Order Number 9414010

Glia-neuron signaling

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Glia-neuron signaling

by

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A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Zoology and Genetics
Interdepartmental Program: Neuroscience
Co-majors: Zoology (Neurobiology)
Neuroscience

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Interdepartmental Program

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1993

to those who are, and
to those who are not

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GENERAL INTRODUCTION

The mammalian nervous system is composed of more than 100 billion neurons surrounded by glial cells. Glial cells are very numerous. There are 10-50 times as many glial cells as neurons. They clearly exceed the neurons not only by their numerical preponderance, but also in the variety of their different types. The major classes of glial cells in the vertebrate central nervous system (CNS) are astrocytes, oligodendrocytes and microglia, whereas Schwann cells are the predominant glial type in the peripheral nervous system (PNS).

Glial cells were first described in 1846 by the German pathologist Rudolf Virchow, who named them neuroglia (Greek, meaning nerve glue). It was proposed that glia serve as "a real cement, which binds the nervous elements together." The fact that in an ascending phylogeny glia:neuron ratio increases and peaks within the human brain, having both relatively and absolutely the greatest number of glia [1], suggests that glia have brain functions in addition to being simply a matrix serving neurons while they are performing their noble neuronal functions. There are several formulated hypotheses assigning different roles to glia:

1. Glia are supporting elements providing structural integrity of brain, a function carried by connective tissue cells in other parts of the body (ref. 2).

2. Cajal proposed a role for glia in the insulation of neurons (ref. 3). The confirmation of his theory came from Hortega's discovery (ref. 3) of the relation of oligodendroglia to myelin and even more by the Geren-Robertson discovery [4,5] that "myelin consists of the overlapped surfaces of the enwrapping oligocytes." This insulation not only provides mechanical protection to neuronal processes, but also increases conduction velocity.

3. Glial cells play a role in repair and regeneration of the nervous system. Microglia behave like a tissue macrophage, phagocyticly removing cellular debris after neuronal death and brain injury. Additionally, macrophages and other glial cell types divide. Astrocytes undergo gliosis, then become swollen and may amitoticaly divide. It is obvious that following injury and consequent neuronal loss, glial division leads to an occupation of the vacant space. If the peripheral nerve is damaged, a peripheral axon can regenerate and has the capacity to reconnect with targets. This axonal growth is along a route marked by residual Schwann cells. However, the differentiated CNS has a very limited capacity to regenerate the interrupted fiber tracts after injury. Why doesn't regeneration take place in the CNS? The reason might be attributed to differences in glial properties between the PNS and CNS. It has been shown that CNS myelin impairs neurite outgrowth and causes growth cone collapse [6-9] whereas PNS myelin is a good substratum for neurite extension. Biochemical studies [6] brought to light two oligodendrocyte-associated neurite outgrowth inhibitors, NI-35 and NI-250, that are responsible for outgrowth inhibition in the CNS. Antibodies raised against these proteins effectively neutralize the inhibitory activity of the entire CNS myelin [10] confirming that NI-35/250 components are the major inhibitory factors of oligodendrocytes and myelin and crucial determinants of the outgrowth inhibitory substratum property of the CNS. On the other hand, experiments in rat retina indicate that extracellular matrix components, particularly laminin, promote neurite outgrowth [11]. Taken together, these findings raise an exciting possibility that axonal outgrowth is regulated by glial cues so that the presence of laminin may mean "Yes," and NI-35/250 may mean "No." It is clear that this possibility can be also applied to the developing brain.

4. Most of neurons in mammalian brain migrate from the location of their final cell division to a position in the CNS where they reside in the adult [12, 13]. During this migration neurons follow the surfaces of adjacent cells. The most prevalent type of migrating cell follows glial pathways

(see review 14). The radial or Bergman glia provide a transient framework for the migration of neurons and leading processes. Even though a few molecules could account for this process, the molecular blueprint has not emerged yet. For example, astrotactin supports neuronal migration on neocortical glial cells as well as on Bergman glia. It seems that binding between neuron and glia surfaces is neither cell- nor region-specific and could be mediated by a single molecule. However, the spatiotemporal order of cell migration can still be achieved since almost exclusively each neuron becomes attached to the neighboring glial shaft [15].

5. In a study of synaptic plasticity after partial denervation, Raisman & Field [16] observed that in the process of collateral reinnervation, astrocytes play an active role. The degenerating presynaptic terminal becomes surrounded by reactive astrocytic processes. After phagocytosis, astocytic processes recognize and give way to the collateral presynaptic neuronal ingrowth which eventually forms a new synaptic terminal. Since astrocytes are able to discriminate different neuronal inputs [17, 18], the astrocyte-neuron recognition seems to be fairly specific. Recently, the experiments involving restoration of plasticity in an adult cat visual cortex further demonstrated that most likely glia play a role in plasticity of an adult brain [19].

6. There is a proposal for a nutritive role of glia and the existence of molecular transfer between glial cells and neurons (ref. 3). In this concept glial cells are metabolic intermediaries between endothelial cells of the capillaries and neurons (vertebrates) or participants in the blood-brain barrier (invertebrates).

7. Glial cells can take up neurotransmitters such as gamma-aminobutyric acid (GABA) [20] and glutamate [21]. Glutamate taken up by the glial cells can be metabolized to glutamine or alpha-ketoglutarate. It is suggested that glia in turn can supply the nerve terminal with glutamine and alpha-

ketoglutarate which can both serve as precursors for glutamate synthesis (see review 22).

8. Release of neurotransmitters from glial cells has been well documented. In chronically denervated skeletal muscle, Schwann cells, which occupy the original site of the nerve terminal, are capable of spontaneous as well as evoked release of acetycholine [23]. Glial cells in sympathetic and dorsal root ganglia can release GABA after depolarization [24]. Müller retinal cells can release glutamate in response to depolarization due to the reversal of the electrogenic glutamate uptake system [25]. In response to osmotic shock, swollen astrocytes can release aspartate [26]. Glia can also release neuroactive reagents including arachidonic acid [27], nerve growth factor [28] and taurine [29].

While several roles are proposed for glia, their physiological contribution to nervous system function is not well defined. The purpose of this dissertation is to examine the physiological roles for glia in releasing neurotransmitter. In the remainder of this introduction, I will discuss signaling in the brain.

Signaling in brain

"Two roads diverged in a wood, and I-
I took the one less traveled by,
And that has made all the difference."
- Robert Frost

Homotypic signaling

Neuron-neuron signaling. Nerve cells have the ability to communicate rapidly with one another. Axonal conduction and synaptic transmission provide means for this rapid communication. Synaptic transmission is central for understanding how the neuronal network works. It can be electrical or chemical. Most synapses use chemical transmitter. However, some are purely electrical. Chemical synaptic transmission is considerably slower than the electrical synaptic transmission

because of significant synaptic delay ranging from at least 0.3 msec up to 1-5 msec, and sometimes even longer. There are two classes of receptors involved in chemical transmission: one that mediates changes in the membrane conductance (ionotropic receptors) and another that mediates metabolic processes (metabotropic receptors) in the postsynaptic cell. Changes in metabolic machinery are achieved mainly through second messenger systems, such as calcium, phosphoinositides, cyclic nucleotides and arachidonic acid. As a consequence of the diversity of postsynaptic receptors, the action of neurotransmitter can last from milliseconds to hours.

Glia-glia signaling. Glial cells are electrically coupled to one another [30]. The conduction properties of such coupled cells are much the same as they would be if the cells were a syncitium. If several glial cells are depolarized by an increased concentration of extracellular potassium, they will draw a charge from neighboring cells creating a current flow. The current flow created by glial cells contributes to the electroencephalogram (EEG). Müller cell potentials have a time course similar to a component in the electoretinogram (ERG), and it is likely that they are responsible for the b wave [31] in the standard ERG. Glial cells also exhibit a newly recognized form of cell signaling, Ca^{2+} waves. Ca^{2+} waves can be elicited by neurotransmitters [32-35] or by direct mechanical stimulation [34]. Ca^{2+} waves propagate nondecrementaly and their intercellular propagation requires gap junctions [35]. Even though the waves are rather slow in comparison to axonal conduction, typically ranging between 5-200 μm/s, they may represent a type of signaling in the CNS. Intercellular calcium waves may also be important in pathophysiological conditions such as Leao's spreading depression or epilepsy [32].

Heterotypic signaling

Neuron-glia signaling. The pioneering work of Orkand et al. [36] has shown the existence of neuron-glia signaling. Using an optic nerve of the mud puppy as a model, they have shown that electrical impulses delivered to nerve fiber caused depolarization of surrounding glia. The potential changes recorded in glia are physiological since the authors obtained similar results in anesthetized mud

puppies. Glial depolarization during neuronal activity has also been detected in the leech CNS [37] and in mammals [38]. The mechanism underling this type of signaling is an accumulation of potassium in the extracellular space as a result of neuronal activity. Recently, Dani et al. [39], using hippocampal slice preparation, have demonstrated that neuronal activity can trigger calcium waves in an astrocyte network and that this action is mediated by glutamate. Since glia respond to many exogenous applied neurotransmitters with calcium mobilization [33, 34, 40-44], this raises possibility of widespread neuron-glia signaling in the CNS.

Glia-neuron signaling. The observation that glia can release the neurotransmitter glutamate [25] and the fact that most neurons possess glutamate receptors raise the hypothesis that glia signal to neurons through neurotransmitter. To date there has been no published test of this hypothesis. Experiments performed in Paper 2 of this dissertation directly test this hypothesis.

Explanation of dissertation format

This dissertation is composed of two papers. The papers are preceded by a general introduction and followed by a general summary which discuss the entire body of work. General references cited in the general introduction and general summary follow the general summary. Paper 1 has been performed in collaboration with Jeftinija's laboratory. This paper is submitted to *Science* by Parpura, V., Liu, F., Jeftinija, K., Jeftinija, S. and Haydon, P.G. I designed and performed all experiments involving calcium imaging and also participated in the design of glutamate release experiments which were performed by Liu, F., Jeftinija, K. and Jeftinija, S. Paper 2 has been submitted to *Nature* by Parpura, V., Basarsky, T.A., Jeftinija, S. and Haydon, P.G. I performed all experiments included in this paper.

PAPER 1. CALCIUM-DEPENDENT RELEASE OF EXCITATORY AMINO ACIDS FROM GLIA

"There is nothing like a dream
to create the future."
- Victor Hugo

INTRODUCTION

It has been well established that glia have multiple roles in the nervous system which include the regulation of external K^+ and the uptake of the excitatory amino acids, glutamate and aspartate, which are released from neurons into the extracellular space. The demonstration that depolarization causes a calcium-dependent release of the amino acid taurine from glia (1) and that glial calcium levels are regulated by neurotransmitters (2) prompted us to determine whether neuroligands can stimulate the calcium-dependent release of excitatory amino acids (EAAs) from glia.

RESULTS AND DISCUSSION

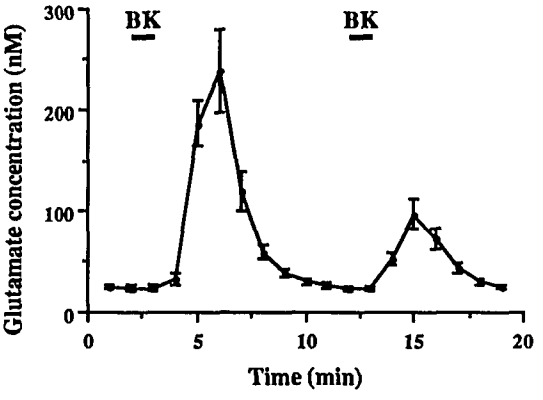
Primary glial cultures of dorsal root ganglia (DRG) were obtained using a modification of the organotypic procedure (3). The established cultures were free of neurons and contained at least 74% Schwann cells. The release of EAAs from these cultures was assayed using high-performance liquid chromatography (HPLC) on the superfusate (4). The basal release of glutamate and aspartate into superfusate produced levels of 26 $\pm$ 2 nM (mean $\pm$ SEM; n= 13) and 6 $\pm$ 1 nM, respectively. Addition of bradykinin caused a dose-dependent increase in release of the EAAs, glutamate and aspartate, from glial cultures. The threshold concentration of bradykinin was 1 nM. 10 nM bradykinin caused a greater than nine-fold increase in the release of glutamate to 239 $\pm$ 42 nM and a greater than three-fold increase in aspartate to 21 $\pm$ 3 nM (n=12; Fig. 1A, B). A second application of bradykinin (n=12) similarly caused EAAs release although the magnitude of this response was attenuated (Fig. 1A, B). This action is receptor-mediated since the B_2 receptor antagonist, (D-Arg^0, Hyp^3, β-Thi^{5,8}, D-Phe^7)-bradykinin (5 μM), reversibly blocked the stimulatory action of bradykinin (n=6; Fig. 1C). However, bradykinin did not significantly affect the release of serine from glial cultures. The basal level of serine was 14 $\pm$ 2 nM compared to 16 $\pm$ 2 nM in the presence of bradykinin (n=13; $p>0.5$). These data demonstrate that the nonapeptide bradykinin potently causes the release of the EAAs glutamate and aspartate from glia.

The release of neurotransmitter at neuronal synapses is calcium-dependent. To ask whether calcium may play a role in bradykinin-induced EAAs release from glia, we used ratiometric imaging techniques to monitor glial calcium levels. Glia were loaded with the membrane permeant calcium indicator fura-2 AM (5). Cells were imaged at low plating density, where they did not form a confluent monolayer. In resting conditions, the cytoplasmic calcium level of glia was 102 $\pm$ 2 nM (n=136). Bradykinin (10 nM) reliably raised the cytoplasmic level of free calcium in 72% of cells tested (n=136 of 188). This increase of intracellular calcium reached the peak level of 747 $\pm$ 24 nM (n=136; Fig. 2A) about 20 seconds after the onset of bradykinin application. In all subsequent experiments, we have reported

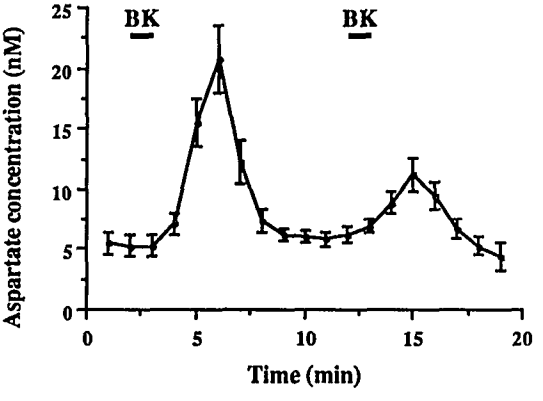
Figure 1. Bradykinin (BK) causes a receptor-mediated release of the EAAs glutamate and aspartate from glia

Using HPLC, the amounts of glutamate and aspartate were determined in superfusate from rat DRG glia cultures. Addition of bradykinin (10 nM) caused a 9-fold elevation of glutamate release (A) and a 3-fold elevation of aspartate release (B). The B_2 receptor antagonist, (D-Arg^0, Hyp^3, ß-Thi^{5,8}, D-Phe^7)-bradykinin, blocked the secretory action of bradykinin (C). After washout of the antagonist, bradykinin reliably increased glutamate release. Points represent means $\pm$ SEM.

A


BK
BK
Glutamate concentration (nM)
300
200
100
0
0
5
10
15
20
Time (min)

B


BK
BK
Aspartate concentration (nM)
25
20
15
10
5
0
0
5
10
15
20
Time (min)

C

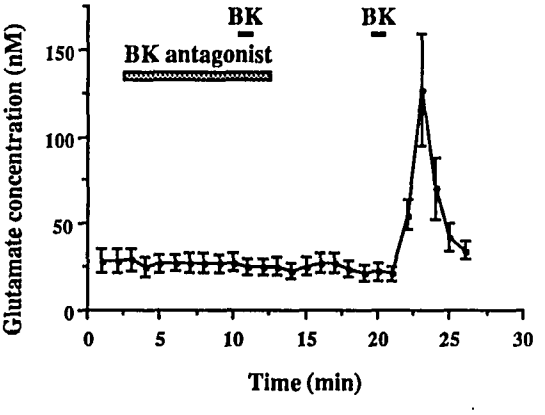
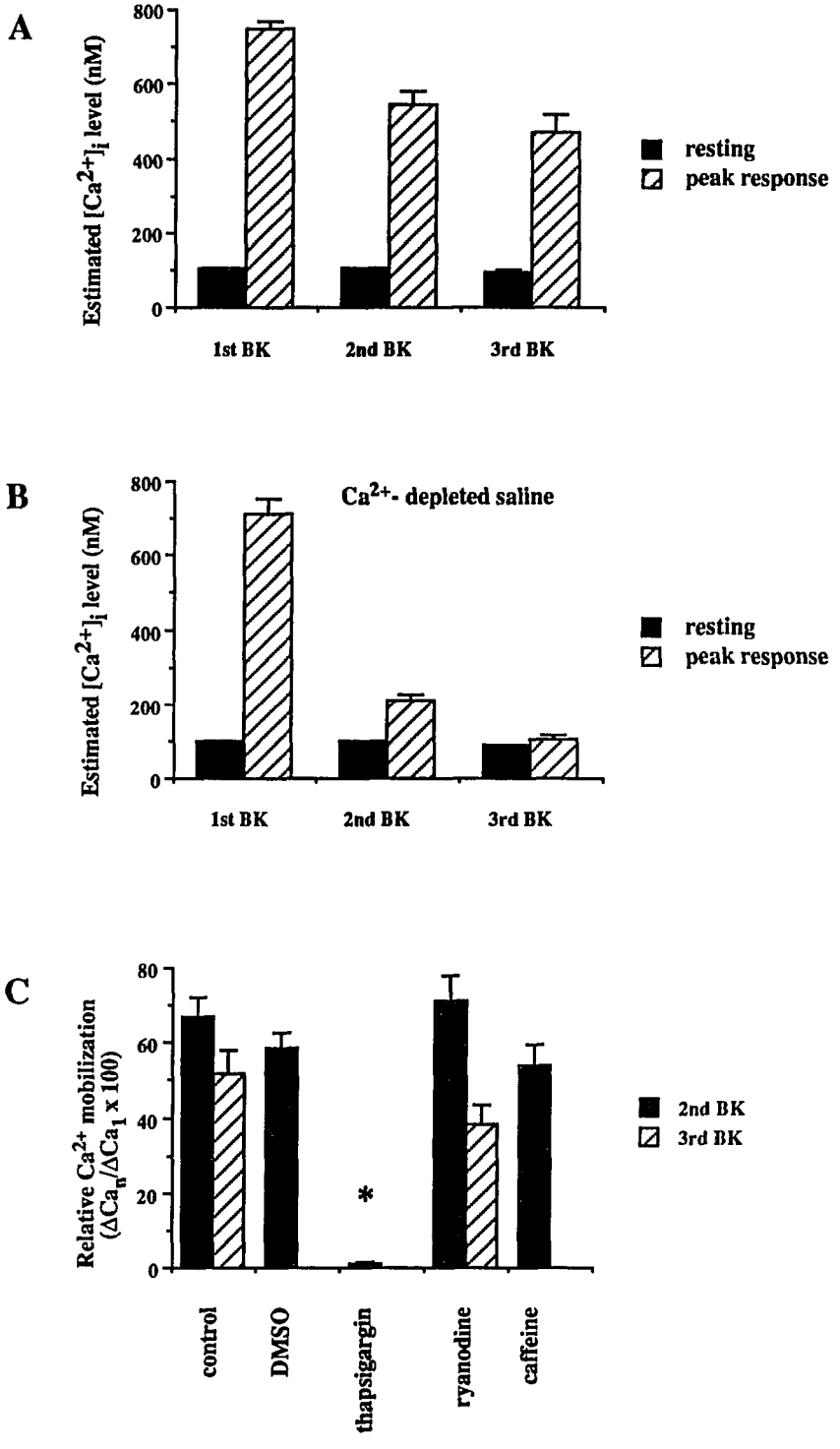

BK
BK
BK antagonist
Glutamate concentration (nM)
150
100
50
0
0
5
10
15
20
25
30
Time (min)

Figure 2. Bradykinin (BK) mobilizes Ca^{2+} from internal stores in DRG glia

In (A) repeated applications of bradykinin (10 nM) caused an elevation of glial cytoplasmic calcium levels. In (B) the external calcium was reduced by exchange of normal saline for calcium-depleted saline. In this saline bradykinin was able to elevate calcium from its resting level indicating that calcium is mobilized from internal stores. However, a second and third addition of bradykinin had little effect on internal calcium levels (compare A with B). (C) shows the effect of pharmacological manipulations on the ability of bradykinin to cause Ca^{2+} mobilization. Relative Ca^{2+} mobilization is expressed as percentage values of $[Ca^{2+}]_i$ accumulation (peak value subtracted from resting; ΔCa) during the 2nd and 3rd applications of bradykinin (ΔCa_n) compared to the 1st (ΔCa_1) (relative calcium mobilization $=\Delta Ca_n/\Delta Ca_1 \times 100$). The presence of ryanodine (10 μM; Molecular Probes) or caffeine (10 mM; Sigma) in the bathing solution did not prevent bradykinin from elevating $[Ca^{2+}]_i$. However, thapsigargin (1 μM; Sigma), but not its DMSO (0.1 % v/v) carrier, applied to the bath following the first addition of bradykinin, reduced the bradykinin-induced increase of $[Ca^{2+}]_i$ during the second addition of bradykinin. Taken together, these data demonstrate that bradykinin mobilizes calcium from internal stores and reloading of these stores requires the presence of external calcium. Bars represent means $\pm$ SEM. To compare effects of different drugs on effect of bradykinin, one-way ANOVA with *post hoc* Scheffeé's comparison was used. Significance was established at $p<0.001$ (*).


A
Estimated $[Ca^{2+}]_i$ level (nM)
800
600
400
200
0
1st BK
2nd BK
3rd BK
resting
peak response
B
Ca^{2+}- depleted saline
Estimated $[Ca^{2+}]_i$ level (nM)
800
600
400
200
0
1st BK
2nd BK
3rd BK
resting
peak response
C
Relative Ca^{2+} mobilization ($\Delta Ca_n/\Delta Ca_1$ x 100)
80
60
40
20
0
*
control
DMSO
thapsigargin
ryanodine
caffeine
2nd BK
3rd BK

the calcium levels for only those cells that responded to bradykinin (5). These bradykinin responsive cells had the morphology of Schwann cells. A second application of bradykinin caused an increase in the level of intracellular calcium from 103 $\pm$ 2 to 544 $\pm$ 34 nM (n=42; Fig. 2A). The B_2 receptor antagonist, (D-Arg^0, Hyp^3, β-Thi^{5,8}, D-Phe^7)-bradykinin (5 μM), reversibly attenuated the stimulatory action of bradykinin on calcium mobilization. In the presence of the B_2 receptor antagonist, the average resting calcium level (107 $\pm$ 3 nM) was moderately increased by application of bradykinin (151 $\pm$ 11 nM; n=32). However, after washout of the antagonist, bradykinin increased the cytoplasmic calcium level to 480 $\pm$ 24 nM (n=32), a value significantly greater than in the presence of the B_2 receptor antagonist (Scheffeé's test, $p<0.001$).

Removal of external calcium from the bathing medium did not prevent the initial calcium mobilizing action of bradykinin (Fig. 2B). Cells were bathed in a calcium-depleted saline containing 0.2 mM calcium with the addition of 1 mM EGTA to yield an estimated free extracellular calcium level of 24 nM (6). We found that it was necessary to have some calcium in the bathing medium otherwise cells detached from the culture substrate. In this calcium-depleted saline, bradykinin elevated cytoplasmic free calcium from 98 $\pm$ 2 nM to 713 $\pm$ 38 nM (n=36). However, subsequent additions of bradykinin in calcium-depleted bathing medium had little calcium mobilizing action (n=36 and n=23 for the second and third applications, respectively; Fig. 2B). These data suggest that bradykinin mobilizes calcium from internal stores and that these stores must refill with calcium from the extracellular medium.

Calcium mobilization from an internal store is further supported by observations using thapsigargin, an inhibitor of the Ca^{2+}-ATPase of internal calcium stores. Following the first addition of bradykinin, thapsigargin (1 μM) was added to the culture to prevent reloading of internal stores with calcium. Addition of thapsigargin alone caused a transient increase in calcium from 90 $\pm$ 5 to 320 $\pm$ 42 nM (n=15; Student t-test, $p<0.0001$). In the presence of thapsigargin, a second application of bradykinin failed to mobilize internal calcium as compared to matched control cells (n=26, Scheffeé's test, $p<0.001$; Fig. 2C) supporting the notion that bradykinin mobilizes calcium from an internal store. To determine the

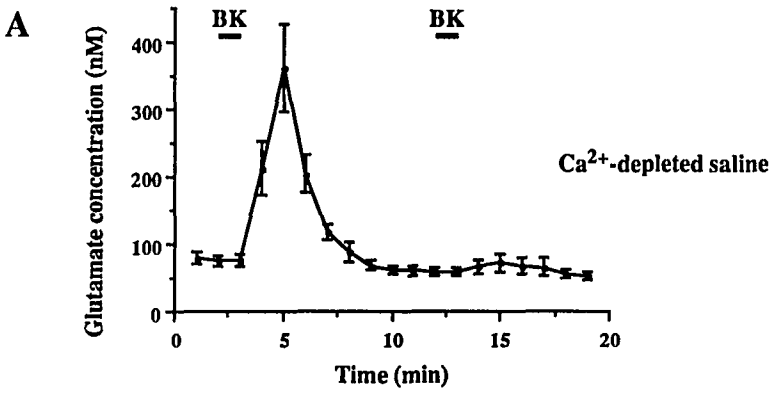
type of internal calcium store which bradykinin acts on, we added ryanodine which can block the release of calcium from the caffeine-sensitive calcium store. Ryanodine (10 μM) did not affect the calcium mobilizing action of repeated applications of bradykinin (n=21 and n=20 for the second and third applications, respectively; Scheffeé's test, $p>0.25$; Fig. 2C). Furthermore, the sustained presence of caffeine (10 mM) did not affect the calcium-mobilizing action of bradykinin (n=23, Scheffeé's test, $p>0.25$; Fig. 2C). Thus, we conclude that bradykinin predominantly mobilizes calcium from a ryanodine/caffeine-insensitive internal calcium store. This suggests that bradykinin's actions are mediated through an IP_3- sensitive calcium store. This is consistent with other studies which have shown that bradykinin causes phosphoinositide hydrolysis in oligodendrocytes and astrocytes (7). In NG108-15 cells, bradykinin has been shown to act through an IP_3-sensitive calcium store to mobilize internal calcium (8).

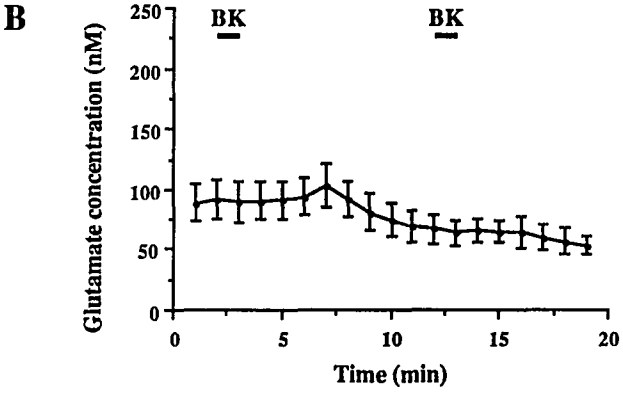
Calcium-imaging data raise the possibility that bradykinin stimulates EAA release through mobilizing internal calcium. To test whether calcium is necessary for glutamate release, we determined whether multiple applications of bradykinin caused glutamate release in calcium-depleted saline (9). Consistent with the ability of bradykinin to mobilize calcium in calcium-depleted saline, the first bradykinin application stimulated the release of glutamate from glial cultures (n=6, Fig. 3A) in calcium-depleted saline. However, a second application of bradykinin did not cause significant release of glutamate (n=6; Student t-test, $p>0.5$; Fig. 3A). To further test the calcium hypothesis for glutamate release, we incubated glial cultures for 30 minutes in 50 μM BAPTA-AM in calcium-containing saline. This membrane permeant calcium chelator blocked the stimulatory action of bradykinin on glutamate release (n=6; Student t-test, $p>0.3$; Fig. 3B).

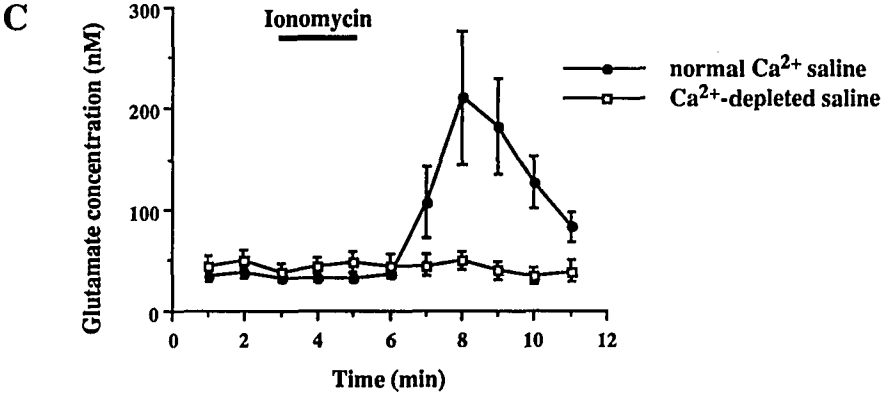
As a critical test of the calcium hypothesis, we exposed cultures to the calcium-ionophore, ionomycin, in calcium-containing saline. Addition of ionomycin (5 μM, Sigma) stimulated the release of both glutamate (n=6; Student t-test, $p<0.025$, Fig. 3C) and aspartate (data not shown). Ionomycin was without stimulatory effects when glia were bathed in calcium-depleted saline (n=6; Student t-test,

Figure 3. Calcium is necessary and sufficient for glutamate release from DRG glia

The first application of bradykinin caused glutamate release from glial cells bathed in calcium-depleted saline. However, a second application of bradykinin (BK) failed to produce significant release of glutamate (A). After incubation with BAPTA-AM (50 μM; Molecular Probes) for 30 minutes, cells were unable to release glutamate in response to bradykinin showing that increase of intracellular calcium is necessary for glutamate release (B). Sufficiency of calcium to induce glutamate release has been established using ionomycin. Cells released glutamate after application of ionomycin when bathed in normal calcium saline, but not when bathed in calcium-depleted saline (C). In both calcium-depleted and BAPTA treated cells, there were slight augmentations of basal glutamate levels in the supernatant. Points represent means $\pm$ SEM.


A
BK
BK
Glutamate concentration (nM)
400
300
200
100
0
0
5
10
15
20
Time (min)
Ca2+-depleted saline


B
BK
BK
Glutamate concentration (nM)
250
200
150
100
50
0
0
5
10
15
20
Time (min)


C
Ionomycin
Glutamate concentration (nM)
300
200
100
0
0
2
4
6
8
10
12
Time (min)
normal Ca2+ saline
Ca2+-depleted saline

p>0.9; Fig. 3C). Taken together, these data demonstrate that calcium is both necessary and sufficient for stimulating the release of EAAs from glial cultures.

The specific mechanism of EAAs release from glial cultures is unclear. Glial cultures were free of immunoreactivity for the synaptic proteins, synaptophysin and synaptotagmin, while axons of DRG explants (separate cultures; data not shown) were immunopositive for these synaptic proteins. Thus, proteins characteristic of neuronal transmitter release apparatus are absent in these secretory glia. Retinal Müller cells can release glutamate in a calcium-independent fashion in response to elevated $[K^+]_o$ due to the reversal of the normal glutamate uptake mechanism (10). This mechanism is unlikely to account for the release of EAAs in response to bradykinin in DRG glia since the action of bradykinin is calcium-dependent. Furthermore, elevated K^+ (50 mM) did not stimulate EAA release from DRG glia (basal glutamate release 48 ± 7 nM, release in elevated external K^+ 46 ± 6 nM; n=6, Student t-test, p>0.7) nor did it cause changes in glial calcium levels (resting calcium 90 ± 3 nM, calcium level in elevated external K^+ 96 ± 3 nM; n=27, Student t-test, p>0.1) (11).

The regulated release of neurotransmitters from glia may be a widespread property. Stimulation of motoneurons leads to the elevation of calcium in the Schwann cell which surrounds the presynaptic terminal (12). After denervation, Schwann cells have been shown capable of releasing acetylcholine (13). Depolarization of hippocampal astrocytes causes the calcium-dependent release of the amino acid taurine, but not glutamate (1). In preliminary studies we have demonstrated that glial cells from the spinal cord and cerebral cortex release aspartate and glutamate in response to bradykinin. Since many neurotransmitters utilized by the nervous system can mobilize calcium in glia (2), it is possible that neurons have in addition to their fast neuron-neuron synaptic actions, a calcium mobilizing action on neighboring glia (14) which in turn leads to the release of other neurotransmitters from glia that may serve secondary roles in glia-neuronal transmission.

The specific roles for release of excitatory amino acids from glial cells remain to be elucidated. However, elevations of external glutamate and aspartate are likely to modulate neuronal properties including excitability and synaptic transmission.

Given the important role of glutamate in the induction of long-term potentiation, it will be important to determine whether glia regulate synaptic plasticity, learning and memory by releasing the neurotransmitter glutamate and whether excessive glutamate released from glia also contributes to neurodegenerative disorders.

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3. Glial cultures from DRG were obtained by modification of the organotypic procedure [B. H. Gähwiler, *Experientia* **40**, 235 (1984)]. Following anesthesia with ether and decapitation, DRG from 1- to 2-day-old Sprague-Dawely rats were rapidly removed and washed in cold (4°C) oxygenated Gey's balanced salt solution (GBSS; Gibco) modified by the addition of 2% glucose. The capsular sheet was carefully removed from the DRG to minimize fibroblast contamination. DRG were then embedded onto a glass coverslip inlayed in a 35 mm dish (for release study) or into a glass-bottomed dish (for imaging) in a plasma-thrombin clot. Cultures were maintained at 36°C in a humidified 5% CO_2/air atmosphere. The culture medium consisted of 25% horse serum (Gibco), 25% Earle's Balanced Salt Solution (Gibco) and 50% Basal Medium Eagle (Gibco) containing 36 mM glucose. The central part of explants which contains neuronal cell bodies was removed from established cultures (4 to 7 days in culture). The residual glial cells were maintained for additional 72 hours when experiments were performed. Neurites degenerated 24 to 48 hours following the removal of the central portion of explants leaving cultures containing only nonneuronal cells . The absence of neuronal elements was confirmed by immunocytochemistry. In some experiments we also used

purified populations of Schwann cells obtained by the Wood (n=6) and Brockes (n=6) methods (for review see 15). These cultures showed similar increase of EAAs glutamate and aspartate in the superfusate after application of bradykinin.

Immunocytochemistry. Antibody visualization was accomplished by using a Vectastain ABC kit (Vector) and the nickel-enchanced DAB method (16). Glial cultures were free of immunoreactivity for the synaptic proteins, synaptophysin (1:1000; clone 7.4a, provided by R. Jahn) and synaptotagmin (1:5000; clone 41.1, provided by R. Jahn), while axons of DRG explants (separate cultures; data not shown) were immunopositive for these synaptic proteins. In contrast with hippocampal glia (not shown), DRG glia could not be identified using an antibody against glial fibrillary acidic protein (GFAP; 1:5000, ICN Immunobiologicals). Using an antibody against low-affinity NGF receptor (1:2000; 192-IgG, provided by E.M. Johnson, Jr.), we have shown that 74% of cells possess NGF receptors characteristically expressed in Schwann cells localized distal to axotomy site (17).

4. The coverslips with glial cultures were mounted onto a 50 µl perfusion chamber. A modified Ringer's perfusion solution containing (in mM) NaCl 128, KCl 1.9, KH_2PO_4 1.2, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26 and glucose 10 (pH=7.4) bubbled with 95% O_2/ 5% CO_2 was used for constant flow through at a rate of 200 µl/min and 35-37°C. After an equilibration period of 40 to 60 minutes, samples of superfusate for amino acid determination were collected every minute. The amino acid content in samples was determined by HPLC with fluorescence detection (18). Prior to injection, aliquots of samples were derivatized with o-phthalic aldehyde (OPA) 2-mercaptoethanol reagent (Pierce). Chromatography was performed on a 15 cm Microsorb-MV HPLC column (Rainin Instrument Co.) using a sodium acetate methanol gradient (pH=5.9). Bradykinin (BK, 10 nM; Sigma) and its antagonist (5 µM; Bachem Bioscience Inc.) were added to a perfusion solution.

5. Cells were loaded with fura-2 AM (4 µM; Molecular Probes) for 40-60 minutes at 37°C. 1µl of 25%(w/w) of Pluronic F-127 (Molecular Probes) was mixed

with every 4 nmol of AM ester to aid solubilization of the ester in aqueous medium. After washing, cells were de-esterified for 30-60 minutes at 37°C. All experiments took place at 22-24°C. All image processing and analysis were performed using QFM software (Quantex Corp.). Background subtracted, ratio images (340/380 nm) were used to calculate the $[Ca^{2+}]_i$ according to Equation 5 of G. Grynkiewicz et al. [*J. Biol. Chem.* **260**, 3440 (1985)]. Calibration was performed *in situ* (19) using the Ca^{2+}-ionophore 4-bromo-A23187 (10 μM, Molecular Probes). Saturation of intracellular dye has been achieved by addition of 4 mM $CaCl_2$. EGTA (10 mM) was used to chelate extracellular Ca^{2+}. An estimate of autofluorescence at each wavelength has been achieved by addition of $MnCl_2$ (20 mM). Cells were included in analysis if the first addition of bradykinin caused a $[Ca^{2+}]_i$ accumulation greater than 50% of the resting calcium level.

Drugs and solutions. Bradykinin (10 nM) was applied to glia for 1 minute by pressure ejection from a puffer pipette (opening diameter 2-3 μm, 10 psi). All other drugs were uniformly applied to the bath by flow through. Repeated applications of bradykinin were 10 minutes apart. In some experiments the second bradykinin application followed the first application after 30 minutes. Ryanodine (10 μM) and caffeine (10 mM) were applied 10 minutes prior to the second application of bradykinin while thapsigargin was applied to the bath 30 minutes prior to the second application of bradykinin. Thapsigargin (1 μM) was dissolved in dry DMSO. Control experiments with 0.1% (v/v) DMSO showed that DMSO did not affect the action of bradykinin. Normal saline contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2 and HEPES 10 (pH 7.4). Calcium-depleted solution contained (in mM): NaCl 128, KCl 1.9, KH_2PO_4 1.2, $CaCl_2$ 0.2, EGTA 1, $MgSO_4$ 2.5, $NaHCO_3$ 26 and glucose 10 (pH=7.4).

6. We use Max Chelator (version 5.6), written by Chris Patton at Stanford University, to calculate free extracellular calcium levels. The calculation includes a correction for extracellular Mg^{2+} ions.

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9. Calcium-depleted solution was superfused over the culture for 30-45 minutes prior to collection of samples for EAAs analysis.

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11. Elevated K^+ (50 mM) solution for HPLC was prepared by modification of a Ringer's solution (see 4) where NaCl was replaced by KCl. Elevated K^+ (50 mM) for calcium imaging was applied to glia for 1 minute by pressure ejection and prepared by modification of a normal solution (see 5).

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PAPER 2. GLUTAMATE-MEDIATED GLIA-NEURON SIGNALING

"What is the use of this new invention?"
someone asked Benjamin Franklin.
"What is the use of a newborn child?"
was his reply.

INTRODUCTION

The regulation of the extracellular levels of glutamate is a complex interplay between release of this neurotransmitter from synaptic terminals and uptake mechanisms into presynaptic terminals and surrounding glial cells [1]. We recently demonstrated an additional process in this regulation in which bradykinin stimulates the calcium-dependent release of glutamate from glia [2]. To determine whether glutamate released from glia signals to adjacent neurons, we monitored bradykinin-induced calcium responses in neocortical neurons. Bradykinin significantly elevated calcium levels in neurons that contact glia, but not in solitary neurons. The general glutamate receptor antagonist, D-glutamylglycine, prevented the bradykinin-induced neuronal calcium elevation. Furthermore, direct photo-stimulation of a single glia caused an increase in calcium levels in unstimulated neurons which was blocked by D-glutamylglycine. Thus, glia can regulate neuronal calcium levels through the action of glutamate.

RESULTS AND DISCUSSION

To determine the effect of bradykinin on $[Ca^{2+}]_i$ in neurons, bradykinin (1 μM) was applied from a puffer pipette to dissociated rat neocortical cultures while monitoring $[Ca^{2+}]_i$ using the calcium indicator fura-2. When neurons were cultured in contact with glia, bradykinin caused a fast elevation in glial calcium levels that was followed by an elevation of neuronal calcium (Fig. 1a, 2a). Bradykinin caused a calcium accumulation in neurons of 101 ± 25 nM (n=25) (Fig. 2c). However, when cultured alone, neurons did not show a significant response to bradykinin (calcium accumulation of 13 ± 2 nM, n=26; Fig. 1b; Fig 2c). Bradykinin has been shown to induce the release of glutamate from dorsal root ganglia (DRG) glia [2] (Parpura et al., submitted) and neocortical glial cells in culture (Jeftinija et al., in preparation). To determine whether bradykinin acts directly on neurons to elevate neuronal calcium levels or acts indirectly through glutamate released from glia, we added a general glutamate receptor antagonist, D-glutamylglycine (DGG), while imaging neurons that contact glia. If glutamate being released from glia causes the neuronal response, then a general glutamate receptor antagonist will block the elevation in neuronal calcium. DGG (1 mM) significantly reduced the bradykinin-induced calcium accumulation in neurons to 15 ± 2 nM (n=31) as compared to 101 ± 25 nM in parallel cultures (Fig. 1c; Fig. 2b, c) without altering the glial ability to respond to bradykinin (see Figure legend 2). Subsequently, DGG was washed out of the bath and glutamate was applied to neurons to determine whether glutamate receptors were present. Addition of glutamate increased neuronal calcium levels in all conditions tested. Taken together, these data suggest that bradykinin elevates neuronal calcium by the action of glutamate, which is known to be released from glia in response to bradykinin [2].

As a further test of glutamate-mediated signaling from glia to neurons, we directly photo-stimulated glial cells to elevate glial calcium levels [3]. A portion of a single glial cell was exposed to focal UV light from a xenon-arc lamp. Calcium levels were monitored in glia and neurons using fluo-3 and confocal microscopy. Photo-stimulation reliably elevated glial calcium levels ($\Delta F/Fo = 202 \pm 19$ %, n=7)

Figure 1. Bradykinin induces an accumulation of calcium in neurons that contact glia

The calcium levels in neocortical neurons and glia were monitored using fura-2. (a) Puffer application of bradykinin (BK) caused an elevation in calcium levels of neurons (circled) and glia (G). However, when neurons were cultured alone (b), bradykinin did not significantly alter their calcium levels. In (c) three neurons are shown on top of glia in the presence of DGG (1mM). Bradykinin elevated the glial calcium levels, but did not change the level of calcium in adjacent neurons. Color scale indicates pseudocolor representation of $[Ca^{2+}]_i$ by fura-2 emission ratio ranging from 0 to 2.0. Scale bar, 10 μm.

METHODS. *Cell Culture.* Visual cortices were dissected from 1- to 4-day-old Sprague-Dawely rats. The dissociation protocol was modification of the Heuttner & Baughman procedure [15] as previously described [16]. Cells were plated into poly-L-Lysine (1mg/mL, MW 100,000; Sigma)-coated glass-bottomed dishes. To suppress proliferation of nonneuronal cells, arabinosylcytosine (Ara-C, 5 μM) was added after 3 days in culture. All experiments were performed on cells that have been in culture for 10-15 days. Using a monoclonal antibody directed against glial fibrillary acidic protein (GFAP, 1:500; ICN Immunobiologicals), we determined that glial cells in this culture were astrocytes (n=159 of 159 tested).

Calcium Imaging. Cells were loaded with fura-2 AM (2 μM; Molecular Probes) for 40 minutes at 37°C. 1 μL of 25% (w/w) Pluronic F-127 (Molecular Probes) in dry DMSO was mixed with 1 mL of 2 μM fura-2 AM ester solution. After washing, cells were de-esterified for 40 minutes at 37°C. All imaging took place at 22-24°C. Image processing and analysis were performed using ratiometric software (Image-1/Fluor, v 1.63g, Universal Imaging Corp., PA). Background subtracted, ratio images (350/380 nm) were used to calculate $[Ca^{2+}]_i$ according to Equation 5 of Grynkiewicz et al. [17]. Calibration was performed *in situ* [18] using 4-bromo-A23187 (20 μM, Molecular Probes). R_{max} was acquired in the presence of 4 mM Ca^{2+} whereas R_{min} was acquired in the absence of Ca^{2+} and with the addition of 10 mM EGTA to the bath. An estimate of autofluorescence was acquired after cell lysis with digitonin (40 μM) and addition of Mn^{2+} (20 mM).

Drugs and solutions. Imaging solution contained (in mM): NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2 and HEPES 10 (pH 7.35). Bradykinin (BK, 1 μM; Sigma) was applied to cells for 75 seconds by pressure ejection from a puffer pipette (~2 μm tip diameter, 10 psi). D-glutamylglycine (DGG, 1 mM; Bachem) was uniformly applied to the culture dish by saline exchange 10 minutes prior to application of bradykinin.

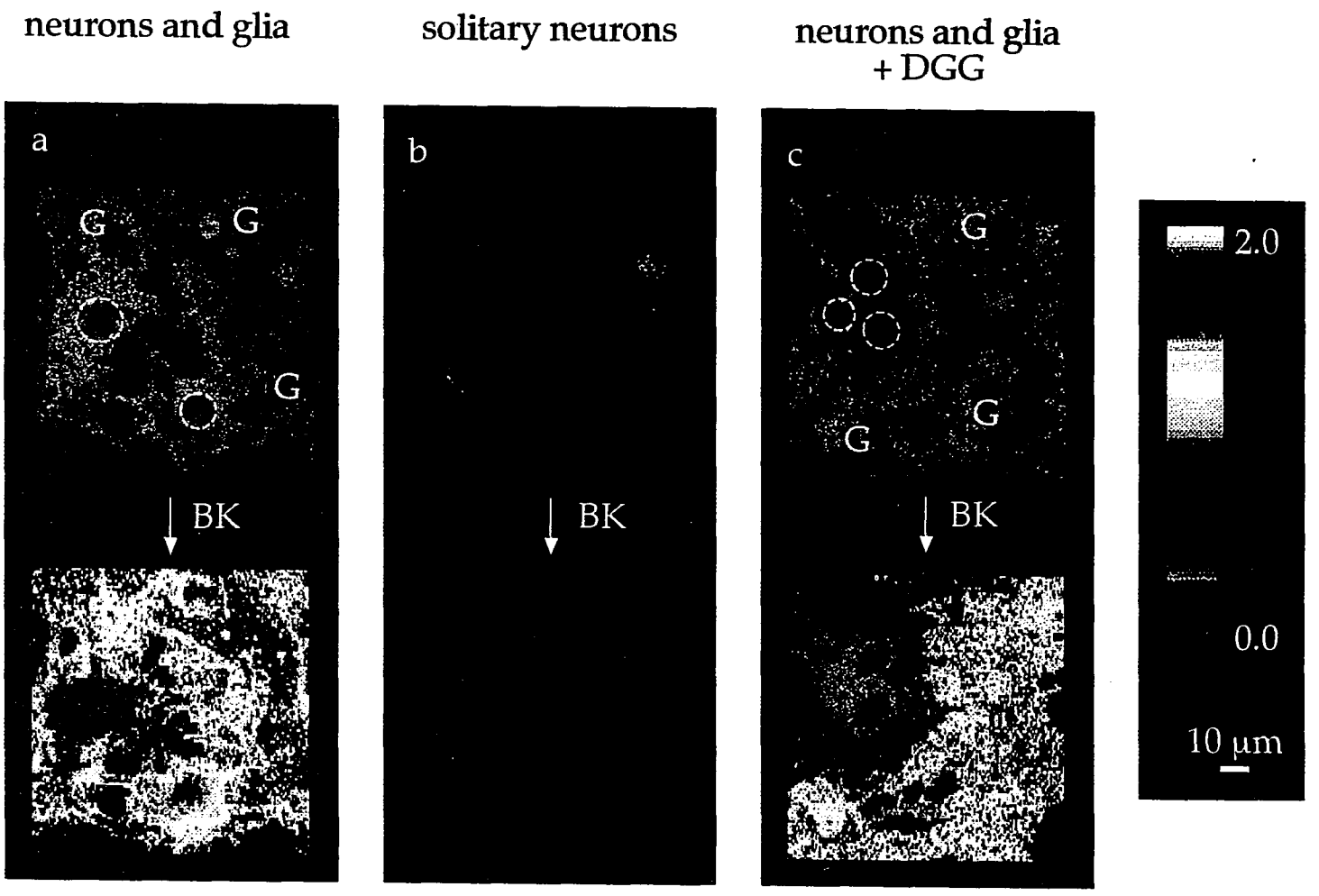
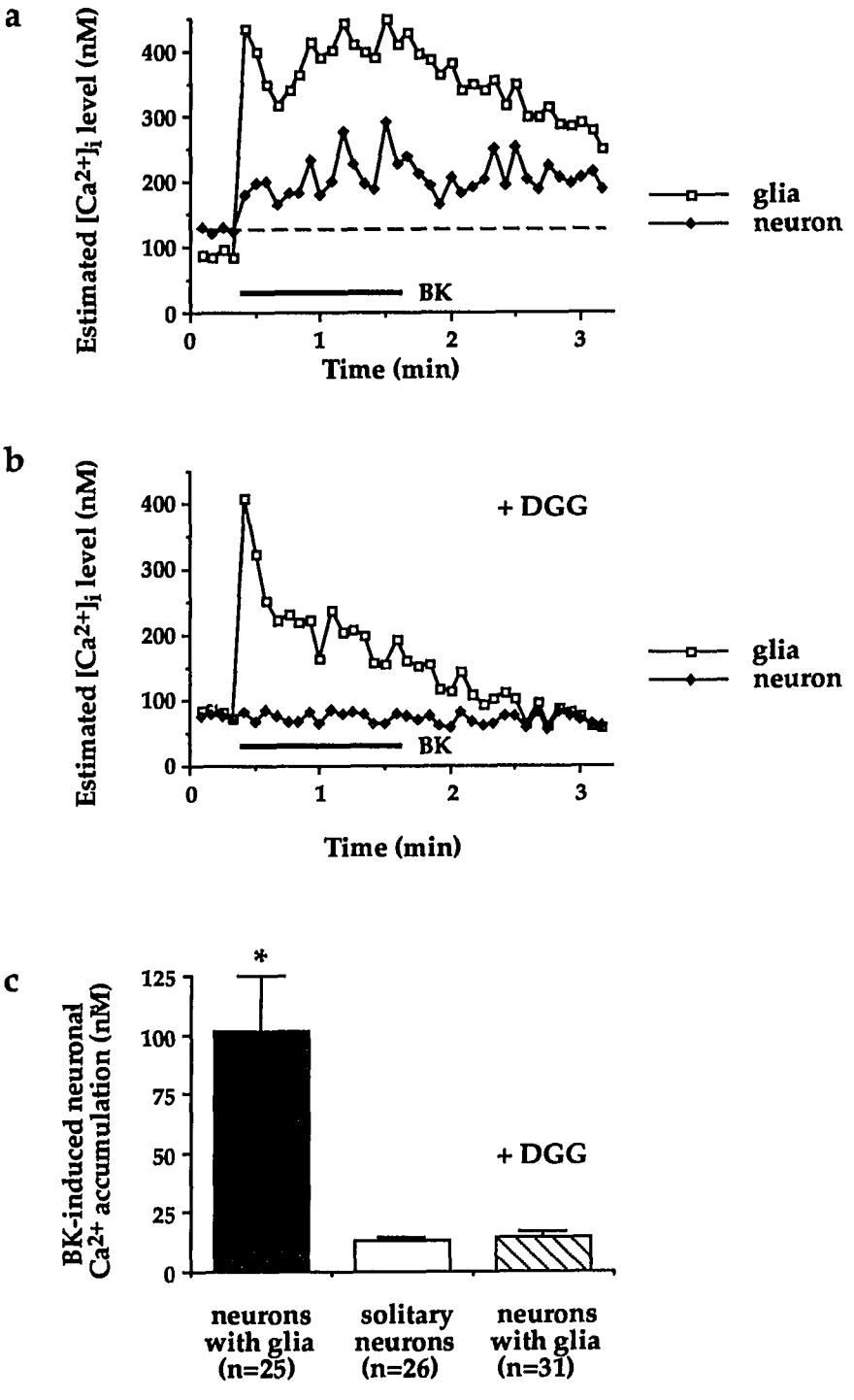

neurons and glia
solitary neurons
neurons and glia
+ DGG
a
b
c
G
G
G
G
G
BK
BK
BK
2.0
0.0
10 µm

Figure 2. The glutamate receptor antagonist, DGG, inhibits bradykinin-evoked calcium accumulation in neurons, but not in glia

In (a) the time course of glial and neuronal calcium responses after addition of bradykinin (BK) for a single pair of cells is shown. An increase in glial $[Ca^{2+}]_i$ is followed by an increase of neuronal $[Ca^{2+}]_i$. Broken line represents the resting $[Ca^{2+}]_i$ of the neuron. (b) In separate experiments performed on a single pair of cells, DGG was added to the external saline. Bradykinin did not change neuronal calcium level in the presence of DGG although glia still responded robustly. The glial change in calcium (peak value subtracted from resting) induced by bradykinin was 469 ± 86 nM and 424 ± 45 nM in DGG (n=20 and n=22, respectively; Student t-test, $p>0.6$). In (c) the mean bradykinin-evoked calcium accumulations (peak value subtracted from resting) in neurons are shown. Bradykinin elevated neuronal calcium when neurons contact glia and DGG blocked this response. After DGG and bradykinin washout, the ability of neurons to respond to glutamate was tested. Neurons from all three groups were similarly capable of responding to glutamate (100 μM; Scheffeé's *post hoc* comparison revealed no significant difference). Bars represent means $\pm$ SEM.
METHODS. *Drugs and solutions.* Glutamate (100 μM) was applied from a puffer pipette for 75 seconds 20 minutes after application of bradykinin and DGG. *Statistical analysis.* One-way ANOVA followed by Scheffeé's test was used. Significance was established at $p<0.01$ (*).

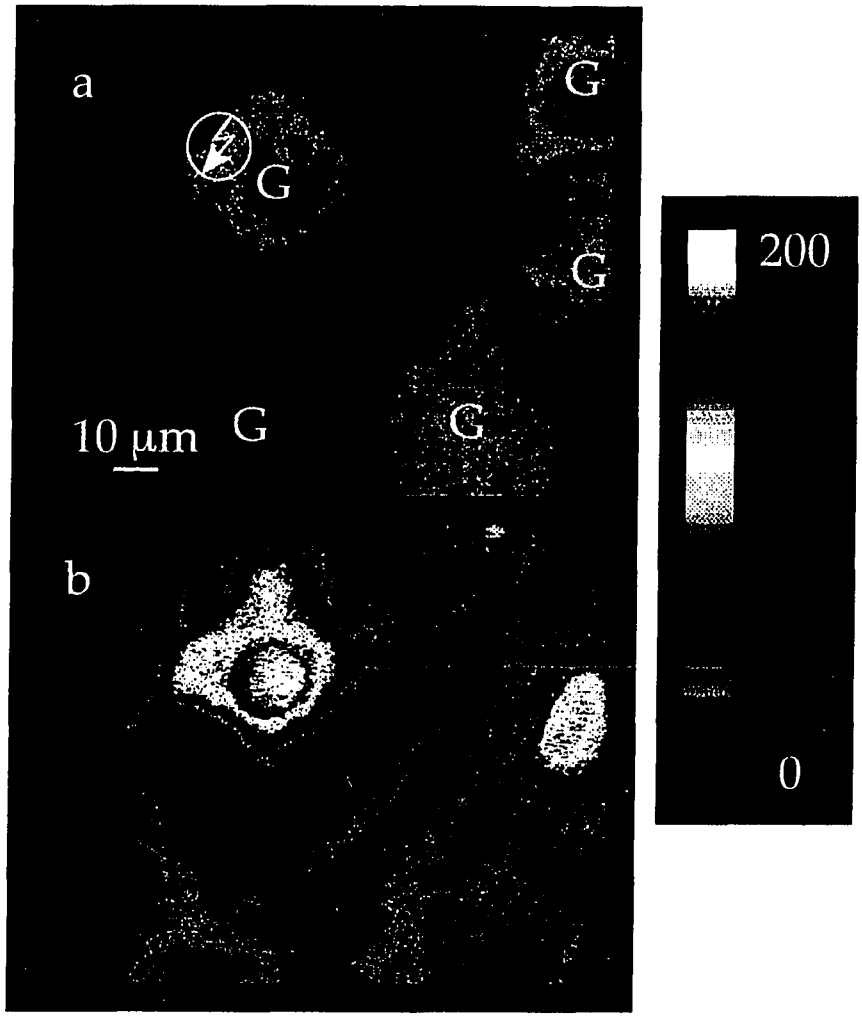

a
Estimated $[Ca^{2+}]_i$ level (nM)
400
300
200
100
0
BK
0
1
2
3
Time (min)
glia
neuron
b
Estimated $[Ca^{2+}]_i$ level (nM)
+ DGG
400
300
200
100
0
BK
0
1
2
3
Time (min)
glia
neuron
c
BK-induced neuronal Ca^{2+} accumulation (nM)
*
125
100
75
50
25
0
+ DGG
neurons with glia (n=25)
solitary neurons (n=26)
neurons with glia (n=31)

which then spread to adjacent glial cells (Fig. 3). Photo-stimulation elevated calcium for several minutes in all glial cells in the field of interest without affecting their morphology or causing a leakage of cytoplasmic components, as judged by the maintenance of the calcium indicator fluo-3. Elevated calcium is sufficient to stimulate the release of glutamate from DRG glia [2] (Parpura et al., submitted) and neocortical glial cells (Jeftinija et al., in preparation). Therefore, we used photo-stimulation to cause the Ca^{2+}-dependent release of glutamate from glia while monitoring neuronal calcium levels. When glial cells were photo-stimulated, the calcium levels of neurons in contact with glia increased (Fig. 4). Photo-stimulation of glia caused a maximal increase in neuronal $\Delta F/Fo$ by 81 ± 20 % (n= 15) which was not significantly altered by addition of tetrodotoxin (1 μM; $\Delta F/Fo = 57 \pm 6$ %, n=15; Scheffeé's test, $p>0.25$) to the medium. However, addition of general glutamate receptor antagonist, DGG, significantly reduced the glia-induced elevation of calcium in adjacent neurons ($\Delta F/Fo = 14 \pm 4$ %, n=15; Scheffeé's test, $p<0.01$; Fig. 4).

Using two distinct stimuli, bradykinin and direct photo-stimulation of glia, this study has demonstrated that the elevation in neuronal calcium level was correlated with glial elevations in calcium. Moreover, the glutamate receptor antagonist, DGG, selectively blocked the calcium elevation in neurons, but not in glia. Taken together, these data lead us to conclude that bradykinin acts on neurons through an intermediate transmitter, glutamate, to elevate neuronal calcium levels. Given the recent demonstrations that bradykinin causes the release of glutamate from glia [2], our data point to a novel form of transmitter signaling in the nervous system in which stimuli that mobilize calcium in glia cause a calcium-dependent release of the excitatory amino acid, glutamate, that can then act on adjacent neurons. Dani et al. [4] have demonstrated that glutamate released at synaptic terminals can trigger calcium waves in an astrocyte network. Since many neurotransmitters within the central nervous system (CNS) can mobilize glial calcium [5-12], it is possible that neurons stimulate neighboring glia to release glutamate which in turn signals back to the neuron. In this case glia could be considered as an integral computational element within the brain. In addition to mediating fast synaptic transmission, glutamate serves a role in synaptic plasticity [13] and, under conditions of excessive release, can cause neuronal damage and

Figure 3. Photo-stimulation of a single glial cell causes an increase in $[Ca^{2+}]_i$ in a glial (G) network

A glial cell in (a) was exposed to UV light (2 min) which caused a sustained increase in $[Ca^{2+}]_i$ in this cell and in unstimulated neighbors (b). A circled lightning bolt represents the location and diameter of UV exposure. Cells neither change their morphology nor leaked dye followed this treatment. Summary of data is shown in (c). Color scale indicates pseudocolor representation of fluorescence intensity ranging from 0 to 200 units. Points represent mean $\pm$ SEM (n=7 for stimulated and n=15 for control group). Scale bar, 10 μm.
METHODS. *Calcium imaging*. Cells were loaded with fluo-3AM (20 μg/mL) for 1 hour at 37°C. 1 μL of 25 % (w/w) Pluronic F-127 was added to 1 mL of 20 μg/mL fluo-3 AM ester solution. After several washes cells were maintained for 1 hour at 37°C to permit de-esterification. Imaging took place at room temperature (22-24°C). Images were acquired using a Noran Odyssey real-time confocal microscope (Noran, WI) equipped with Image-1/AT software (v. 4.0, Universal Imaging Corp., PA). After background subtraction fluo-3 signals were normalized against base-line fluorescence (Fo). The resulting $\Delta F/Fo$ is a sensitive indicator of Ca^{2+} deviations from base-line levels [9].


a
G
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G
G
10 μm
G
b
200
0

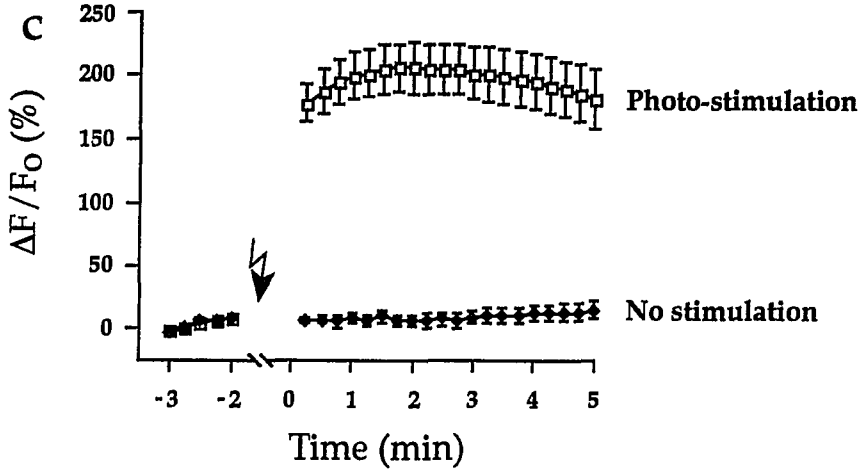
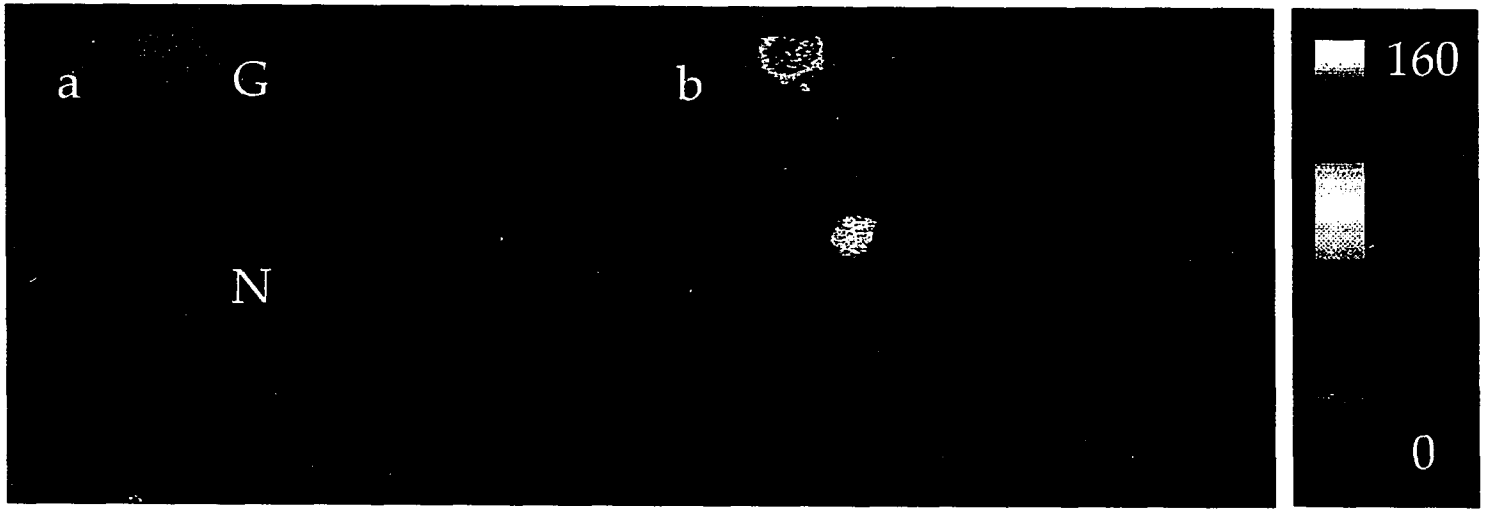
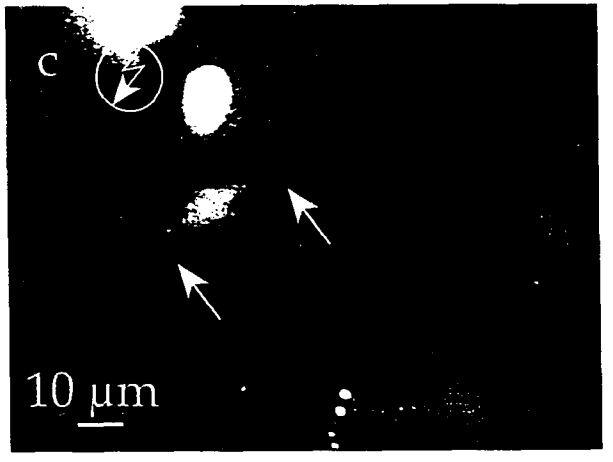

c
250
200
150
100
50
0
ΔF/Fo (%)
Photo-stimulation
No stimulation
-3
-2
0
1
2
3
4
5
Time (min)

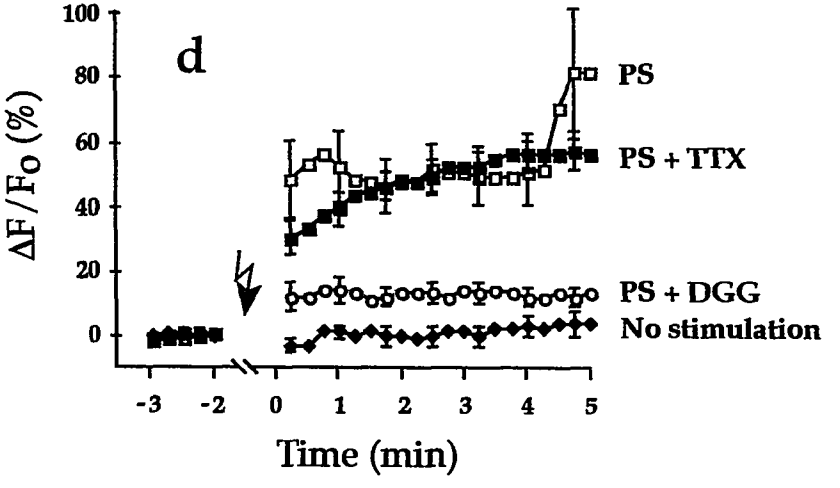
Figure 4. Photo-stimulation of glial cells causes an increase of $[Ca^{2+}]_i$ in adjacent unstimulated neurons due to the action of glutamate

Images represent calcium levels in the confocal plane of the neuronal body before (a) and after (b) UV stimulation of a single glial cell. After stimulation, $[Ca^{2+}]_i$ increased both in the glial cell (G) and in the neuron (N). The increase in neuronal calcium is not due to a direct action of UV exposure because neuronal processes (arrows, c) were not in the path of UV excitation (circled lightning bolt, c). The image in (c) was acquired at the end of the experiment at the level of glial bodies and neuronal processes. The time course of $[Ca^{2+}]_i$ changes in neurons due to photo-stimulation (PS) of glial cells is shown in (d). The presence of DGG (1 mM), but not tetrodotoxin (TTX, 1 μM), greatly reduced the neuronal response. Color scale for (a) and (b) indicates pseudocolor representation of fluorescence intensity ranging from 0 to 160 units. Points represent means ± SEM (n=15 for each group). Scale bar, 10 μm.

METHODS. TTX (1 μM, Sigma) and DGG (1 mM) were uniformly applied to the bath by saline exchange 10-20 minutes prior to UV stimulation.


a
G
N
b
160
0


c
10 µm


d
ΔF/Fo (%)
100
80
60
40
20
0
-3
-2
0
1
2
3
4
5
Time (min)
PS
PS + TTX
PS + DGG
No stimulation

degeneration [14]. This raises the possibility that glia-mediated glutamate release plays an important role in regulating neuronal physiology and pathology.

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GENERAL SUMMARY

This dissertation deals with release of neurotransmitter from glia. It has been demonstrated that bradykinin causes a receptor-mediated release of excitatory amino acids (EAAs), glutamate and aspartate, from glial cultures obtained from dorsal root ganglia (DRG) together with an increase in the cytoplasmic level of glial free calcium. Perturbations which inhibited bradykinin-induced calcium mobilization prevented the release of EAAs from glia. The addition of ionomycin caused a calcium-dependent release of EAAs. Taken together, these data demonstrate that calcium is both necessary and sufficient for stimulating the release of EAAs from DRG glia.

Bradykinin was applied to mixed neuron-glial cultures derived from rat cerebral cortex while monitoring calcium levels. Bradykinin elevated calcium levels in neurons only when neurons contacted glia. The general glutamate receptor antagonist, D-glutamylglycine (DGG), prevented bradykinin-induced neuronal calcium elevation. These data indicate that bradykinin elevates neuronal calcium levels through the action of glutamate that is released from glia. While addition of bradykinin to mixed neuron-glial culture is the simplest experimental method of testing the hypothesis that glia can signal to neurons, a second technique allowed a more direct test of this hypothesis. Direct photo-stimulation of glia was used to increase glial calcium levels. A portion of glia was exposed to focal application of UV light while monitoring the calcium response. Photo-stimulation reliably raised the level of calcium in the glial cell. Since elevated calcium is sufficient to stimulate the release of glutamate from glia, this perturbation induced the calcium-dependent release of glutamate. By monitoring calcium levels from adjacent neurons, it was possible to determine that photo-stimulation of glia caused an elevation in calcium levels of adjacent unstimulated neurons. This effect was greatly reduced by DGG. Thus, glia can regulate neuronal calcium levels through glutamate-mediated actions.

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ACKNOWLEDGMENTS

Here we go. I would like to thank the Big Kahuna, Phil, for all of his support. Also, I would like to thank the Kahuna's tribe and you know who you are. Anyway (alphabetically), Bob, Fred, Helen, Janet, Kerry, Lynn, Lisa, Mark, Sally and Trent, thank you all. Special thanks go to Trent for coping with my mood swings. However, they were nothing in comparison to what I had to go through. Those of you who know Trent, you know what I mean (right Kerry). Fred and Kerry, I really enjoyed tee-times spent in your company. We should get some "we suck big time" shirts printed for next season.

I would like to thank the members of my committee: Eric, a departmental misfit and a probe manic, for introducing me to scanning probe microscopes and Californian vocabulary, Srdija, for Yugo gossip, Sheldon, for calcium-related chats including those about cute girls and Al, for showing up.

Finally, I thank my sister Anja for all the good food that kept me alive and my parents for their intellectual and emotional support through all these years.