters. As a result of the interaction, astrocytes may show calcium responses, which then trigger the production and release of neuroactive compounds, or morphology alteration. Since there are reports describing the blockade of the influences of the responding astrocytes to neurons by the antagonists for glutamate or ATP (Newman, 2003; Pasti et al., 1997), calcium increases in astrocytes could lead to the release of these neurotransmitters. Further investigation is necessary to establish the general implication of such an astrocyte-neuron interaction, which could vary in regions of brain. Nevertheless, it is a fascinating idea that astrocytes are involved in the information processing in the CNS, via calcium responses, since it seems to be the key issue, regulation of neuronal activity in a medium temporal range, which has gone unanswered for a long time in neuroscience. The temporal range of the astrocytic calcium responses is from several tens of seconds to several minutes, which is quite slower than that of the responses of neurons, including synaptic transmission and action potential (several
milliseconds). Therefore, astrocytes could regulate neuronal activity in the medium temporal range, which is not well controlled by the neuron itself.

**Problems in previous calcium imaging studies using cultured astrocytes**

Although evidence of astrocytic calcium responses concerning neuronal activities is accumulating, studies on the topic do not always employ the same specimens or methods for measurement. As such, researchers’ results are often contradictory in terms of pharmacological properties and patterns (transient or oscillatory) (Cai et al., 2000; McCarthy and Salm, 1991; Muller et al., 1997). To reveal the general implication of astrocytes in neuronal activities, it is necessary to readjust the problems caused by the experimental conditions as differences in methodology could have led to differences in the environments for astrocytes. Astrocytes are known to detect the factors reflecting the brain environments, including growth factors and cytokines, and alter their proliferation, morphology and gene expression (Bezzi et al., 2001; Krushel et al., 1998; Rostworowski et al., 1997; Stachowiak et al., 1997; Verkhratsky et al., 1998; Xian and Zhou, 1999). In terms of development, memory formation, pathological processes and aging of the CNS, the production of these environmental factors is known to change (Gomez-Pinilla et al., 1998; Iseki et al., 2002; Rostworowski et al., 1997; Stachowiak et al., 1997; Xian and Zhou, 1999), and the factors will affect brain functions. Although environmental factors are known to affect neuronal activities, including synaptic transmission (Ishiyama et al., 1991; Murray
and Lynch, 1998), the regulating mechanisms have not been fully elucidated. If the 
environmental factors affecting the astrocytic calcium responses were understood, as well as 
proliferation, morphology and gene expression, the novel mechanisms underlying the 
regulation of neuronal activity according to the brain environment, would be provided. In 
order to address these issues, it will be valuable to systematically examine the experimental 
conditions for the study of astrocytic calcium responses, and to determine the effects of the 
environmental factors.

**Purpose of the present study**

The purpose of the present study is to reveal the regulation of astrocytic calcium 
responses to neurotransmitters via environmental factors, and then the implications of 
astrocytes in brain functions. A novel method for the statistical analysis of the calcium 
response patterns in cultured astrocytes has been established (CHAPTER 1), and applied to 
the examination of the effect of the environmental factors, including growth factors and 
pro-inflammatory cytokines for the calcium response of the cultured astrocytes (CHAPTER 
2). In addition, by comparing the calcium response patterns of the astrocytes in culture and 
the neurons in tissue culture preparation, the feasibility of the cultured astrocytes for *in vivo* 
studies was evaluated. Furthermore, by analyzing the effects of the environmental factors to 
the signal transduction pathways of astrocytes, the regulatory mechanisms of the calcium 
response patterns were investigated (CHAPTER 3). The present series of analysis will
provide basic information about the interaction between neurons and astrocytes via astrocytic calcium response, and the mechanisms by which the environmental factors affect neuronal activities.
Figure

**Fig. 0-1**
**Fig. 0-1.** Excitatory post-synaptic currents (EPSC) at parallel fiber synapses of Purkinje cells in slices prepared from normal (+/+) and GLAST knock-out (-/-) mice. (A) Responses to single parallel fiber stimulations. (B-E) Responses to 10 stimuli at 200 Hz in the absence or presence of GLT-1 blocker (dihydrokinate; DHK, 200 µM) or  

$\alpha$-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker 

(6-Nitro-7-sulphamoylbenzo[f]quinoxaline-2,3-dione; NBQX, 25 µM). Stimulus intensities were adjusted to produce an initial EPSC amplitude of $\sim$160 pA (low stim) or $\sim$1100 pA (high stim). (Marcaggi et al., 2003)
MATERIALS

Cultured astrocytes

Astrocytes were isolated from the cerebral cortex of postnatal day 1 Wistar rats, using a modification of the method described by Levison et al. (Levison and McCarthy, 1991). Briefly, brain cells were prepared from the cortices of 10 - 15 brains by trypsinization, trituration, and filtration, and seeded at 1.3 x 10^4 cells / cm² in 75 cm² plastic flasks (Sumitomo Bakelite, Tokyo, Japan). After 12 days’ culture at 37°C in 5% CO₂ humidified air in basal Eagle’s medium containing 10% fetal calf serum (FCS; Equitech-Bio, Ingram, TX) with a medium change every three days, the resulting mixed glia culture was shaken at 260 rpm for 18 h at 37 °C, then rinsed with medium to remove non-astrocytic cells. The adherent cells were subcultured by trypsinization and seeded at a density of 3 x 10^4 cells / cm² in Dulbecco’s Modified Eagle’s medium (DMEM; Asahi Technoglass, Funabashi, Japan) containing 25 mM HEPES, pH 7.4, and 10% FCS on either poly-D-lysine-coated coverslips (for calcium imaging or immunocytochemistry) or on plastic plates (Asahi Technoglass) (for Western blotting or reporter gene assays). After 48 h, the medium was changed to that described in each experiment and culture continued for a further 48 – 96 h before assay. The astrocyte defined medium (ADM), a modified version of that reported by Miller et al
(Miller et al., 1995), consisted of DMEM containing 25 mM HEPES, pH 7.4, 1 mM pyruvate, 2 mM glutamine, 50 mg/ml of human apotransferrin (Gibco-BRL, Gaithersburg, MD), 10 ng/ml of D-biotin, 5.2 ng/ml of sodium selenite, 1.5 mg/ml of bovine fibronectin (Gibco-BRL), 0.5 mg/ml of heparan sulfate, 5 mg/ml of insulin (Gibco-BRL), 10 ng/ml of epidermal growth factor (EGF, Gibco-BRL), and 5 ng/ml of basic fibroblast growth factor (bFGF, Gibco-BRL); growth factor-free ADM (GF-free ADM) had the same composition, but lacked the EGF and bFGF. Rat interleukin-1β (IL-1β) and rat tumor necrosis factor-α (TNFα) were obtained from Peprotech (London, UK).

**Organotypic culture of hippocampus**

Organotypic slice cultures of rat hippocampus were prepared as described by Sakaguchi et al. (Sakaguchi et al., 1994). The hippocampi were removed from postnatal day 5 – 7 Wistar rats and 300 µm slices prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering Co.). Each slice was placed on a filter (Millipore, Millicell-CM, 30 mm), which was then placed in a 6-well microplate (Iwaki Glass; code 3810-006) containing 1.0 ml of culture medium (Eagle's minimal essential medium, Gibco) supplemented with 25% heat-inactivated horse serum (Gibco). Three slices were placed in each well and cultured for 1 week before being used for the following experiments.

**PC12h cells**

PC12h cells (Hatanaka, 1981) were seeded on 12 mm diameter coverslips coated
with polyethyleneimine (1 mg/ml) in DMEM-high glucose (Asahi Technoglass, Funabashi, Japan) containing 5% horse serum (HS; Gibco) and 5% semi-fetal calf serum (Mitsubishu Kagaku, Tokyo, Japan).

**Antibodies**

Primary antibodies were obtained as follows; a rabbit polyclonal anti-GFAP antibody (DAKO, Hamburg, Germany), rabbit polyclonal anti-mGluR5 antibody (Upstate, Lake Placid, NY), mouse monoclonal anti-actin antibody (Chemicon, Temecula, CA), mouse monoclonal antibody against phosphorylated ERK (Sigma, St. Louis, MO) and rabbit polyclonal anti-ERK antibody (Sigma). For immunocytochemistry, rhodamine-conjugated goat second antibody (Chemicon) was used as secondary antibody. For Western blotting analysis, horseradish peroxidase-conjugated secondary antibody from Amersham (Buckinghamshire, UK) was used to detect all bound primary antibodies.

**Recombinant DNA**

As a reporter gene vector, the promoter region of the rat early growth response gene-1 (egr-1) gene (-525 to +117) (Changelian et al., 1989) was obtained by PCR and subcloned into pGL3-Basic (Promega, Madison, WI). The expression vector for the fusion protein of green fluorescence protein (GFP) and pleckstrin homology domain from rat phospholipase C-δ1 (PLC-δ1), which is designated as GFP-PHD, was constructed as reported by Hirose et al. (Hirose et al., 1999). The DNA fragment encoding amino acids 11-140 of rat
PLC-δ1 was amplified from rat brain cDNA by PCR and inserted to the C-terminus of GFP obtained from Clontech, Inc. (Palo Alto, CA). The DNA fragment encoding the fusion protein was inserted into an expression vector containing the SRα promoter (Takebe et al., 1988). The expression vectors of cyan and yellow variant of GFP-PHD (CFP-PHD and YFP-PHD) were constructed similarly.

Chemicals

Carbachol was obtained from Wako Chemicals (Tokyo, Japan) and A23187 from Calbiochem (La Jolla, CA). All other chemicals were from Sigma (St. Louis, MO).
A novel method to quantify calcium response pattern and oscillation using Fura2 and acridine orange
1.1 Introduction

Calcium imaging, based on the use of calcium fluorescence indicators such as Fura2 (Gryniewicz et al., 1985), is a powerful means to analyze various cellular responses, including those of neurotransmitters and hormones. These indicators form acetoxymethyl esters, which stain cultured cells (Tsien et al., 1982), thus enabling the identification of various patterns of calcium response, including transient, sustained and oscillatory responses (Berridge et al., 2003; Carafoli, 2002). This response pattern has been found to be dependent on cell type, culture conditions and stimulus paradigms (Berridge et al., 2003; Carafoli, 2002), and several molecular mechanisms have been proposed to explain the calcium dynamics (Luo et al., 2001; Miyakawa et al., 1999).

In studying calcium response patterns in various cultured cells, I have found that the response of a cell population cannot be easily characterized, inasmuch as this response is a combination of various responses and individual cells are indistinguishable in imaging data. When a population contains cells that show calcium oscillation at various peak amplitudes and frequencies, quantification and statistical analysis of each cell’s response is necessary to characterize the response of the population. It is not always possible, however, to identify individual cells in images of cultured cells filled with calcium indicator. When cells are flat and in contact with each other or seeded at high cell density, the images may be smeary or
uneven, and the borders of cells may be indistinguishable. If individual cells cannot be identified, the response of each cell and the number of unresponsive cells cannot be accurately determined, and statistical characterization of the calcium response in the population is impossible.

To address this issue, a novel method to visualize the nuclei of cells filled with a fluorescent calcium indicator, Fura2 has been developed. By visualizing nuclei of cells subjected to calcium imaging, areas inside cell bodies could be determined, thus enabling us to comprehensively characterize the calcium response pattern in cell populations to a degree accurate enough for statistical analysis. I also applied this nuclear visualization method to the analysis of concentration dependent alteration of calcium oscillation of rat neonatal cerebral astrocytes in response to glutamate.
1.2 Experimental Procedures

1.21 Calcium imaging

Astrocytes were prepared and cultured as described in MATERIALS. For calcium imaging, the cells were washed three times, then loaded for 45 min at 37 °C with 7.5 µM Fura2AM (Dojin, Kumamoto, Japan) in basal salt saline (BSS) consisting of (in mM): NaCl 129, KCl 4, MgCl₂ 1, CaCl₂ 2, glucose 4.2, and HEPES 10, the pH being adjusted to 7.4 with NaOH. In all steps after loading, 100 µM sulfinpyrazone was added to the BSS. After washing and incubation at room temperature for 20 min, the coverslips were mounted in a chamber (RC-26; Warner Instruments, Hamden, CT) and perfused at a rate of 1.5 ml/min. Calcium imaging was performed using an IX70 inverted microscope, a UApo/340 40x/1.15w objective, and an OSP-EXA filter exchanger (all from Olympus, Tokyo, Japan) equipped with a C6790 CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Fluorescence images were acquired and ratio images (R340/380) calculated using AQUACOSMOS software (Hamamatsu Photonics). For quantitative analysis of calcium response patterns, nuclei were stained by perfusion of BSS containing 1 µM acridine orange (Molecular Probe, Eugene, OR) for five min and illuminated for three min with light emitted by a xenon lamp and reflected by a dichroic mirror (DM500), after calcium imaging. In each imaging experiment, 20 - 40 cells were identified; each histogram shows the results from six imaging experiments.
from two series of cultures prepared from two different preparations of cells.
1.3 Results

1.3.1 Assignment of cell bodies in calcium imaging data using nuclear identification by acridine orange staining and light illumination

In calcium imaging studies of cultured cells, it is not always easy to distinguish each cell and describe its response, especially when cells are in contact with each other. To determine areas corresponding to cell bodies in calcium imaging data, I have developed a method for visualizing nuclei of cells using the fluorescence indicator acridine orange. Loading of neonatal rat cerebral astrocytes with Fura-2 produced a fluorescent image in which individual cells were hardly distinguished (Fig 1A). When these same cells were stained with acridine orange (1 µM, 5 min) and illuminated for 3 min with light emitted from a xenon lamp and reflected by a dichroic mirror (DM500 nm), the fluorescence in the cytosol was reduced, making the fluorescence in the nucleus more prominent (Fig. 1C), such that the number of cells could be counted and the areas inside cell bodies could be determined. In the absence of xenon lamp illumination, the fluorescence image of acridine orange stained astrocytes was as smeary as that of the Fura-2 filled cells (Fig. 1B).

I proceeded to use this nuclear visualization method in the analysis of calcium imaging data. After the calcium imaging to determine the responses to 30 µM glutamate, Fura2-loaded astrocytes were stained with acridine orange, and individual cells were
identified using the nuclear visualization method. The calcium response of each cell was calculated by averaging the ratio (340/380) of the change in area inside the cell body. A fluorescence image of acridine orange positive nuclei and ratio images of Fura2-based calcium imaging before and during glutamate stimulation were taken (Fig. 2A), and time dependent changes in intracellular calcium in five areas determined as being inside cell bodies were plotted (Fig. 2B). These results indicated that the areas inside the cell body corresponded well to the areas responsive to glutamate.

1.3.2 Application of the nuclear visualization method to analyzing calcium oscillation of astrocytes in response to glutamate

The feasibility of the nuclear visualization method for characterizing the calcium response of a cell population was assessed by analyzing calcium oscillation of astrocytes in response to various concentrations of glutamate. Astrocytes were cultured in serum-free defined medium, which promotes calcium oscillation in response to various reagents, including glutamate, as described in CHAPTER2. When cells were treated with glutamate, at concentrations ranging from 10 µM to 1000 µM, we found that calcium oscillation was most frequent and continuous at 30 µM glutamate (Fig. 3). The calcium response was minimal at 10 µM glutamate, whereas, at concentrations higher than 30 µM, glutamate induced a large calcium response initially, which quickly faded during the stimulation. The maximum amplitude of calcium response was dependent on glutamate concentration, but it was almost
saturated at 100 μM. These concentration-dependent alterations in calcium oscillation were further analyzed quantitatively using the nuclear visualization method (Fig 4). Using this method, we observed similar results in that the frequency of calcium oscillation peaked at 30 μM glutamate and the maximum calcium response was concentration dependent and almost saturated at 100 μM glutamate.

The nuclear visualization method also enabled us to quantify the contribution of cells without calcium response. At 10 μM glutamate, 59.9% of cells were unresponsive, but this number decreased at concentrations higher than 30 μM (Fig. 4).
1.4 Discussion

In order to comprehensively characterize the calcium response pattern in a population of cultured cells, I developed a novel nuclear visualization method that can identify individual cells in calcium imaging data. Staining of Fura2-loaded cells with acridine orange, followed by light illumination, was successful in distinguishing individual nuclei. I evaluated the applicability of this nuclear visualization method by analyzing calcium imaging data and calcium oscillation of glutamate-treated astrocytes. I found that the areas visualized as nuclei were identical to the inside cell bodies responding to glutamate. Moreover, statistical analysis of the response patterns, which were calculated using the fluorescence ratio values in these areas, enabled me to quantify these patterns, which correlated well with the representative ones. Taken together, these findings indicate that this nuclear visualization method can be utilized for comprehensive quantitative characterization of response patterns in calcium imaging data obtained from cell populations.

In screening fluorescent dyes that bind nucleic acid, I found acridine orange the most appropriate for the visualization of nuclei of cells filled with the calcium indicator Fura2. This latter reagent has advantages over other calcium indicators for the accurate measurement of cellular calcium, including high quantum efficiency, availability of ratio imaging and abundant accumulation of references (Hayashi and Miyata, 1994; June and
Rabinovitch, 1994; Roe et al., 1990). There are, however, other cell permeable fluorescent dyes for nucleic acid, which can be excited with ultraviolet (UV) or visible light. Hoechst 33258 and DAPI, which belong to the group of dyes excited with UV light, are generally used for visualization of nuclei (Haugland, 2002). We found these dyes inappropriate in this study, however, since their fluorescence spectra are similar to that of Fura2. A series of cell-permeable dyes for nucleic acid excited with visible light was recently developed by Molecular Probe Inc. (Haugland, 2002), but I found that these dyes bind to both nuclear DNA and cytosolic RNA and are therefore inappropriate for nuclear visualization. In contrast, acridine orange, which belongs to the group of dyes excited with visible light, has a unique property as a nucleic acid binding dye in that it has one fluorescence wavelength for DNA binding (520 nm) and another for RNA binding (650 nm) (Darzynkiewicz, 1994; Haugland, 2002), a property that enables optical discrimination of nuclear fluorescence.

Nuclear visualization with acridine orange required prolonged illumination with light emitted from a xenon lamp and reflected by a dichroic mirror (DM500 nm), which probably causes cell damage. Acridine orange is known to visualize acidic organelles, such as lysosomes (Palmgren, 1991), which I found to be prominently labeled in the cells before light illumination. In addition, acridine orange is known not to intercalate into nuclear DNA that is in the form of condensed chromatin (Delic et al., 1991). Thus light damage to lysosomes would result in their loss of proton gradients, reducing the fluorescence from
these organelles; whereas, light damage of nuclear DNA would decrease the amount of condensed chromatin, increasing the accessibility of acridine orange to nuclear DNA. My finding, that acridine orange staining in combination with light illumination resulted in the visualization of nuclei, suggests the occurrence of this or a similar mechanism. Since the illumination with the visible light, that was in the range of the absorption spectrum of acridine orange, was ineffective for the visualization (data not shown), the light damage described above is assumed to have been caused by the UV light, but not by the photodynamic effects of the excited acridine orange.

Calcium oscillation of astrocytes was used to evaluate the nuclear visualization. Cultured astrocytes have a flat morphology, and individual cells are indistinguishable when seeded at a high density. Although the calcium response patterns are various in cultured astrocytes (Yoshida et al., 2003), it has been shown that they are to be controlled using defined medium (see CHAPTER2). In the present study, when cultured in defined medium lacking growth factors at high cell density ($9 \times 10^4$ cells/cm$^2$), the cells were a mixture of transiently and oscillatory responding cells, but when cultured in the presence of growth factors at moderate cell density ($3 \times 10^4$ cells/cm$^2$), most of the response was converted to oscillation. At low concentrations of glutamate, calcium oscillation was continuous, but as the concentration increased, the initial response became larger and the following peaks faded quickly. These results suggest that massive receptor activation depletes cellular stores of
calcium. Similar concentration dependent alterations in the frequency of muscarinic acetylcholine receptor-induced calcium oscillation of pancreatic acinar cells have also been reported (Petersen et al., 1991).

Recently, glutamate induced calcium oscillation in CHO cells, which is inhibited by protein kinase C (PKC) inhibitor, was shown to be dependent on the level of expression of metabotropic glutamate receptor (mGluR5). In addition, a second type of calcium oscillation in these cells was found to be dependent on the glutamate concentration that remains after the downregulation of PKC (Nash et al., 2002). I have found that astrocytes express mGluR5 and that the level of expression is not affected by culture conditions (see CHAPTER 2). The present finding, that the level of calcium oscillation is dependent on glutamate concentration, is probably analogous to the PKC independent component found in CHO cells.

The nuclear visualization method described in this chapter would be useful for determining factors affecting the calcium response pattern of cells, which has been difficult to study quantitatively. This method would also be useful for evaluating reagents that affect calcium response patterns, and it may contribute to the development of drugs affecting muscle contraction, secretion and gene regulation.
1.5 Figures

Fig. 1-1
Fig. 1-1. Visualization of nuclei of Fura2-loaded astrocytes, using acridine orange staining in combination with light illumination. Astrocytes were seeded at a density of $9 \times 10^4$ cells/cm$^2$ and cultured in growth factor free ADM. After calcium imaging using Fura2, astrocytes were stained with acridine orange (1 µM, 5 min) in perfusion chambers and illuminated for 3 min with light of wavelength shorter than 500 nm. (A) Fura2 fluorescence (Ex/Em = 380 nm/520 nm) after calcium imaging. (B) Acridine orange fluorescence (Ex/Em = 480 nm/520 nm) before light illumination. (C) Acridine orange fluorescence after light illumination. Scale bar, 50 µm
Fig. 1-2
Fig. 1-2. Analysis of calcium imaging data using the image of nuclei. The calcium imaging data and the image of nuclei obtained as described in the legend to Fig. 1-1 were analyzed for time-dependent changes in cellular calcium. Each area corresponding to a nucleus was analyzed as an area inside cell body in calcium imaging data. (A) Representative imaging data of nuclear identification and calcium imaging. Five nuclei were identified in an acridine orange image (left). Calcium imaging data were analyzed using the five identified cell bodies, with the ratio image (340/380) before (middle) and during (right) stimulation with 30 µM glutamate. Scale bar, 50 µm. (B) Calcium responses of the five identified cell bodies. Glutamate was applied as shown by an under bar. Vertical scale bar, Ratio (340/380) = 0.1; horizontal scale bar, 30 sec.
Fig. 1-3
Fig. 1-3. Concentration-dependent alteration of calcium oscillation of cultured astrocytes in response to glutamate. Astrocytes were seeded at a density of $3 \times 10^4$ cells/cm$^2$ and cultured in ADM. For calcium imaging, cells were subjected to five min stimulation (under bar) with (A) 10 µM, (B) 30 µM, (C) 100 µM, (D) 300 µM and (E) 1000 µM glutamate. Each set of traces represents the calcium responses of two representative cell bodies of astrocytes assigned using the nuclear identification method. Vertical scale bar, ratio (340/380) = 0.1; horizontal scale bar, 30 sec.
Fig. 1-4
**Fig. 1-4.** Frequency and maximum amplitude of calcium oscillation of astrocytes in response to glutamate. Astrocytes were treated as described in the legend to Fig. 1-3, stimulated for five min with (A) 10 µM, (B) 30 µM, (C) 100 µM, (D) 300 µM and (E) 1000 µM glutamate, and the images were analyzed as described in the legend to Fig. 1-2. Each area was quantified, and histograms for frequency (number of peaks/five min, left) and maximum amplitude (ΔRatio (340/380), right) of calcium oscillation was plotted. The white columns indicate cells showing no calcium response. The upper numerical values in each panel are the mean ± S.E.M. and the total number of cells (n) examined (responders plus non-responders), while the lower values (shown in parenthesis) are those for responding cells.
CHAPTER 2

Dual regulation of calcium oscillation in astrocytes by growth factors and pro-inflammatory cytokines
2.1 Introduction

Calcium imaging and the identification of receptor genes have provided useful information about the astrocytic calcium response to neurotransmitters (Verkhratsky et al., 1998) and have suggested a dynamic role for astrocytes in brain function (Haydon, 2001). Astrocytes, which form the largest population of non-excitable cells in the mammalian central nervous system (CNS), adhere closely to neurons and blood vessels and fill the space between them (Zonta et al., 2003). This anatomical property is advantageous not only in the regulation of CNS metabolism, but also for the interaction of astrocytes with neurotransmitters released from nerve terminals. During neuronal activity, astrocytes respond by an increase in intracellular calcium levels (Pasti et al., 1997), but many aspects of this response have not been well studied, for instance, the degree of neuronal activity required and the consequences for brain function.

In this Chapter, I cultured astrocytes in serum-free defined medium and examined the effects of growth factors and cytokines on calcium dynamics. Although astrocytes have been studied by calcium imaging for more than ten years, their reported pharmacological properties and response pattern vary (transient or oscillatory), depending on the species, brain region, and age of the animal (Cai et al., 2000; McCarthy and Salm, 1991; Muller et al., 1997), and this complexity makes it difficult to determine the physiological role of these
phenomena. I propose that this variability could reflect an important function of astrocytes, namely that they can respond to factors, such as ions, neurotransmitters, bioactive lipid metabolites, growth factors, cytokines, and adhesion molecules, in the CNS environment, and can alter the environment by changing their morphology and metabolism, including the expression of enzymes and other factors (Bezzi et al., 2001; Krushel et al., 1998; Rostworowski et al., 1997; Stachowiak et al., 1997; Verkhratsky et al., 1998; Xian and Zhou, 1999). These responses are important in maintaining CNS homeostasis, but would cause significant variation in the results of studies on cultured astrocytes. Small differences in culture conditions, such as cell density, serum, and the presence of other cell populations, affect astrocyte metabolism by the presence of soluble factors or by cell adhesion, leading to further changes in the medium. Because of this synergistic effect, initial small differences can produce markedly different results. To avoid these issues and to examine the real properties of astrocytes, it is necessary to culture them in well defined conditions, for which serum-free defined medium is ideal. The use of defined medium allows the effect of the environment on the physiological properties of astrocytes to be systematically studied and should provide important new information on astrocyte function. Calcium oscillation is reported to generate repetitive glutamate release from astrocytes which then affects surrounding neurons (Pasti et al., 1997; Pasti et al., 2001), suggesting a feed-back mechanism from the astrocytes to the neuronal network. I therefore studied calcium
oscillation in astrocytes cultured in defined medium and compared these responses to those in tissue slice preparations to determine whether our culture system results could be applied to in vivo studies.
2.2 Experimental procedures

2.21 Calcium imaging of cultured astrocytes

Astrocytes were prepared as described in MATERIALS and cultured as indicated. Calcium imaging was performed as described in CHAPTER 1.

2.22 Immunological detection

Immunocytochemical staining for glial fibrillary acidic protein (GFAP) was performed using a rabbit polyclonal anti-GFAP antibody (5 µg/ml). For counter-staining of the nucleus, Hoechst 33258 (1 µM) was added to the second antibody solution. To assess mGluR5 expression, Western blotting was performed using rabbit polyclonal anti-mGluR5 antibody (2 µg/ml) or mouse monoclonal anti-actin antibody (1:1000 dilution).

2.23 Calcium imaging of slice cultures

Slice cultures were prepared, as described in MATERIALS, and cultured for 7 - 14 days before calcium imaging. BSS containing 0.1 mM ascorbic acid and 0.5 mM inositol was used throughout, and sulfinpyrazone was included as described for the cell culture experiments. The cells were incubated with 50 µM MK801 for 30 min before, and during, loading for 1 h at 37 °C with Fluo4AM (Molecular Probe, Eugene, OR) in BSS containing 0.005% Cremophore. After three washes, the slices were incubated for 30 min at room temperature in medium without MK801, then were transferred for 5 min to BSS containing
100 mM mannitol, which suppresses swelling during pharmacological stimulation. Calcium imaging was performed using an E600FN upright microscope and a Fluor 40x/0.8w objective (all from Nikon, Tokyo, Japan) equipped with a CSU-10 laser confocal scanning unit (Yokokawa, Tokyo, Japan), 532R-BS-A04 argon laser, (Melles Griot, Irvine, CA), and a C6790 CCD camera (Hamamatsu). Fluorescence images were acquired using AQUACOSMOS software (Hamamatsu) and the fluorescence ration (F/Fo) calculated from the average intensity of the indicated areas.
2.3 Results

2.31 Growth factor-induced calcium oscillation

As in a previous report (Jensen and Chiu, 1990), astrocytes cultured in medium containing 10% FCS, a commonly used additive, were found to consist of a mixture of two populations, the proportions of which varied between cultures. One of these showed a transient response, and the other an oscillatory response, to glutamate (Glu, 30 µM) or ATP (100 µM) (Fig. 2-1A, top panels); the percentage of responding cells showing oscillatory responses to glutamate or ATP, respectively, was 33.3% (n = 42) and 18.9% (n = 58). In contrast, after culture for 48–96 h in serum-free defined medium containing EGF and bFGF (ADM), almost all the responding cells showed calcium oscillation (center panels). Typical imaging data for the calcium oscillation in response to glutamate are shown in Movie 1.

Furthermore, these cells showed a similar oscillatory response to thimerosal (10 µM), which affects the redox state of the inositol-1,4,5 trisphosphate (IP_3) receptor and induces calcium release (Swann, 1991). In contrast, cells in growth factor-free ADM (GF-free ADM) gave a transient response to all three stimuli (bottom panels). The percentage of responding cells showing oscillatory responses to glutamate, ATP, or thimerosal, respectively, was 10.3% (n = 156), 8.3% (n = 60), and 3.6% (n = 56) in GF-free ADM and 75.0% (n = 212), 74% (n = 85), and 80.0% (n = 128) in ADM.
Although it has been previously reported that this same set of growth factors increases astrocytic mGluR5 expression, enhances phosphoinositide hydrolysis and the calcium response, and converts the calcium response to oscillatory (Miller et al., 1995; Nakahara et al., 1997), Western blotting showed no significant increase in mGluR5 protein levels in the presence of growth factors over the same time-course (Fig. 2-1B). These results show that the mixed calcium response seen in serum-containing medium could be converted to an entirely transient or entirely oscillatory response in serum-free medium depending on the absence or presence, respectively, of growth factors, and that this conversion was mediated by changes in some calcium controlling mechanism. Because the effects of defined medium required more than 48 h to become apparent (data not shown), I hypothesized that regulation of gene expression was involved, and that the candidate genes would be those coding for proteins regulating intracellular calcium dynamics, such as calcium channels, pumps, exchangers, and buffer proteins.

2.32 Inhibition by cytokines or a MEK inhibitor

Growth factor production in the CNS changes during development, under different pathological conditions, and during functions such as memory formation, and has been shown to affect astrocytic proliferation and their differentiation to reactive astrocytes (Stachowiak et al., 1997; Xian and Zhou, 1999). Production of pro-inflammatory cytokines, such as IL1β and TNFα, also changes with pathology and stress, and is known to affect
astrocytic proliferation, morphology, and metabolism (Herx and Yong, 2001; Murray and Lynch, 1998; Rostworowski et al., 1997). These two groups of factors (growth factors and pro-inflammatory cytokines) competitively regulate the production of S100β or growth inhibitory factor by astrocytes (Hinkle et al., 1998; Uchida, 1999), and are produced with different time-courses in brain injury, cytokine levels increasing within several hours after insult (Rostworowski et al., 1997), whereas growth factor levels reach a maximum only after a week, when the scar of astrocytes becomes mature (Iseki et al., 2002). On the basis of these results, I hypothesized that these soluble factors may have opposing effects, and examined the effect of cytokines on the growth factor-induced calcium oscillation in astrocytes. I also examined the effects of LPS, which is known to activate a similar cell signaling cascade to that activated by pro-inflammatory cytokines (Raetz and Whitfield, 2002), and those of a mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor, U0126, which attenuates the MAP kinase cascade, one of the main pathways activated by growth factors (Favata et al., 1998).

Fig. 2-2A shows calcium responses of 3 representative cells cultured in ADM containing a pro-inflammatory cytokine (IL1β or TNFα), LPS, or a MEK inhibitor, all of which suppressed the calcium oscillation induced by the growth factors. To analyze these results quantitatively, individual cells were identified by nuclear staining with acridine orange after calcium imaging with Fura2AM, and their calcium responses to 30 µM
glutamate measured and plotted as frequency and amplitude histograms (Figs. 2-2B, C). The number of peaks seen during 2 min stimulation with glutamate was 4.12 ± 0.20 (mean ± S.E.M., n = 228) and 1.04 ± 0.05 (n = 178) in ADM and GF-free ADM, respectively, indicating that the growth factors caused a marked change in the pattern. Although the various agents tested gave different effects, giving frequencies between 0.90 and 2.04 peaks/2 min, in all cases, fewer peaks were seen than in ADM alone. The amplitude of the calcium response was also suppressed in parallel with the calcium oscillation. Since the percentage of cells showing no response increased from 7.0% in ADM and 9.8% in GF-free ADM to 24.9% in ADM+LPS, 35.5% in ADM+IL1β, 56.7% in ADM+TNFα, and 47.4% in ADM+U0126, the calcium responses were recalculated using only the responding cells, with similar results (shown in parenthesis).

2.33 Morphology and calcium response

Under certain pathological conditions, astrocytes proliferate and become morphologically hypertrophic; this is known as differentiation into reactive astrocytes, a process in which growth factors and pro-inflammatory cytokines are thought to be involved (Iseki et al., 2002; Rostworowski et al., 1997). To investigate the relationship between differentiation and changes in the calcium response, I carried out an immunocytochemical study using anti-GFAP antibody and Hoechst nuclear staining and examined astrocytic morphology and proliferation under different culture conditions. As shown in Fig. 2-3A,
cells cultured in ADM bore more fibers staining strongly for GFAP, whereas those cultured in GF-free ADM were flat and showed mesh-like GFAP staining in the peri-nuclear region. IL1β or LPS partially suppressed the effect of growth factors, i.e., the fibrous morphology and mesh-like structure were intermediate between those in ADM and those in GF-free ADM. The effect of the MEK inhibitor was more marked, the cells being flat, as in GF-free ADM, with mesh-like GFAP fibers surrounded the nuclei. Proliferation was quantified by calculating the cell density (Fig. 2-3B). The growth factors promoted astrocyte proliferation, the density of cells cultured in GF-free ADM being only 65% of that of cells cultured in ADM. The densities of cells cultured in ADM containing ILβ, LPS, or the MEK inhibitor were 61%, 73%, or 73%, respectively, of that of cells grown in ADM, indicating suppression of the growth factor-induced proliferation by these compounds. These results show a correlation between proliferation and calcium oscillation of astrocytes. Expression of GFAP, which increases in the reactive astrocyte in situ (Brock and O'Callaghan, 1987), was measured under the different culture conditions using Western blotting, but no significant differences were detected (data not shown). These results show that the growth factors promoted proliferation and a hypertrophic morphology of astrocytes via the MAP kinase cascade, and that these effects were blocked by pro-inflammatory cytokines and LPS. These changes correlated well with calcium oscillation, suggesting that calcium oscillation is a characteristic of a differentiation state of the astrocyte, possibly a reactive astrocyte. In
addition, the results shown in Fig. 2-3B support the idea that astrocytes giving either
transient or oscillatory responses are both derived from a single cell population. Although it
is possible that the astrocytes with different responding patterns belong to different cell
populations and that the growth factors affect the proliferation of each population to a
different extent, our results suggest this is not the case. The cell density, which was \(3 \times 10^4\)
cells / cm\(^2\) at seeding, increased to \(4.8 \pm 0.2 \times 10^4\) cells / cm\(^2\) or \(7.4 \pm 0.3 \times 10^4\) cells / cm\(^2\), in
the absence or presence of growth factors, respectively. Since the percentage of cells
(responders and nonresponders) showing a non-oscillatory response decreased from about
90% to 30% in the presence of growth factors (Fig. 2-2), the growth factors caused a
decrease in the density of non-oscillatory cells from \(4.3 \times 10^4\) cells / cm\(^2\) to \(2.2 \times 10^4\) cells /
cm\(^2\). As growth factors caused no significant cell death, and growth factors would not be
expected to suppress the proliferation, this reduction indicates that the growth factors
converted cells showing a non-oscillatory response to cells showing an oscillatory response.

2.34 Calcium oscillation in slice preparations

To elucidate the role of the astrocytic calcium response in brain function, I
investigated changes in neuronal network activity following astrocytic stimulation. In
hippocampal acute slice preparations, an mGluR agonist,

\((1S,3R)-1\)-aminocyclopentane-1,3-dicarboxylic acid (tACPD), induces a calcium response in
astrocytes, then in neighboring neurons, and, since ionotropic glutamate receptor (iGluR)
agonists only inhibit the neuronal calcium increase, it was suggested that tACPD induces both a calcium increase in, and glutamate release by, astrocytes, and that these astrocytic responses affect the surrounding neurons via neuronal iGluRs (Haydon, 2001; Pasti et al., 1997). In the present study, the NMDA receptor antagonist, MK801, was used to reduce the excitotoxicity of calcium indicator loading of slice preparations, in which the detergent, cremophore, was used. However, I also noted that the washing out of MK801 after loading induced spontaneous, fast, synchronous calcium increases in neurons, in accordance with a previous report that NMDA receptor blockade increases NMDA receptor expression and that the removal of the blocker induces epileptic neuronal activity (Goldin et al., 2001), and was therefore able to use these neuronal calcium increases to investigate the effect of mGluR activation. I therefore used calcium imaging of individual pyramidal neurons in slice culture preparations of the neonatal rat hippocampal CA1 region to examine the effect of agents which induce an astrocyte calcium response. Fig. 2-4 shows representative fluorescence images (Fig. 2-4A) and time-dependent fluorescence changes in the eight pyramidal neurons marked 1-8 in Fig. 2-4Ae (Fig. 2-4B-D). Fluorescence images before, and at the peak of, the spontaneous, fast, synchronous calcium increase seen after MK801 washout, derived from imaging data in Movie 2, are shown in Fig. 2-4A-a and b, respectively. The time-dependent changes in the spontaneous responses before mGluR activation (Fig. 2-4B; shown on an expanded scale in Fig. 2-4C) also show the synchronous spike-like properties of these
calcium increases. Because the spontaneous, fast calcium increase was blocked either by a combination of two iGluR antagonists, 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX, 50 µM) and D-(-)-2-amino-5-phosphonopentanoic acid (APV, 50 µM), or by the sodium channel blocker, tetrodotoxin (1 µM), (data not shown), it was probably attributable to the synchronous activity of neurons as a result of synaptic transmission. Activation of mGluRs by tACPD significantly affected the calcium response, i.e. the synchronized fast calcium increase was converted to an asynchronous slow calcium oscillation (Fig. 2-4B). Two representative fluorescence images of the calcium oscillation derived from Mov. 3 are shown in Fig. 2-4Ac and 2-4Ad. These results show that tACPD treatment reduced the frequency of the calcium response and made each response independent. Subsequent application of a high concentration of glutamate (1 mM) induced a global calcium increase (Fig. 2-4A-e, D), and most of the neurons could be identified. The frequency of the slow calcium increase in the neurons in the slice preparation after tACPD application (1.71 ± 0.11 peaks / min, n=8 cells) was similar to that seen in astrocytes cultured in ADM, as described above (2.05 ± 0.14 peaks / min, n=228 cells). These results suggest that the intrinsic calcium oscillation in astrocytes influences neuronal activity, and supports the idea that the astrocytic calcium response to tACPD results in glutamate release, which induces a calcium response in neighboring neurons via iGluRs, as proposed on the basis of similar experiments using acute slice preparations (Pasti et al., 1997). Furthermore, the present results show that mGluR
activation converts the pattern of neuronal activity and suggest that astrocytes play a dominant role in the neuronal calcium increase after mGluR activation.
2.4 Discussion

The above-described regulation of calcium oscillation in the astrocyte by growth factors and pro-inflammatory cytokines is the first evidence for the dual regulation of calcium dynamics by soluble factors and could be the mechanism by which the astrocyte detects changes in the CNS environment and regulates brain activities, such as the processes of inflammation, regeneration, and memory formation, under various physiological conditions. Since the growth factors promoted proliferation and a hypertrophic morphology, in addition to calcium oscillation, an oscillatory calcium response to neurotransmitters could be a property of reactive astrocytes. If this is the case, neurodegeneration during gliosis could be attributed to this calcium oscillation of the astrocyte, which would result in increased glutamate release and cause excitotoxicity. However, I cannot definitely conclude that the properties of astrocytes cultured in ADM reflect those of reactive astrocytes, because the growth factors did not cause increased expression of GFAP, which is reported to be increased in reactive astrocytes (Brock and O'Callaghan, 1987), and it is known that both growth factors and pro-inflammatory cytokines are involved in the differentiation of reactive astrocytes (Iseki et al., 2002; Rostworowski et al., 1997). Growth factors are produced to some extent in the CNS under normal physiological conditions and act as tropic factors, and their concentrations are altered in response to physical and psychological conditions.
(Gomez-Pinilla et al., 1998; Stachowiak et al., 1997; Xian and Zhou, 1999). In contrast, pro-inflammatory cytokine production is suppressed until triggered by events such as brain damage, psychological stress, or aging (Murray and Lynch, 1998; Rostworowski et al., 1997). Based on these two lines of evidence, the percentage of astrocytes showing an oscillatory calcium response is assumed to vary in the normal CNS, mostly depending on the production of growth factors, as seen in cells cultured in the presence of 10% FCS. This flexibility in the calcium response could be part of the regulatory mechanism of memory formation, as the astrocytic calcium response to neuronal activity, especially tetanic stimulation, is reported to affect synaptic plasticity (Kang et al., 1998). This notion is in good agreement with the evidence that synaptic transmission is promoted by growth factors (Ishiyama et al., 1991), but reduced by pro-inflammatory cytokines (Murray and Lynch, 1998). For both sets of factors, the astrocyte would be the main target for regulation of higher brain function.

Most mGluRs have a 10-fold higher affinity than iGluRs for glutamate (Conn and Pin, 1997), thus allowing a long lasting calcium response (including calcium oscillation) by the astrocyte to glutamate leaked from the synaptic cleft following massive neuronal activation, e.g. by tetanic stimulation (Porter and McCarthy, 1996). Furthermore, since neuronal mGluRs are localized in the peri-synaptic region (Baude et al., 1993; Lujan et al., 1997), they are probably activated by the leaked glutamate (Lujan et al., 1997; Mitchell and Silver, 2000). In the present study on slice cultures, the mGluR agonist, tACPD, converted
the spontaneous, fast, synchronous calcium increase of pyramidal neurons to slow, asynchronous calcium oscillation. Furthermore, the frequency of the calcium oscillation in the neurons in the slice cultures was similar to that of cultured astrocytes, suggesting that the intrinsic calcium oscillation in astrocytes influences neighboring neurons via glutamate release. These results imply that the astrocyte plays a dominant role in neuronal activity following mGluR activation. Further examination of the effects of soluble factors on calcium responses in slice cultures using serum-free medium should reveal how they affect neuronal activity via changes in the astrocyte. Although calcium-dependent glutamate release from astrocytes has been shown both in culture and slice preparation (Bezzi et al., 2001), it is reported to have both excitatory and inhibitory effects on neuronal activity (Araque et al., 1998; Porter and McCarthy, 1996), and its real consequence has still to be determined. It is reasonable to assume that the response of neurons to glutamate released by astrocytes is dependent on the subtype of glutamate receptor, which can vary outside synapses, and that the inhibitory effects are due to inhibitory mGluRs (groups II and III). If this were the case, the physiological role of the calcium response and glutamate release by the astrocyte would vary, depending on the structure and topology of the glutamate release site on the astrocyte and the glutamate-receptive site on the neuron. In conclusion, I propose that the soluble factor-mediated regulation of astrocyte calcium dynamics is a novel mechanism for sensing the state of the CNS environment and responding to it by altering the physiology and
pathology of the CNS. Further studies on this regulatory mechanism should provide

significant information on how neuron-glial interaction regulates brain functions.
2.5 Figures

Fig. 2-1
**Fig. 2-1.** Calcium responses of astrocytes under various culture conditions. (A)

Representative calcium responses from three cells under each of the experimental conditions.

Neonatal rat cerebral cortex astrocytes were cultured for 48 h in 10% FCS, ADM, or growth factor free ADM (GF-free ADM), then stimulated with Glu (30 µM), ATP (100 µM), or thimerosal (10 µM) and their calcium responses measured. (B) mGluR5 expression in astrocytes in serum-free defined medium. Western blots of extracts from astrocytes cultured for 48 h in ADM or GF-free ADM using anti-mGluR5 antibody or anti-actin antibody.
Fig. 2-2
Fig. 2-2. Factors affecting the astrocytic calcium response. Calcium responses of astrocytes to 30 µM glutamate after 48 - 72 h culture in various media. The effects of two pro-inflammatory cytokines, IL-1β (IL1, 5 ng / ml) and TNFα (TNF, 20 ng / ml), LPS (0.01 µg / ml), or the MEK inhibitor, U0126 (U, 20 µM) were examined in cells cultured in ADM. (A) Representative calcium responses from three cells under the different experimental conditions. (B) Histograms showing the frequency of calcium oscillation. (C) Histograms showing the maximum amplitude of the calcium response. The white columns indicate cells showing no calcium response. The upper numerical values in each panel are the mean ± S.E.M. and number of cells (n) for all cells (responders plus non-responders), while the lower values (shown in parenthesis) are those for responding cells.
Fig. 2-3
Fig. 2-3. Morphology and proliferation of astrocytes cultured in various media. (A)

Fluorescent labeling of GFAP and nuclei, using anti-GFAP antibody and Hoechst stain. Scale bars, 20 µm. (B) Density of astrocytes grown in different media. The values were calculated from the number of nuclei in an area of 278 µm² obtained from three Hoechst-stained images and are expressed as the mean ± S.E.M. The results are representative of those from three independent experiments using different series of cultures.
Fig. 2-4
Fig. 2-4. Generation of an oscillatory calcium response in neurons in the CA1 region of rat hippocampal slice preparations, similar to that seen in cultured astrocytes. (A) Calcium increase in neurons monitored by confocal microscopy; (a, b) Spontaneous, fast, synchronous calcium increase seen on MK801 washout; (c, d) calcium oscillation following application of 30 µM tACPD; (e), transient and plateau calcium response to 1 mM glutamate. Scale bar, 50 µm. (B-D) Spontaneous and tACPD-induced calcium responses of the cells marked 1-8 in (e). (B) Conversion of the calcium response following tACPD application, indicated by the line below the traces. (C) The spontaneous calcium increase before tACPD application on an expanded time-scale. (D) The calcium increase in response to 1 mM glutamate. Scale bars: horizontal, 10 sec; vertical (F/Fo) 0.1 in B and C and 0.2 in D.
2.6 Legends for movies in CD

**Movie 1.** Calcium oscillation in cultured astrocytes. The movie shows the imaging ratio (340/380) for astrocytes in ADM stimulated with 30 µM glutamate for 2 min. 160 sec of imaging data is played in 16 sec.

**Movie 2.** Fast, synchronous calcium increase in a slice preparation before tACPD stimulation. 100 sec of fluorescent imaging data is played in 10 sec.

**Movie 3.** Calcium oscillation in a slice preparation in response to 30 µM tACPD. 100 sec of fluorescent imaging data is played in 10 sec.
CHAPTER 3

Cellular mechanisms regulating the calcium oscillation in astrocytes
3.1 Introduction

The dual regulation of the astrocytic calcium response patterns to neurotransmitters by growth factors and pro-inflammatory cytokines is shown in CHAPTER 2. The oscillatory calcium response was promoted by growth factor treatment for more than 48 h, and the effect of the growth factors was suppressed by pro-inflammatory cytokines and an inhibitor for MAP kinase cascade. Therefore it is assumed that MAP kinase cascade regulates some gene expressions, and converts the calcium response patterns. In order to reveal the cellular mechanisms underlying this conversion, the effects of the soluble factors to the signal transduction pathways in astrocytes, especially the MAP kinase cascade and the calcium mobilization via phosphatidyl inositol metabolism were investigated in the present chapter.

It is well established that growth factors promote proliferation and gene expression via p42/p44 extracellular signal-regulated kinases (ERKs), which belong to the MAP kinase super family (Johnson and Lapadat, 2002). In contrast, the effect of pro-inflammatory cytokines to this cascade have been found to vary depending on cell type (Kyriakis, 1999). Although it has been reported that the activation of ERKs is required for the pro-inflammatory cytokine-induced expression of inducible nitric oxide synthase in astrocytes (Marcus et al., 2003), it has also been reported that pro-inflammatory cytokines affect astrocytes via signal transduction pathways besides ERKs (Saklatvala et al., 1996). In
the present study, the activation of the MAP kinase cascade was examined at two different levels, the ERK phosphorylation and the activation of the immediate early gene (IEG). ERKs are phosphorylated and become active forms, which induce gene expressions via serum response element (SRE) (Shaw and Saxton, 2003; Yordy and Muise-Helmericks, 2000). The gene activation was monitored by a reporter gene assay using the IEG promoter. The early growth response gene-1 (egr-1), which has six SREs and two cAMP response elements in the promoter region and encodes a transcription factor that is known to control bFGF production via the MAP kinase cascade in astrocytes, was used to construct the reporter gene vector (Biesiada et al., 1996; Changelian et al., 1989; Harada et al., 1996).

In astrocytes, glutamate induces calcium responses via mGluRs, since calcium responses were induced by neither kainic acid nor N-methyl-D-aspartate, NMDA, which act on iGluRs, but were instead induced by the group I mGluR agonist, (S)-3,5-dihydroxyphenylglycine, DHPG, or the more selective mGluR5 agonist, (RS)-2-chloro-5-hydroxyphenylglycine, CHPG, in the present preparation (data not shown). The group I mGluRs have been found to couple to Gq and utilize phosphatidyl inositol metabolism and the resulting calcium release from calcium stores to induce cellular responses (Berridge et al., 2000; Conn and Pin, 1997; Irvine and Schell, 2001). It has also been reported that initial step in the phosphatidyl inositol metabolism is the hydrolysis of phosphatidyl inositols by phospholipase C (PLC) (Rhee, 2001). When the substrate is
phosphatidyl inositol-4,5 diphosphate (PIP₂), it has been found that one of the products, IP₃,
induces calcium release from the intracellular calcium store via the IP₃ receptor, known as
IP₃-induced calcium release (IICR) (Berridge et al., 2000). IICR shows various patterns,
including calcium transient and oscillation, which are attributable to the complex regulatory
mechanism of enzymes in the phosphatidyl inositol metabolism by calcium and the
metabolite (Carafoli, 2002; Irvine and Schell, 2001). Because direct activation of the IP₃
receptor with thimerosal was sufficient to induce an oscillatory calcium response in
astrocytes, the regulatory mechanisms of intracellular calcium dynamics were assumed to be
the main target of factors affecting calcium oscillation, and therefore the changes in the
calcium store were investigated.

The recent establishment of imaging methods for cellular IP₃, using a series of
fusion proteins of green fluorescence protein (GFP) variants and pleckstrin homology
domain (PHD) from phospholipase C-δ₁ (Hirose et al., 1999; Stauffer et al., 1998), revealed
the synchronized oscillation of cellular calcium and IP₃ in a number of cells (Hirose et al.,
1999; Young et al., 2003). GFP-PHD exists as a membrane-bound PIP₂-binding form, which
is converted to a soluble IP₃-binding form following activation of phosphatidyl inositol
metabolism. This conversion has been observed as a redistribution of fluorescence from the
plasma membrane to the cytosol and the effect can be used to monitor the IP₃ production
(Hirose et al., 1999). In the present study, two variations of IP₃ imaging based on GFP-PHD
were evaluated, then the appropriate method was applied to astrocytes, in order to investigate the dynamics of the phosphatidyl inositol metabolism during calcium oscillation and the effects of the soluble factors affecting the calcium response patterns. One of the imaging methods evaluated was simultaneous imaging of intracellular calcium and IP$_3$ levels using a combination of GFP-PHD and a fluorescent calcium indicator, FuraRed, and the other was ratio imaging of intracellular IP$_3$ level using the fluorescence resonance energy transfer (FRET) between cyan and yellow variants of GFP-PHD (CFP-PHD and YFP-PHF).

Evaluation of the method was performed using muscarinic acetylcholine receptor (mAChR) induced phosphatidyl inositol metabolism in PC12h cells. PC12h (Hatanaka, 1981) is a subclone of PC12, which has been studied extensively on the calcium release via phosphatidyl inositol metabolism by calcium imaging and biochemically (Arslan et al., 2000; Fasolato et al., 1990; Vicentini et al., 1985).
3.2 Experimental Procedures

3.21 Cell culture and calcium imaging

Astrocytes and PC12h cells were cultured as described in MATERIALS. Calcium imaging was performed as described in CHAPTER 1.

3.22 Immunological detection

To assess ERK phosphorylation, cells on six well plates were cultured in GF-free ADM for 48 h and pretreated either overnight with pro-inflammatory cytokines or lipopolysaccharide (LPS), or pretreated for 5 min with the MEK inhibitor. Then growth factors were added for 5 min and Western blotting was performed using mouse monoclonal antibody against phosphorylated ERK (2 µg/ml, Sigma) or rabbit polyclonal anti-ERK antibody (0.6 µg/ml, Sigma).

3.23 Reporter gene assay.

A reporter gene vector containing an egr-1 promoter was constructed as described in MATERIALS. The reporter gene vector was transfected, using TransFast (Promega), into astrocytes which had been grown for 48 h in DMEM containing 25 mM HEPES, pH 7.4, and 1% FCS. After 24 h, the medium was changed to GF-free ADM, then, after 48 h culture, with or without pretreatment, as described for the Western blot experiments above, growth factors were added for 6 h, and luciferase activity assayed using PicaGene (Nippon Gene, Tokyo, Japan).
3.24 IP$_3$ imaging

The expression vectors of GFP-PHD and its variants were constructed as described in MATERIALS. These expression vectors were transfected to PC12h cells using TransFast, and to astrocytes as described for the reporter gene assay. Cells were used for imaging studies after being cultured for 48-72 h. For simultaneous imaging of IP$_3$ and calcium, the PC12h cells were loaded with Fura Red-AM (10 µM; Molecular Probe, Eugene, OR) in the presence of 0.1% cremophore, using the same protocol for calcium imaging with Fura2. Fluorescence images were obtained using an E600FN upright microscope and a Fluor 40x/0.8w objective (all from Nikon, Tokyo, Japan) equipped with a W-View dichronic mirror system (Hamamatsu Photonics, Hamamatsu, Japan), a CSU-10 laser confocal scanning unit (Yokokawa, Tokyo, Japan), a 532R-BS-A04 argon laser, (Melles Griot, Irvine, CA) and a C6790 CCD camera (Hamamatsu). The fluorescence was split using a dichronic mirror (590LP) and 535DF35 and 600EFLP barrier filters were used to obtain, respectively, the GFP-PHD and FuraRed images, which were acquired and analyzed using AQUACOSMOS software (Hamamatsu). For ratio imaging of IP3, the fluorescence was split by a W-View dichroic mirror system (Hamamatsu ) equipped with a dichroic mirror (510LP) and barrier filters, 480DF30 and 535DF25, for YFP-PHD and CFP-PHD, respectively. IP$_3$ increases were expressed as the change in the ratio ($\Delta R$), which was calculated by dividing the fluorescent intensity of YFP-PHD (535 nm) by that of CFP-PHD (489 nm).
3.3 Results

3.3.1 Effects of growth factors and pro-inflammatory cytokines on the MAP kinase cascade and downstream immediate early gene activation in astrocytes

To examine the involvement of the MAP kinase cascade in the changes of the astrocytic calcium response patterns, its activation was examined at two different levels, ERK phosphorylation and activation of the IEG, in astrocytes cultured in the presence of factors affecting calcium dynamics. As shown in Fig. 3-1A, in the presence of the growth factors, ERKs were phosphorylated within 5 min; this effect was slightly enhanced by the cytokines and LPS, which did not activate the ERKs, although they have been reported to activate this cascade in astrocytes (Molina-Holgado et al., 2000). However, the phosphorylation of ERKs by growth factors was completely abolished by pretreatment with the MEK inhibitor. Furthermore, gene activation via the MAP kinase cascade was monitored in a reporter gene assay using the promoter of *egr-1*, which is an IEG responsible to MAP kinase cascade. As shown in Fig. 3-1B, the growth factors caused gene activation within 6 h and this was suppressed by pretreatment with the pro-inflammatory cytokines, LPS, or the MEK inhibitor. As in the ERK phosphorylation experiment, the cytokines or LPS did not activate the reporter gene (data not shown). These results show that the growth factors activated the MAP kinase cascade and subsequent IEG expression, and that the cytokines and LPS affected these processes downstream of
ERK activation and suppressed gene regulation in the cascade.

3.32 Correlation between enlargement of calcium stores and calcium oscillation

In order to compare the sizes of the calcium stores involved in the astrocytic calcium response to glutamate, the cells were treated with ionomycin in the absence of extracellular calcium and the amount of released calcium measured; this treatment abolished the glutamate-induced calcium release (Fig. 3-2A, left), showing that it depleted the store required for calcium oscillation. In the absence of ionomycin treatment, astrocytes retained the ability to release calcium even after six minutes in the absence of extracellular calcium (Fig 3-2A, right). Fig. 3-1B shows calcium release in astrocytes under different culture conditions. When this method was used to compare the size of the stores under each of these conditions, the calcium release in the presence of growth factors was about twice that seen in their absence (Fig. 3-2C) and was decreased to an intermediate level by the presence of pro-inflammatory cytokines, LPS, or the MEK inhibitor, indicating that the enlargement of the calcium store by the growth factors was suppressed parallel to the calcium oscillation.

3.33 Evaluation of methods for IP$_3$ imaging in PC12h cells

Simultaneous imaging of cellular IP$_3$ and calcium levels was evaluated by quantitative analysis of the fluorescence changes of PC12h cells labeled with GFP-PHD and FuraRed, in response to mAChR activation. Cellular calcium and IP$_3$ increases were induced using carbachol.
(100 µM), as this chemical specifically activates mAChR and induces IP₃ production in PC12 cells even at 500 µM (Vicentini et al., 1985). As shown in Fig. 3-3A, in response to treatment with carbachol, the FuraRed fluorescence decreased rapidly, indicating a rapid increase in calcium, while the GFP-PHD fluorescence gradually redistributed from the plasma membrane to the cytosol, indicating a change from the PIP₂-binding form to the IP₃-binding form. Fig. 3-3B shows the time-course of the changes. The fluorescent intensity of FuraRed (F/F₀) in the cytosol decreased transiently following the carbachol stimulation, this effect being reversed following wash-out of carbachol. In contrast, the GFP-PHD gradually redistributed from plasma membrane to cytosol following carbachol stimulation. The redistribution, which was quantified by the coefficient of variance (FSD/FAVE) of the GFP fluorescence over the cellular area except nucleus, reached a minimum at a time-point later than the calcium peak and was then maintained at this level throughout stimulation, then returned slowly to baseline levels during wash-out.

The FRET-based ratio imaging of the cellular IP₃ was evaluated by the quantitative analysis of the fluorescence changes of PC12h cells labeled with CFP-PHD and YFP-PHD. Without the mAChR activation, CFP-PHD and YFP-PHD interacted with each other as PIP₂ binding forms, and the interaction was measured using the fluorescence property reflecting FRET between CFP-PHD and YFP-PHD, namely, the increase of the fluorescence of YFP-PHD and the decrease of that of CFP-PHD at the excitation of CFP-PHD. The activation of mAChR converts the fusion
proteins to IP$_3$ binding form and changes the FRET-based fluorescence. Fig. 3-3C shows the changes in the normalized fluorescent intensities of CFP-PHD (480 nm) and YFP-PHD (535 nm), together with the corresponding ratio (YFP/CFP). Following carbachol stimulation, the fluorescent intensity of the FRET donor (CFP-PHD) increased, while the intensity of the corresponding acceptor (YFP-PHD) decreased, causing a subsequent decrease in the overall (YFP/CFP) ratio. The fluorescent intensities were found to gradually fall back to their original values after complete removal of the carbachol. Fig. 3-3D shows the averaged responses of cellular IP$_3$ and calcium increases, which were measured using FRET-based IP$_3$ imaging and calcium-based (Fura2 indicator) imaging, respectively. The mAChR activation was found to induce both a transient and sustained calcium increase, together with a sustained IP$_3$ increase, each of which peaked over a similar time.

3.34 IP$_3$ production of astrocytes in response to glutamate and calcium ionophore

The FRET-based ratio imaging of IP$_3$ had a better temporal correlation between cellular calcium and IP$_3$ levels. Although the simultaneous imaging of calcium and IP$_3$ was advantageous in measuring the dynamics of phosphatidyl inositol metabolism and cellular calcium in the same cell, temporal resolution of this method was assumed insufficient to measure the IP$_3$ dynamics in the calcium oscillation of astrocytes. Therefore, the FRET-based ratio imaging was applied to investigate the dynamics of the cellular IP$_3$ during the calcium responses of astrocytes. Cellular calcium was measured separately using the cells in the same preparation series and stimulation paradigm. Fig.
3-4A shows the calcium and IP₃ responses of astrocytes cultured in ADM, following glutamate (30 µM) and ionomycin (2.5 µM) treatment. Although glutamate induced oscillatory calcium response as described in Chapter 2, it did not induce detectable IP₃ production. Even at higher concentration (up to 1 mM), glutamate failed to induce detectable IP₃ production by itself (data not shown). However, the addition of ionomycin in the presence of glutamate induced detectable IP₃ production, as well as a substantial and sustained calcium increase. As shown in Fig. 3-4B, ionomycin treatment alone failed to induce IP₃ production, although it induced a substantial calcium increase. A similar tendency of responses was observed in astrocytes cultured in GF-free ADM (Fig. 3-4C and D).
3.4 Discussion

In parallel with calcium oscillation, the growth factors and the pro-inflammatory cytokines regulated the MAP kinase cascade in opposite directions. The growth factors induced the ERK phosphorylation and the following IEG activation. Although the pro-inflammatory cytokines and LPS did not affect the growth factor induced ERK phosphorylation, they suppressed the IEG activation mediated by the MAK kinase cascade. These results suggest that the pro-inflammatory cytokine utilize a novel regulatory mechanism in this cascade. The presently known negative regulation mechanisms for the MAP kinase cascade affect signaling between receptors and ERKs, such as MAP kinase phosphatases (Takaki et al., 2001) and sprouty (Hanafusa et al., 2002). Therefore the pro-inflammatory cytokines could affect signaling between ERKs and gene regulation by unidentified mechanisms.

A broad spectrum of non-excitable cells show calcium oscillation. The bell-shaped calcium dependency of the IP$_3$ receptor (Miyakawa et al., 1999) and the dual regulation of RGS4 by calcium and phosphatidyl inositol trisphosphate (PIP$_3$) (Luo et al., 2001) have been suggested as possible mechanisms, but no general model explaining all the phenomena has been presented. Furthermore, there are reports that oscillations in intracellular IP$_3$ levels are synchronized with calcium oscillations (Hirose et al., 1999) and that spontaneous oscillation of calcium release from the
intracellular calcium store is directly stimulated by a low IP₃ concentration (Hajnoczky and Thomas, 1997). The present results show that the size of the calcium store, but not mGluR levels, is crucial in generating calcium oscillation in astrocytes. Since the growth factors altered the calcium responses to both glutamate and ATP and did not affect mGluR5 expression, this shows that their effect was independent of the type and level of expression of receptors. Furthermore, the calcium response induced by direct activation of IP₃ receptors by thimerosal was also converted from transient to oscillatory by the growth factors, suggesting that the growth factors affected the properties of the calcium store or some controlling mechanism of calcium dynamics. Measurement of the size of the calcium store using ionomycin showed that enlargement of the calcium store correlated with the generation of the oscillatory calcium response. A similar correlation has been reported in mouse oocytes during maturation (Jones et al., 1995), suggesting that this is a common mechanism for converting the response pattern under physiological conditions. I assume that growth factors increase the size of the calcium store, then increase the duration or total amount of calcium release, which finally affects the local calcium concentration around the IP₃ receptor. Since the IP₃ receptor is regulated by calcium both in a positive and negative manner (Miyakawa et al., 1999), growth factors may affect IP₃ receptor function via the local calcium concentration and produce synchronized calcium release. Another possible explanation for the calcium oscillation is that, when growth factors increase the size and possibly the distribution of the calcium stores, this may enable the propagation
of a calcium wave which is thought to be one mechanism involved in calcium oscillation (Carafoli, 2002). If enlargement of the calcium store resulted in a larger region of the astrocyte being involved in the calcium response, it is likely that the local calcium increase propagates as a calcium wave.

Some cases of calcium oscillation have been explained as a result of repetitive propagation of calcium waves (Miyazaki et al., 1992; Strahonja-Packard and Sanderson, 1999), and propagation of the calcium increase was observed during calcium oscillation (see Movie 1). Further analysis of the calcium store in astrocytes, including calcium concentration in the store in both the resting and stimulated state, the morphology of the endoplasmic reticulum, and the localization of the IP₃ receptor, would provide useful information for examining these two possibilities.

The imaging of IP₃ with fluorescently-labeled PHD fusion proteins, essentially relies on one of two reported methods; analysis of the cellular localization of fluorescent fusion proteins by confocal microscopy (Hirose et al., 1999; Varnai and Balla, 1998), and the measurement of the interaction between the fusion proteins using FRET (van der Wal et al., 2001). Both methods are dependent on the redistribution of fusion proteins from plasma membrane to cytosol following the induction of phosphatidyl inositol metabolism. In the present evaluation of simultaneous imaging of cellular calcium and IP₃ using confocal microscopy, mAChR activation resulted in the production of a redistribution peak which was significantly delayed with respect to the increase in calcium levels. This is in stark contrast to the FRET-based ratio imaging of cellular IP₃ in which IP₃ and calcium
assume a similar time frame. FRET is considered to be the more accurate of the two abovementioned analytical methods with respect to time dependency, since the interaction between fusion proteins changes almost instantaneously following the disruption of PIP$_2$ binding. Therefore, the observed delay in my simultaneous imaging would indicate that the release of the fusion proteins from the plasma membrane require several tens of seconds in order to migrate to the cytosol. A similar delay has also been reported in CHO cells (Nash et al., 2001), where the time taken was typically shorter than that observed in PC12h cells. This implies that either the diffusion constant of the fusion proteins, or the spatial organization between the plasma membrane and the confocal plane, vary among cell types; effects which are typically avoided with FRET.

Since the FRET-based ratio imaging was shown to possess a similar temporal resolution to calcium imaging, it was applied to astrocytes in order to reveal the calcium and IP$_3$ dynamics during the calcium oscillation. As a result, astrocytes, cultured either in ADM or GF-free ADM, failed to show detectable IP$_3$ production, in response to glutamate or ionomycin alone, however their simultaneous stimulation induced detectable IP$_3$ production. Since most subtypes of PLC have calcium binding domain and show calcium dependency (Rhee, 2001), the above-described calcium dependent potentiation of the IP$_3$ production would be attributed to the properties of these enzymes. Although the astrocytic calcium response to glutamate was ascribed to Gq coupling group I mGluR, glutamate stimulation was not sufficient to induce detectable IP$_3$ production even at high
concentrations. As shown in CHAPTER 1, high concentrations of glutamate induce large but transient (quickly restoring) calcium responses in astrocytes, probably because the group I mGluRs desensitize following the phosphorylation by protein kinase C, which is activated in the phosphatidylinositol metabolism as well (Dale et al., 2001). As the group I mGluR is inactivated by desensitization, it is assumed that the cellular calcium concentration will not reach a sufficient level, which is realized using ionomycin, for the detectable IP$_3$ production at any glutamate concentrations. In previous biochemical studies using radio-labeled inositol, glutamate has been shown to induce much smaller IP$_3$ production in cultured astrocytes than adenosine or catechol amine, which activate Gi coupled receptors (Biber et al., 1997). Therefore, the level of IP$_3$ production largely depends on the properties of receptors. In preliminary studies in our laboratory, we have found that ATP does not induce detectable IP$_3$ production in astrocytes, but the exogenous Dorosophila octopamine receptor, which produces transient calcium responses alone even in the astrocytes cultured in ADM, induces both calcium and IP$_3$ responses in the same astrocyte.
3.5 Figures

Fig. 3-1
Fig. 3-1. Activation of the MAP kinase cascade by growth factors and inhibition by pro-inflammatory cytokines. Astrocytes cultured in GF-free ADM (-) were stimulated with growth factors (GF) for 5 min for Western blot experiments or for 6 h for reporter gene assays, with or without 5 min pretreatment with MEK inhibitor U0126 (U) or overnight pretreatment with pro-inflammatory cytokines (IL1 or TNF) or LPS. (A) ERK activation monitored by Western blotting using anti-phosphorylated ERK antibody and anti-ERK antibody. (B) Activation of the immediate early gene, egr-1 assessed by the reporter gene assay.
Fig. 3-2
**Fig. 3-2.** The size of the calcium store was altered by factors affecting calcium oscillation. (A)

Procedure for assessing the size of the calcium store. Astrocytes cultured in ADM were transferred to calcium-free medium at the start of the trace, then treated either with ionomycin (Iono, 2.5 µM) followed by glutamate (Glu, 30 µM) (**left panel**) or with glutamate alone (**right panel**). Ionomycin causes calcium release, which represents the size of the calcium store, and abolishes the calcium response to glutamate. (B) Ionomycin-induced calcium release of astrocytes cultured under different conditions; ionomycin treatment is indicated by the bar underneath the traces. Each trace is the mean ± S.E.M. for three cells in a single imaging experiment. The effects of cytokines (**left panel**) and the MEK inhibitor (**right panel**) were compared in the same series of cultures from a single batch of cells. (C) Quantitative comparison of calcium release. The ionomycin-induced calcium release from cells cultured in different media is expressed relative to that of cells cultured in ADM. The values are the mean ± S.E.M. for 21 cells from three independent imaging experiments using the same series of cultures as in (B).
Fig. 3-3
Fig. 3-3. Comparison of imaging methods for cellular IP₃, using the response of PC12h cells to carbachol (100 µM, bars). Simultaneous imaging of intracellular calcium and IP₃ levels (A, B), and FRET-based ratio imaging of IP₃ (C,D) were compared to evaluate their accuracy. (A): FuraRed and GFP-PHD fluorescence images in PC12h cells on addition and wash-out of carbachol. (a-d): Different time-points before, during and after stimulation: (a) before stimulation, (b) after 7.8 sec of stimulation, (c) after 42.5 sec of stimulation, (d) after 45.1 sec of washout. (B): The change in fluorescence in a representative cell, indicated by an asterisk in A, was quantified in terms of fluorescent intensity (ΔF/F₀) or coefficient of variance (ΔFSD/FAVE) of the fluorescence distribution for FuraRed and EGFP-PH, respectively. (C) A PC12h cell expressing CFP-PHD and YFP-PHD was stimulated with carbachol (1min). The fluorescence ratio (YFP/CFP) (red line) was calculated from the corresponding fluorescent changes (F/F₀) in CFP (blue line) and YFP (green line). (D) Cellular calcium (upper traces) and IP₃ (lower traces) were measured using PC12h cells containing either Fura2, or expressing CFP-PHD and YFP-PHD, respectively. Cells were stimulated with carbachol, and responses are shown as means ± S.E.M [n=7 (upper trace), n=8 (lower trace)].
Fig. 3-4
**Fig. 3-4.** Calcium and IP$_3$ responses of astrocytes to glutamate (30 µM, bars) and ionomycin (2.5 µM, dashed bars). Cellular calcium (upper traces) and IP$_3$ (lower traces) were measured using astrocytes containing either Fura2, or expressing CFP-PHD and YFP-PHD, respectively. Astrocytes were cultured in ADM (A, B) or GF-free ADM (C, D). The glutamate (A, C), or the ionomycin stimulation (B, D) proceeded respectively.
CONCLUSIONS

On the basis of the above findings, I propose the model shown in Fig. 4-1, in which the astrocytic calcium response is altered by soluble factors, the production of which is affected by the CNS environment, and this change in response then affects the activity of neighboring neurons. The growth factors, EGF and bFGF, increase the size of the intracellular calcium store in the astrocyte, which leads to conversion of the neurotransmitter-induced calcium response from transient to oscillatory, and promotes proliferation and a hypertrophic morphology, all of which involve the MAP kinase cascade. The calcium oscillation generated in the astrocyte in response to glutamate leaking from the synaptic cleft during neuronal activity causes repetitive glutamate release, which gives back repetitive excitatory inputs to the neuronal network. In contrast, pro-inflammatory cytokines suppress these changes in the calcium response and morphology by inhibiting the expression of genes downstream of the MAP kinase cascade. This leads to a reduction in excitatory input from astrocytes to neurons which is influenced by the frequency of glutamate release and contact between the two types of cell.
Fig. 4-1
**Figure**

**Fig. 4-1** Proposed scheme showing the effect of astrocytes on neuronal activity.
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