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Neuroligand-evoked release of excitatory neurotransmitters from cultured glial cells

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Liu, Fang, Ph.D.

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Neuroligand-evoked release of excitatory neurotransmitters from cultured glial cells

by

Fang Liu

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

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DOCTOR OF PHILOSOPHY

Interdepartmental Program: Neuroscience
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For the Graduate College

Iowa State University
Ames, Iowa

1994

DEDICATION

To my husband and my parents.

Without their support this would not be possible.

Specially to my lovely son, David.

You make it all worthwhile.

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GENERAL INTRODUCTION

The human brain, which consists of about 10^{11} nerve cells and 10^{12} glial cells, continually receives information, elaborates and perceives it, and makes decisions. To carry out this task, the nervous system possesses an immense number of lines of communication provided by the nerve cells (neurons). Each neuron makes specific contact with many target cells such as different types of neurons, gland cells, or muscle cells.

In order for neurons to communicate with each other, they need to have points of "contact" with each other. These are called synapses, named by the English physiologist Charles Sherrington in 1897. The term synapse is derived from the Greek, meaning to clasp, connect or join. Sherrington thought of it, anatomically as a site of "surfaces of separation", but he always emphasized that it was first and foremost a *functional* connection. In 1911, Ramón y Cajal found that there were three elements for a synapse: a presynaptic terminal, a postsynaptic target site and the synaptic cleft, the space between the pre- and post-synaptic plasma membranes. These three elements underline the ability of neurons to communicate with one another, the process of synaptic transmission.

Literature Review

Synaptic Vesicle and Neurotransmitter Release

It has been widely accepted that there are two major modes of synaptic transmission: electrical, which depends on current through gap junctions that

bridge the cytoplasm of pre- and post-synaptic cells (Furshpan and Potter, 1957; reviewed by Bennett et al., 1991); and chemical, in which pre- and post-synaptic cells have no direct continuity (Katz, 1966; Changeux, 1981; Karlin, 1990). Synaptic transmission across the majority of synapses in the brain is mediated by the interaction of chemical signals released from the presynaptic terminal with postsynaptic receptors (reviewed by Jessell and Kandel, 1993).

In the resting state, neurotransmitters such as glutamate, γ-aminobutyric acid (GABA) and acetylcholine (ACh) are stored in uniformly sized small organelles of 40-50 nm diameter. When an action potential reaches the nerve terminal, the presynaptic plasma membrane depolarizes and voltage-gated calcium channels open at the active zone. The ensuing rise in intracellular calcium triggers exocytosis of synaptic vesicles, resulting in the release of the neurotransmitter (Katz, 1966). The synaptic vesicle membranes are reclaimed from the plasma membrane by endocytosis, and the vesicles eventually refill with neurotransmitter (Südhof and Reinhard, 1991). To summarize this process, Kelly (1993) indicates that transmitter release can be considered to involve at least four steps: the transport of vesicles from the reserve pool to the releasable pool at the active zones, the docking of vesicles to their release sites at the active zones, the fusion of the synaptic vesicle membrane with the plasma membrane during exocytosis in response to an increase in intracellular Ca^{2+}, and the retrieval and the recycling of vesicle membrane following exocytosis.

Synaptic vesicles have two fundamentally different functions: they take up and store neurotransmitters, and they fuse with and bud from other membranes, most notably the presynaptic plasma membrane. These functions must be performed by the proteins of synaptic vesicles, either alone or in concert with

other components of the nerve terminal. A major advance in the analysis of transmitter release mechanisms has been the cloning and characterization of several proteins that may participate in different aspects of the release cycle. As might be expected from the steps involved in release, these proteins fall into four groups: proteins such as the synapsins that are thought to control the mobilization of vesicles and thereby to regulate their availability for release (De Camilli et al., 1990); proteins such as Rab_3, a member of the p21ras superfamily, thought to be involved in the intracellular trafficking and docking of synaptic vesicles (Zahraoui et al., 1989); proteins such as synaptotagmin, synaptobrevin, neurexins, and syntaxins, which appear to be involved more specifically in docking vesicles into release sites on the presynaptic membrane (Bennett et al., 1991; Trimble et al., 1991; Schiavo et al., 1992); and proteins such as synaptophysin, thought to be involved in the formation of the vesicle fusion pore or in recycling (Almers and Tse, 1990; Südhof and Jahn, 1991; White, 1992).

The Central Role of Ca^{2+} in Neurotransmitter Release

As mentioned above, the elevation of the intracellular calcium level following calcium influx through voltage-gated calcium channels is a critical step in action potential evoked synaptic transmission (Augustine et al., 1987). However, in a number of experimental conditions neurotransmitter can also be released in the absence of extracellular calcium (Adam-Vizi et al., 1984; Hochner et al., 1989). Neurotransmitter release in the absence of calcium may be derived from a different intracellular pool than that in the presence of calcium (Adam-Vizi et al, 1990). The concentration of calcium in the nerve terminal is controlled by a complex system of soluble buffers, organellar stores and a membrane

transport system with varying calcium transport affinities and capacities; distortion will result in a modulation of transmitter release (Adam-Vizi and Ashley, 1987). Calcium is sequestered inside the nerve terminal in the smooth endoplasmic reticulum, synaptic vesicles, mitochondria, and possibly also calcisomes. Furthermore, there exist intracellular buffering systems like calcium-binding proteins (Blaustein, 1988; Meldolesi, 1988).

Inositol polyphosphates have been assigned a key role in calcium intracellular homeostasis. Inositol 1,4,5-trisphosphate (IP_3) is formed by receptor-mediated and G-protein-controlled turnover of phosphatidylinositol 4,5-bisphosphate together with diacylglycerol (DAG), an activator of protein kinase C (PKC). The inositol trisphosphate functions via specific receptors in the release of calcium from intracellular stores, presumably the endoplasmic reticulum and the calcisome (Nahorski, 1988). In addition to the IP_3-sensitive pool, there is an IP_3-insensitive calcium store, which is located in the peripheral region of the cell away from the nucleus (Hardie, 1991). Calcium in the IP_3-insensitive pool can be released by the drug caffeine, which also activates the Ca^{2+}-release channels of sarcoplasmic reticulum (ryanodine receptors). It is believed that, in axon terminals, receptor-mediated turnover of synaptic phosphoinositides for mobilizing calcium from internal stores may serve mainly as a modulatory mechanism.

Mechanisms of Glutamate Release

It is well established that glutamate and aspartate mediate the excitatory transactions taking place between nerve cells in the vertebrate central nervous system (CNS). Glutamate is probably the main excitatory amino acid (EAA)

neurotransmitter in the mammalian CNS. It works along with other acidic amino acids, particularly aspartate, homocysteate and cysteine sulfinate (Mayer and Westbook, 1987). Since the first proposal of the vesicular hypothesis more than 30 years ago (Del Castillo and Katz, 1957), the release of numerous neurotransmitters has been assumed to be due to the exocytosis of synaptic vesicles in response to an increase in the cytoplasmic calcium concentration in nerve terminals. Many arguments favor such a release mechanism for glutamate. These include the calcium dependency of glutamate release (Nicholls and Sihra, 1986), the presence of glutamate-like compounds in synaptic vesicles (Stom-Mathisen et al., 1983), and the massive and rapid accumulation of glutamate in highly purified synaptic vesicles (Naito and Ueda, 1983, 1985).

However, glutamate is transported by a reversible, sodium dependent (Kanner and Sharon, 1978; Kanner and Marva, 1982; Erecinska et al., 1983, 1987; Wilson and Pastuszko, 1986), and electrogenic (Brew and Attwell, 1987; Szatkowski et al., 1990) uptake mechanism, so it remains possible that the reversal of the EAA uptake system could account, at least in part, for the release of EAAs. Such a mechanism may explain the calcium independent EAA release reported, for example, in the retina (Miller and Schwartz, 1983).

There is the third situation for the accumulation of extracellular glutamate. Swelling of isolated cells and many vertebrate and invertebrate tissues leads to release of glutamate, aspartate, taurine, and other amino acids (Kimelberg and Ransom, 1986). This release is part of the process of regulatory volume decrease by which swollen cells regain their normal volume (Gilles et al., 1987). Swelling-induced release of glutamate is inhibited by a number of anion transport blockers

such as furosemide and L-644, 711, a fluorenyl derivative of ethacrynic acid (Kimelberg et al., 1987).

Types of Glutamate Receptors

Glutamate interacts with at least five classes of membrane receptors, each with significantly distinct functions (reviewed by Monaghan, 1989). Three have been defined by the depolarizing actions of selective agonists (N-methyl-D-Aspartate, NMDA; kainate; quisqualate or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid, AMPA) and their blockade by selective antagonists (Watkins and Olverman, 1987). A fourth, the AP4 receptor (L-2-amino-4-phosphonobutyrate) appears to represent an inhibitory autoreceptor (Koerner and Cotman, 1981; Davies and Watkins, 1982; Evans et al., 1982). The fifth receptor, activated by trans-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), modifies inositol phosphate metabolism (Baudry et al., 1986; Nicoletti et al., 1986; Sladeczek et al., 1985).

The NMDA receptor is best defined. This receptor opens a distinctive membrane channel characterized by high conductance (main state about 50 pS), voltage-dependent Mg^{2+} blockade and permeability to both Ca^{2+} and Na^{+} (Nicoll et al., 1988; Ascher and Nowak, 1988; MacDermott and Dale, 1987; Mayer and Westbrook, 1987). Voltage-clamp studies of cultured neurons injected with a calcium-sensitive dye indicate that Ca^{2+} can enter cells after NMDA application in a manner independent of the voltage-dependent calcium channels (MacDermott et al., 1987; Mayer et al., 1987). The increase in intracellular Ca^{2+} ions is thought to initiate the biochemical processes responsible for both NMDA receptor-induced plasticity observed in developing and adult animals (Lynch et

al., 1983; Collingridge et al., 1987; Cotman et al., 1988) and NMDA receptor-mediated excitotoxic cell death (Rothman and Olney, 1986; Cotman et al., 1988).

Although NMDA receptors play a critical role in synaptic function, they do not mediate the excitatory postsynaptic potential resulting from a unitary synaptic activation. Pharmacological studies indicate that kainate and/or quisqualate receptors are responsible for the voltage-independent portion of the synaptic response in many neuronal pathways (reviewed by Mayer and Westbrook, 1987; Watkins and Evans, 1981). The kainate/quisqualate antagonist, 6-cyano-7-nitroquinoxaline-2,3-done (CNQX), blocks synaptic transmission at low concentration (Honoré et al., 1988; Blake et al., 1988).

Glutamate-induced excitatory transmission appears to involve actions mediated by one or more combination of glutamate receptors.

Neuroglia

Glial cells are intimately associated with most neurons. The neuroglial cells used to be called arachnoid cells because of their resemblance to spiders. They were also described as Deiter's cells in honor of the researcher who discovered them. The main classes of glial cells in the vertebrate central nervous system are astrocytes, oligodendrocytes and microglia, whereas Schwann cells are the predominant glial type in the peripheral nervous system.

Neurobiologists have wondered for 150 years what glial cells do. There were several hypotheses about the functions of neuroglial cells. One of them was Golgi's nutritive theory. This theory was based on the fact that the dendrites of neurons are in contact with the capillaries or with neuroglial cells. The capillaries, neuroglial cells and dendrites thus form a solid functional unit the

role of which is to carry to the cell body of neurons the nutritive elements that are necessary for it. The second hypothesis was Weigert's "filling-out" theory that the role of neuroglia is simply passive, to fill out the empty spaces left by the neuronal elements. The third one was the isolation theory favored by Cajal. He proposed that neuroglial cells form a resistance field of passage for nerve conduction.

Although there are still different hypotheses about the physiological roles of neuroglial cells, it has been widely accepted that neuroglia serve as supporting and protective material to the neurons and to the capillary blood vessels. However, recent findings suggest that glial cells may be more actively involved in brain function than had been previously thought. New studies, made possible with patch-clamp recording, have shown that glial cells in vitro and in situ possess most of the same kinds of voltage-dependent ion channels that are found in neurons (reviewed in Barres et al., 1990; Bevan, 1990). Glial cells in culture respond to a variety of neurotransmitters with changes in membrane potential (reviewed by Bevan, 1990). Electrophysiological studies show that cultured glial cells are indeed depolarized by glutamate (Kettenman et al., 1984a; Browman and Kimelberg, 1984; Kettenmanne et al., 1984b).

The glial membrane is sensitive not only to glutamate but also to some of the glutamate analogs used to define the subtypes of neuronal glutamate receptors. Cultured glia are depolarized by kainate which acts on kainate receptors, and they are also depolarized by quisqualate and AMPA, which are selective agonists of neuronal quisqualate-AMPA receptors (Teichberg, 1991). As a general rule, NMDA and other agonists acting at neuronal NMDA receptors do not depolarize the glial membrane (Backus et al., 1989; Usowicz et al., 1989; Barres et al., 1990b).

The presence of glutamate receptors on glia suggests that signaling via glutamate between neurons and glia might occur. In support of this theory, squid giant axons have been found to signal their Schwann cells (Lieberman et al., 1989; Lieberman, 1991). The other example of neuron to glial signaling mediated by neurotransmitters has been studied in the mammalian brain slice preparation (Dani et al., 1990). Calcium waves in astrocytes can be elicited by the application of NMDA in an organotypic hippocampal slice preparation. Since the astrocytes are believed to express only non-NMDA receptors, the calcium waves are most likely induced by an NMDA-evoked glutamate release from neurons.

Glia-neuron Signaling

Can glial cells signal neurons? Glial cells synthesize and, in some cases, release neurotransmitters. Schwann cells of the squid giant axon normally synthesize and release ACh (Heumann et al., 1981). The neurotransmitter GABA can be detected, using HPLC (high-pressure liquid chromatography), in O2A glial cells cultured in medium lacking any source of GABA (Barres et al, 1990b). Astrocytes have been shown to directly modulate the free cytosolic calcium and signal their neighboring neurons through intercellular connections (Nedergaard, 1994).

Electrogenic glutamate uptake is a commonly occurring system on glia and a major current carrier. Its likely functions are to terminate the transmitter action of glutamate and to prevent it from exerting its neurotoxic properties. It may also play a role in synaptic plasticity (Barbour et al., 1989). Although it has been suggested that glutamate release from glia is by "reversed uptake" in special

experimental conditions (Schwartz, 1987)., there is not yet convincing evidence to show the mechanism of glutamate release from glia.

Neuroligands

The research described in this thesis focused on the effects of neuroligand capsaicin on cultured peptidergic sensory neurons and the mechanism of neuroligand bradykinin- and adenosine 5'-triphosphate-induced release of glutamate from glial cultures.

CAPSAICIN Capsaicin (8-methyl-N-vanillyl-6-nonemamide), the pungent ingredient found in peppers of the capsicum family, has a variety of effects on the C-type sensory neurons responsible for the transmission of nociceptive information (Fitzgerald, 1983). The "hot" sensation caused by peppers is due to excitation of afferent nerve endings in the oral cavity by capsaicin. Similar excitatory actions occur in the skin, in the airways and in many visceral organs. This widespread irritancy is due to highly selective excitation of a sub-class of somatovisceral afferents with unmyelinated axons. Capsaicin selectively depolarizes dorsal root ganglion (DRG) neurons and excites nociceptive primary afferents (Heyman and Rang, 1985). The depolarization produced by capsaicin is due to a non-specific permeability increase for sodium and calcium (Marsh et al.; 1987, Wood et al., 1988). The C-type sensory neurons activated by capsaicin contain substance P (SP) and calcitonin gene-related peptide (CGRP) and participate in pain perception, thermoregulation and in neurogenic inflammation (Holzer, 1988). In neonatal rats and mice, the systemic injection of capsaicin leads to large reductions in numbers of C-fibers and DRG neuronal cell bodies (Buck and Burks, 1986; Holzer et al., 1988). Levels of all peptides found in small afferent

neurons supplying somatic and visceral tissues are reduced by capsaicin treatment (Jessell et al., 1978). Although many experiments have been done with capsaicin, it is unclear how capsaicin can stimulate the transmission of nociceptive information. In the first paper of this thesis, we determined the effects of capsaicin on peptidergic neurons in cultures.

BRADYKININ (BK) The neuroligand bradykinin is a potent inflammatory nonapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) whose generation in tissues and body fluids elicits numerous responses including vasodilation, edema, smooth muscle spasm, as well as pain and hyperalgesia via stimulation of C- and Aδ-fibers. There is substantial evidence that BK contributes to the inflammatory response in acute and chronic diseases including allergic reaction, arthritis, asthma, sepsis, viral rhinitis, and inflammatory bowel diseases. There are at least two types of BK receptor, B_1 and B_2. The B_1 receptor is not expressed to any significant extent in normal tissues. It may be of greater significance in pathophysiological conditions. Most action of BK is mediated through the B_2 receptor (Dray, 1993). BK produces an immediate depolarization of sensory neurons and nociceptive fibers (Dray and Perkins, 1993). It also stimulates membrane phospholipase C (PLC) to generate IP_3 and DAG which elevate intracellular calcium and activate intracellular PKC, respectively (Burgess et al., 1989; McGuirk and Dolphin, 1992).

The objectives of the second paper in this thesis were to determine whether bradykinin can induce release of EAAs from cultured Schwann cells and the mechanism of bradykinin-mediated EAA release from Schwann cells. Furthermore, in the fourth paper, we demonstrated that the release of the

excitatory amino acid glutamate from astrocytes plays an important role in astrocyte-neuron signalling.

ADENOSINE 5'-TRIPHOSPHATE (ATP) The significance of intracellular ATP has been recognized for a long time, but it is only recently that an extracellular role for this nucleotide has been accepted. It has been demonstrated that micromolar concentrations of ATP affect several biological processes including neurotransmission (peripheral and central), cardiac function, muscle contraction and relaxation, platelet aggregation, vascular tone, secretion of hormones and other factors, immune responses and cell growth (reviewed by Gordon, 1986; Burnstock, 1990). Of particular interest is the finding that ATP can be released from neurons and may be an important chemical mediator of synaptic transmission in the central nervous system (White, 1977; Richardson and Brown, 1987; Wieraszko et al.,1989). Although calcium as a second messenger plays an important role in a number of signal transduction-regulated functions in neurons (Miller, 1987), relatively little is known about signaling in glia and the processes that are influenced by increases in intracellular calcium in these cells. The purpose of the third study in this thesis was to determine whether ATP induces the release of EAAs from cultured glia and whether calciam is involved in the ATP-induced EAA release.

Significance of Research

As we know, the release of excitatory amino acids from nerve terminals plays an important role in synaptic transmission. Increasing extracellular glutamate and aspartate are likely to modulate neuronal properties such as learning and memory, developmental plasticity and epileptogenesis. Excessive glutamate

release from glia may also lead to neuron death or neurodegeneratory disorders. Do neuroglial cells play roles in these physiological or pathological states? The specific effects of excitatory amino acids released from glial cells need to be elucidated.

Studying the mechanism of neuroligand-induced release of EAAs from glial cultures will help us to understand the signal transduction pathways of the neurotransmitter release process in glia. By using this information and combining it with other research results, we may be able to prevent or attenuate some brain diseases in the future.

Dissertation Organization

This dissertation is composed of four papers. The papers are preceded by a general introduction and followed by a general conclusion which include a summary and discussion of the entire body of work. References cited in the general introduction and the general conclusion follow the general conclusion. Most of the experiments were performed in the Department of Veterinary Anatomy under the guidance of Dr. Srdija Jeftinija.

The experiments involving calcium imaging (chapter 2 and chapter 4) were performed by Vladimir Parpura in the laboratory of Dr. Philip Haydon.

CHAPTER 1. EFFECT OF CAPSAICIN AND RESINIFERATOXIN ON PEPTIDERGIC NEURONS IN CULTURED DORSAL ROOT GANGLION

A paper published in Regulatory Peptides

Srdija Jeftinija, Fang Liu, Ksenija Jeftinija and Laszlo Urban

Summary

The neurotoxic effect of capsaicin has been shown to be selective on a subpopulation of small dorsal root ganglion neurons in newborn animals. The aim of this study was to provide evidence of the long lasting effect of capsaicin and its ultrapotent analog resiniferatoxin (RTX) on sensory peptidergic neurons maintained in organotypic cultures. The effects of the two irritants were examined on neurons that contained substance P (SP) and calcitonin gene-related peptide (CGRP). Exposure of the cultures to 10μM capsaicin and 100nM RTX for periods of 2 days or longer resulted in almost complete elimination of SP-immunoreactive (IR) neurites and reduction, but not elimination, of CGRP-IR neurites. In addition, both 10μM capsaicin and 100nM RTX significantly reduced the number of SP- and CGRP-IR cell bodies within DRG explants. Capsaicin in 100μM concentration produced complete elimination of SP-IR fibers and a greater decrease in the number of CGRP-IR fibers, but failed to completely eliminate IR cell bodies. Exposure of the cultures to the irritants in the same concentrations for 90 min did not produce a measurable effect on SP- or CGRP-IR in neurites or cell bodies. It is important to establish that the effect of

capsaicin and RTX on cultured neurons was of long duration (longer than 4 days) and is therefore different from depletion of peptides.

These findings demonstrate that processes of cultured sensory neurons are much more sensitive to capsaicin and RTX than cell bodies. Furthermore, our results show that SP-IR neuronal elements are more sensitive to capsaicin than CGRP-IR elements. These data suggest that cultured sensory neurons express the functional properties of differentiated sensory neurons in vivo.

Key words: Primary sensory neuron, Neurotoxicity, Organotypic culture, Substance P, Calcitonin gene-related peptide

Introduction

Capsaicin (8-methyl-N-vanillyl-6-nonemamide), the pungent substance found in plants of the capsicum family, has a variety of effects on the small unmyelinated and myelinated sensory neurons responsible for the transmission of nociceptive information [2,8]. Capsaicin selectively depolarizes dorsal root ganglion (DRG) neurons [18], excites nociceptive primary afferents [9], and inhibits voltage activated Ca^{2+} current by evoking intracellular Ca ion accumulation [38]. The C-type sensory neurons activated by capsaicin contain substance P (SP) and calcitonin gene-related peptide (CGRP), and participate in nociception, thermoregulation and in neurogenic inflammation [3, 19]. Capsaicin induces release of CGRP and SP from both peripheral [10, 11, 20, 37] and central terminals [13, 14, 26, 27]. Capsaicin also evokes release of excitatory amino acids (glutamate and aspartate) from cultured DRG neurons [25].

Depletion of neuropeptides occurs after administration of higher doses of capsaicin to intact animals as well as after exposure of isolated tissues to higher concentrations of capsaicin [30, 36]. Depletion of releasable peptide pool or accumulation of toxic levels of intracellular calcium ions might be involved in the sensory neuron blocking and neurotoxicity of capsaicin [16,31]. Neurotoxic effect of capsaicin is restricted to a subpopulation of DRG neurons and is age related; when administered to neonatal rats, capsaicin causes a large reduction in the number of C-fibers and "small dark" neurons [23, 24, 35].

Resiniferatoxin (RTX) is a natural diterpen structurally related to the phorbol esters that is neither tumor promoting nor does it compete for binding to protein kinase at concentrations required for the capsaicin-like effects [7,39]. It has been shown that RTX mimics the effects of capsaicin in stimulating and disensitizing certain peptidergic unmyelinated primary afferents [6, 32]. A common capsaicin-RTX-binding site discovered by Szallasi and Blumberg [33, 34] might correspond to the capsaicin "receptor" whose presence was predicted [22, 32].

The aim of this study was to provide evidence on the effect of capsaicin and its ultrapotent analog RTX on peptidergic DRG neurons harvested from two day old animals and maintained in culture. By utilizing cultures we were able to study the effect of irritant when acting for long time, conditions similar to <u>in vivo</u> injection. In order to evaluate the irreversible effect of capsaicin and RTX we chose to monitor changes in SP and CGRP immunoreactivity of DRG somata and neurite-outgrowth of DRG neurons.

Materials and Methods

Preparation of cultures

Organotypic DRG cultures were prepared according to a modification of methods described by Gahwiler [12] and Delfs et al. [5]. Two day old Sprague-Dawley rats were decapitated and following laminectomy DRGs were aseptically dissected out and transferred into chill Gay's balanced salt solution (BSS). Culturing was carried out on glass coverslips which were specially prepared by soaking 24 hours in xylene, 4 hours in acetone, 24 hours in absolute alcohol, and dried for 30 min at 80°C. Explants were placed on glass coverslips which were coated with chicken plasma. One drop of thrombin solution was added to keep explants in place. Glass coverslips with explants were placed into Petri dishes where the culturing medium (25% horse serum, 25% Earls BSS, 50% Basal Medium Eagles with glucose 6,4 mg/ml) was changed every second day. Cultures were kept in an incubator at 36 °C in 95% O_2 and 5% CO_2.

Four exposure schedules were designed in order to study the time course of capsaicin or RTX's neurotoxic effect: 1) Capsaicin (10, and 100 μM) or RTX (10 and 100 nM) were present in the growing media for the whole culturing period. 2) Capsaicin or RTX in the concentrations listed above were added to the culture on day 1 and were removed 48 hours later. The cultures were maintained for next 4-5 days in normal media. 3) Capsaicin or RTX (in above concentrations) were added to established 4 day old cultures and were present in the media for 2 days. 4) Capsaicin or RTX (in listed concentrations) were added to newly started cultures or to 4 day old established cultures for 90 min and then cultures were kept in normal medial for the rest of culturing.

Stock solutions of capsaicin (8-methyl-N-vanillyl-6-noneaneamide; Sigma) and RTX (resiniferatoxin, Sigma) were made in ethanol or dimethylsulphoxide (Sigma) and diluted in appropriate growing media.

Immunocytochemistry (ICC)

Cultures were fixed in 4% paraformaldehyde for 1 hour. After fixation, cultures were washed in 50 mM potassium phosphate buffer (KPBS) and held in KPBS until starting ICC. The ICC procedure was performed by using a modification of the avidin-biotin-peroxidase complex (ABC) technique [21]. Cultures were washed 3 times for 10 min each in 50 mM KPBS. Inactivation of endogenous hydrogen peroxidase was achieved by rinsing cultures in 0.3% hydrogen peroxide in 50 mM KPBS for 15 min. The cultures were incubated for 30 min in 3% normal goat serum in 50 mM KPBS, 1% bovine serum albumin (BSA) and 0.4% Triton X-100. SP and CGRP antiserum were diluted to 1:5000 in 50 mM KPBS, 1% BSA and 0.4% Triton X-100. After incubation with primary antiserum for 24 hours at 4°C (for CGRP) or at room temperature (for SP), the cultures were adequately washed. Following washing, the cultures were incubated in goat anti-rabbit IgG (Vector; 1:600) for one hour at room temperature and than reacted with avidin-biotin complex (Vector; 1:200) for additional hour at room temperature. Staining was performed by exposing cultures to 0.04% 3-3'diaminobenzidine tetrahydrochloride (DAB, Sigma), 2.5% nickel sulfate (Fisher Scientific) and 0.01% hydrogen peroxide dissolved in 0.1M sodium acetate. Controls were processed by omitting the specific antiserum or using antiserum preabsorbed with 1μM concentration of the immunogen. No positive immunostaining was detected on the control tissues.

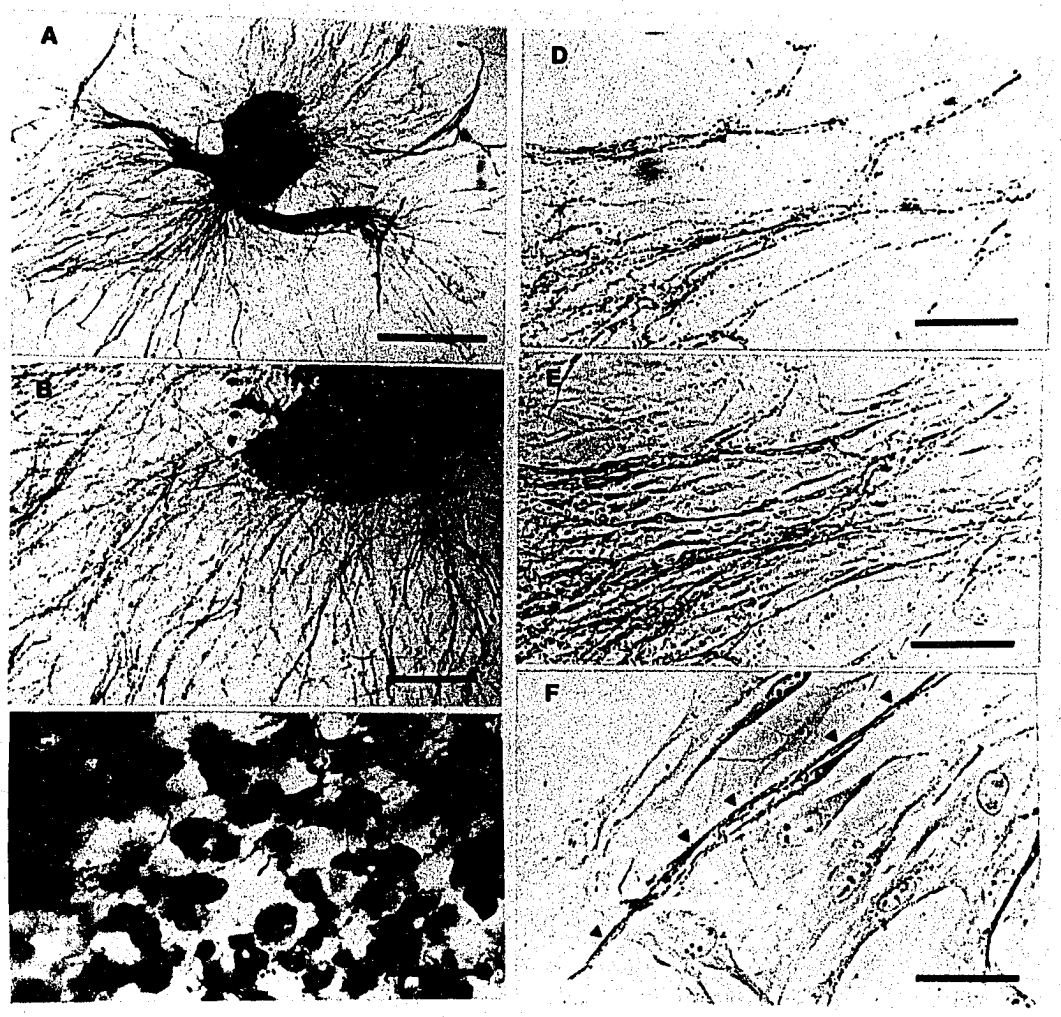
SP-IR and CGRP-IR neurons were counted in single ganglia under the light microscope and averaged. Images of IR neurites were quantified by image analysis system (Zeiss-Kontron; IBAS version 2.00). Samples were viewed with a Zeiss axiophot microscope at 6.25x magnification (2.5x by 2.5x optivar). Images were captured with a Sony 3CCD color video camera. The internal scaling feature of the image analysis software was calibrated to measure in millimeters. The neurite images underwent normalization (histogram stretching) and contour enhancement prior to interactive discrimination. The resulting images were measured to obtain the area of immunoreactive neurites present in the field. Four fields, evenly spaced around the DRG explant, were measured from each culture. The total area measured for each field was 1.15mm^2. Data were analyzed by Student's t- test.

Results

Data are based on the experiments performed on total of 210 DRG cultures. Of these cultures 90 were used as control and in each experimental group there were at least 6 DRG cultures.

Dorsal root ganglion explants from 2 day old rats established an extensive growth of neuronal and non-neuronal elements when cultured for 1 week. Explants had a tendency to flatten and spread but migration of DRG neurons into the growing zone surrounding DRG explant was not recorded (Figs 1A,B and 4 A,B). Normal SP- or CGRP-IR in 8 day old cultures is illustrated in Figs. 1 and 4 A,B,C. The number of SP-IR DRG neurons was significantly larger than number of DRG neurons immunostained for CGRP ($P<0.001$, Student's t test). The growth of immunoreactive neurites was radial (Fig. 1A and 4A) and well

Figure 1. Substance P -IR fibers and neurons in cultured DRG. A. Low power bright field micrograph of a 6 day old DRG organotypic culture started from a 2 day old rat and immunoreacted to SP. Numerous SP-IR cell bodies are present in the DRG explant and radially oriented fibers are surrounding the explant. No positive cell bodies are noticable outside the explant. B and C. Higher power micrographs of the same culture with easily visible cell bodies and fibers within and outside the DRG explant. D . A higher power bright field micrograph of a region outside the explant with numerous positive neurites. E. Phase contrast micrograph of the field shown in D with presentation of relation between positive fibers and nonneuronal elements. F. High power phase contrast micrograph of SP-IR fibers (arrow heads) around nonneuronal elements. Bars A,500μm, B,D and E, 160μm; C and F, 50μm.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supported by non-neronal elements in undergrowth (see Fig 1E,F). Both SP-IR and CGRP-IR fibers showed extensive branching and immunopositive boutons were abundant (Figs 1 D,F and 4 C).

Effects of capsaicin on SP-IR sensory neurons

In concentration of 1μM, capsaicin produced a decrease in the number of SP-IR neurites. The effect was noticeable but was such that it had to be quantified with image analysis system (Table 1) and is not illustrated. Exposure of the cultures to 10μM capsaicin resulted in a severe decrease of SP-IR from neurites surrounding DRG explant (Fig.2). By using predetermined 4 fields, evenly spaced around the ganglia, we were not able to find any SP-IR fibers with image analysis system (Table 1). No obvious depressant effect of capsaicin was observed on the growth of non-neuronal elements in the growing zone surrounding the DRG explant (Fig. 2E). No SP-IR fibers were found in cultures exposed to 100μM capsaicin. The effect of capsaicin on SP-IR cell bodies in the DRG explant proper was manifested as a decrease in the number of positive cell bodies and decrease or elimination of positive fibers (Fig. 2A,B). In concentration of 10 μM capsaicin produced a 35% decrease of SP-IR neurons in cultures (Table 2).

The effect of RTX on SP-IR fibers was dose dependent and quite similar to the effect of capsaicin. While the effect of 10nM RTX on SP-IR neurites was significant (Table 1), 100nM RTX almost completely eliminated SP-IR fibers (Fig. 3F and G). Similar to capsaicin, RTX in 10 and 100nM concentrations decreased the number of SP-IR neurons in DRG explants. 100 nM RTX for a period of 6

Table 1. The effect of capsaicin and RTX on the density of SP-IR and CGRP-IR neurites in DRG organotypic cultures. Images of SP or CGRP immunoreactive neurites were captured with a vide camera and measured in 4 fields (1.15mm^2 each) evenly spaced around the ganglia (there were 6 ganglia in each group). The immunoreactive images underwent normalization (histogram stretching) and contour enhancement prior to interactive discrimination. The presented numbers are average areas in mm^2 (±SEM) of IR fibers present in the field after background values were subtracted.

	control	capsaicin (μM)			RTX (nM)		
		1	10/90 min	10	10	100/90min	100
SP	0.039±0.007	0.025±0.005*	0.038±0.006	0	0.0149±0.005*	0.0434±0.009	0
CGRP	0.263±0.018	0.186±0.025*	-	0.112±0.014*	0.159±0.022*	-	0.0322±0.007*

* significantly different from control at P<0.001
** significantly different from control at P<0.01

Figure 2. Morphological changes produced by capsaicin. 48 hours exposure to 10μM capsaicin decreased the number of SP-IR cell bodies (A and B) and drastically decreased number of SP-IR fibers in the growing zone of the culture. C. SP-IR soma of sensory neurons in a culture exposed to 100μM capsaicin for 6 days. D and E. Photomicrographs of the field shown in in bright field (in D) and phase contrast (in E) illustrating the almost complete elimination of SP-IR fibers in a culture exposed to 10μM capsaicin for 6 days and absence of a similar effect on nonneuronal elements. F. A high power micrograph of a short segment of SP-IR fiber marked with arrowheads in D and E. Bars; A 160μm, B,C,D and E 100μm: F 50μm.

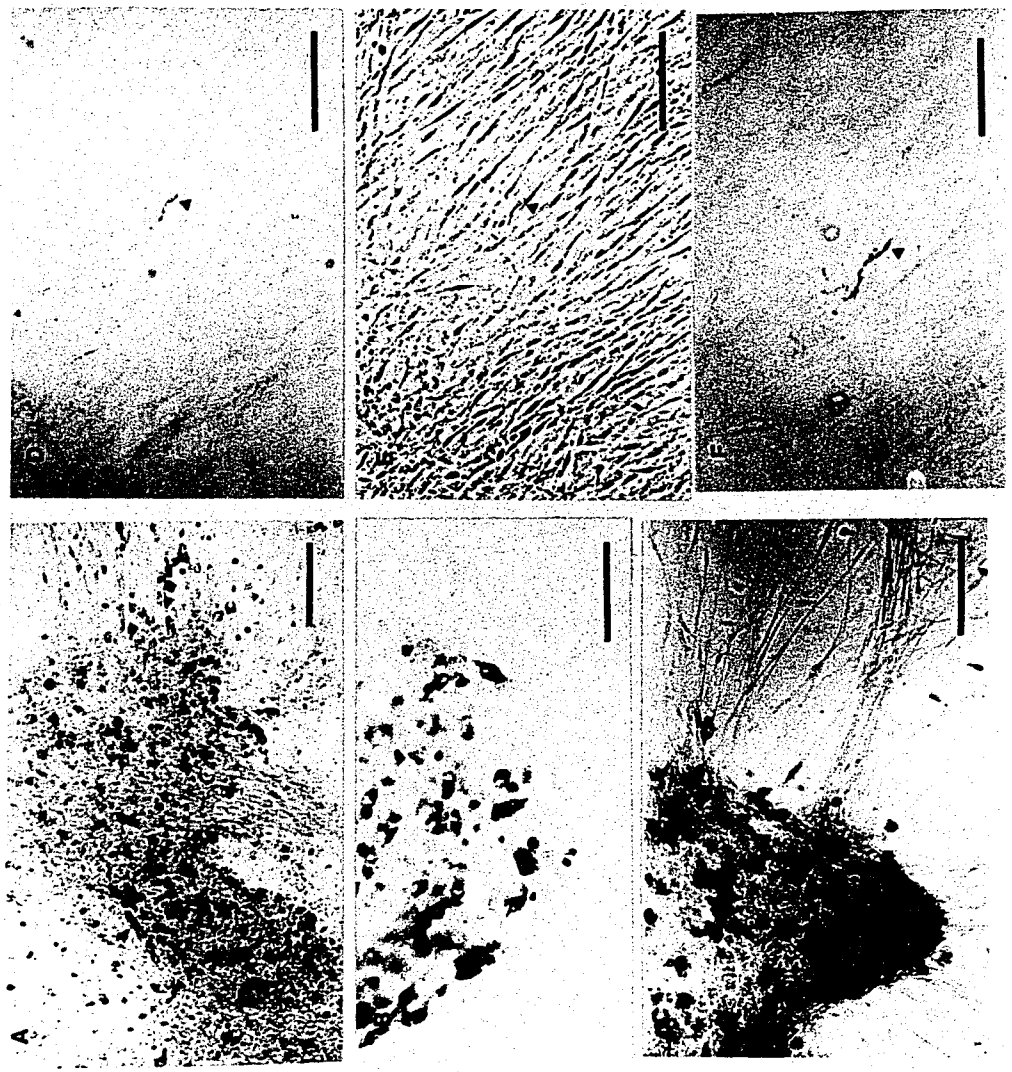
B
C
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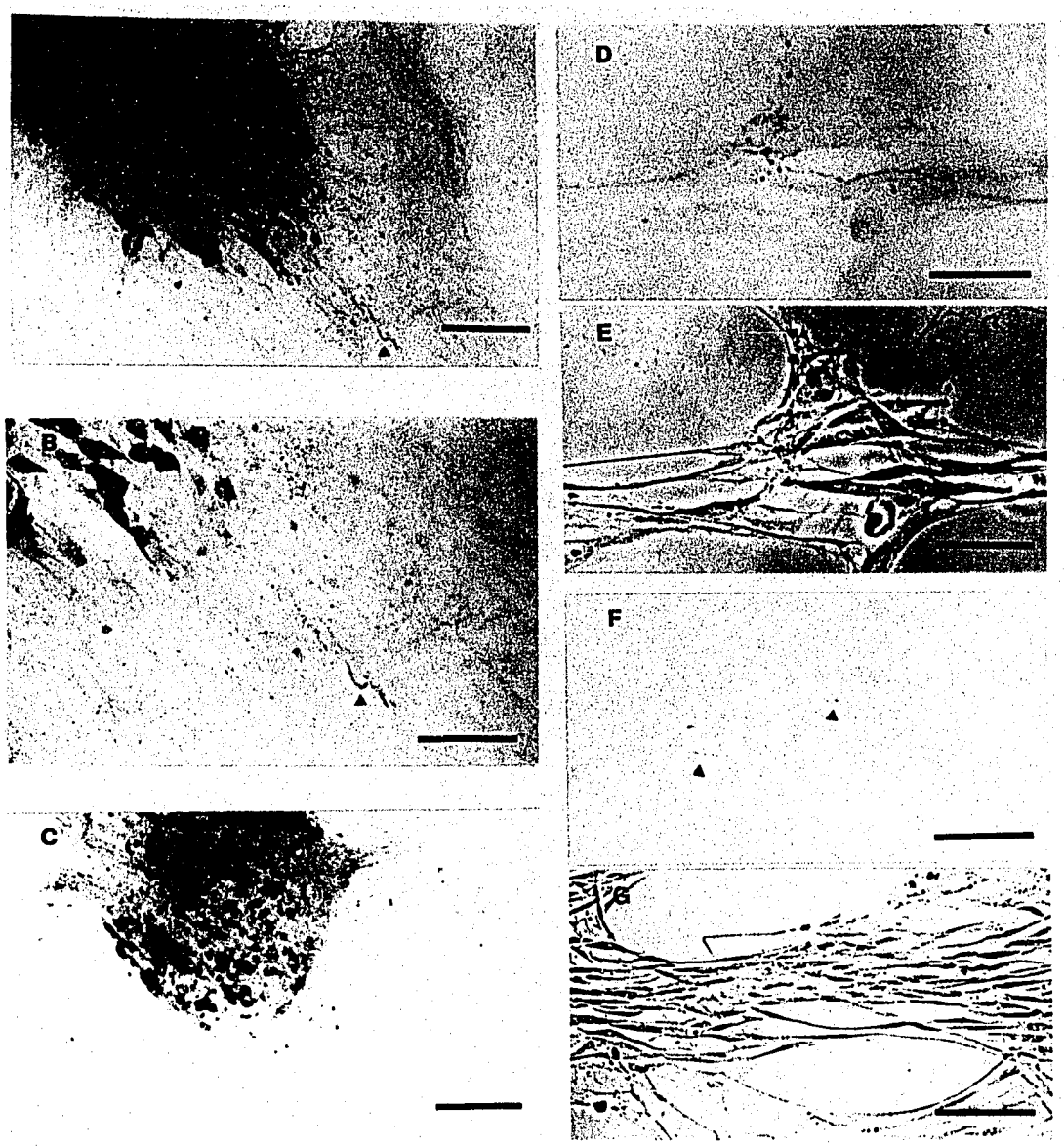
Table 2. The effect of capsaicin and RTX on the number of SP-IR and CGRP-IR cell bodies in DRG organotypic cultures

	control		10 μM caps		100nM RTX	
	n	mean±SEM	n	mean±SEM	n	mean±SEM
SP-IR neurons	21	138±6	20	89±7*	20	64±7*
CGRP-IR neurons	23	97±6**	21	47±4*	20	53±5*

* significantly different from control at P<0.001

** significantly lower than the number of SP-IR neurons at P<0.001

Figure 3. The effects of RTX on SP-IR cultured sensory neurons. A. Low power micrograph of a culture exposed to 100nM RTX for 48 hours. B. A higher power magnification of the same culture with SP-IR cell bodies inside the explant and several SP-IR fibers. C. 100nM RTX for 6 days eliminated all SP-IR fibers in the growing area surrounding the DRG explant while many SP-IR cells are visible. D and F. Bright field and phase contrast high power micrographs of SP-IR fibers and nonneuronal immunonegative elements in a culture exposed to 100nM RTX for two days. 100nM RTX almost completely eliminated all SP-IR fibers (F, arrow heads are pointing towards structures that may be SP-IR segments of neurites) without an similarly severe effect on nonneuronal elements in the same field (G). Bars, A and C, 160μm; B,D,E,F and G, 100μm.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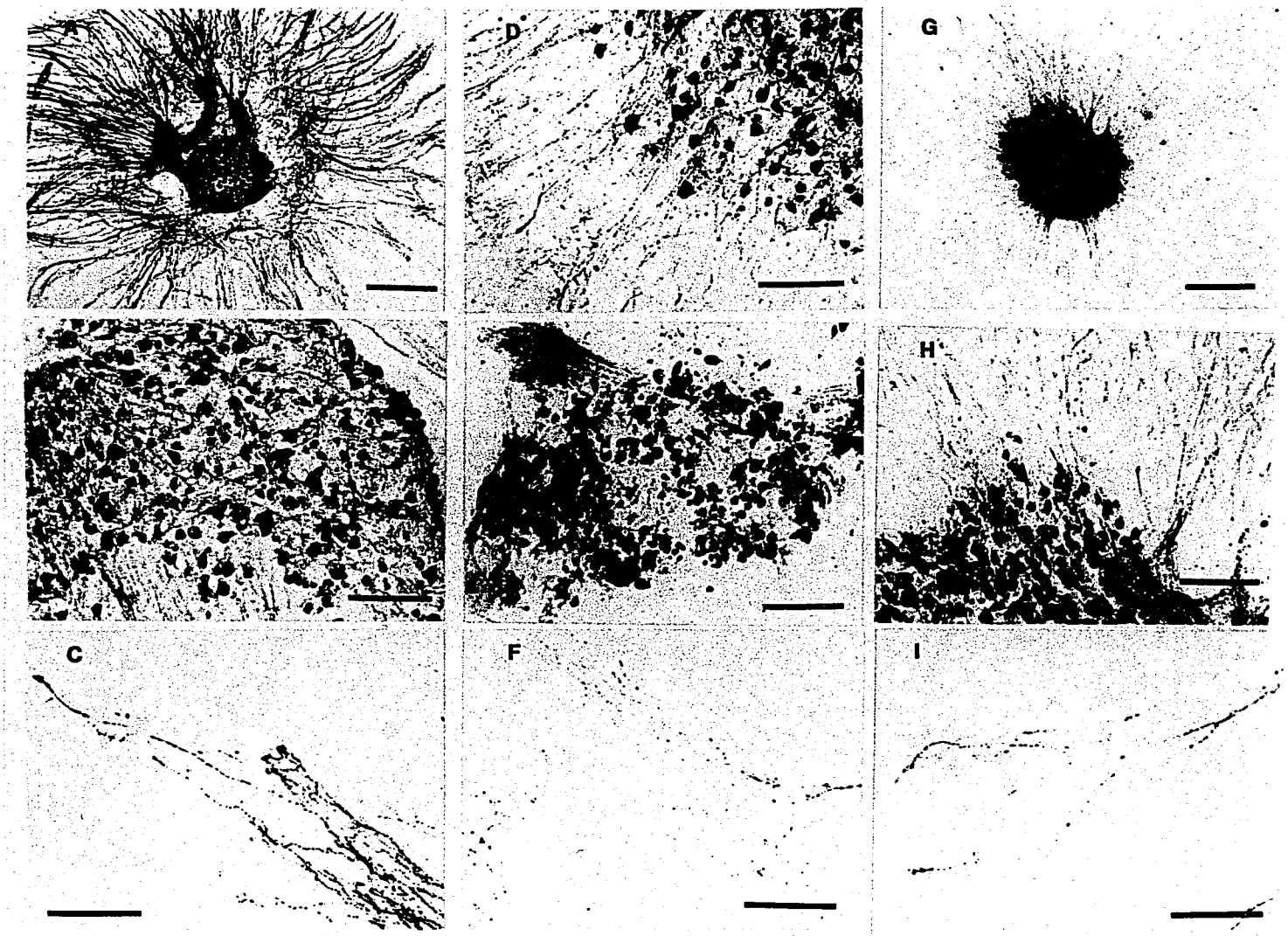
days produced about a 50% decrease in SP-IR cell bodies in DRG explants (Table 2).

In order to establish the time dependence of capsaicin and RTX effect, we exposed tissue to the compounds in different schedules and recorded the effect. In cultures that were exposed to 100μM capsaicin for 2 days a complete elimination of SP-IR fibers and significant decrease in the number of SP-IR cell bodies in DRG explant were observed (Table 2). 10μM capsaicin for 48 hours produced a drastic decrease in the number of SP-IR fibers and 6 day exposures resulted in almost a complete elimination of SP-IR fibers (Fig,2 D,E and F). 1μM capsaicin for 7 days capsaicin produced a significant decrease of SP-IR neurites. However, 10μM capsaicin (n=18) or 100nM RTX (n=28) failed to produce an effect , determined by examining immunoreactivity to peptides 6 days after exposure, when presented to cultures for 45 to 90 min (Table 1). Exposure of the cultures to RTX or capsaicin vehicle, in a concentration equal to one used with the irritants, was without any obvious effect.

Effect of capsaicin and RTX on CGRP-IR neurons

Exposure of the cultures to 10 and 100 μM capsaicin resulted in an obvious concentration-dependent decrease of CGRP-IR neurites surrounding DRG explant (Table 1, Fig. 4 C,D,E). 10μM capsaicin produced a noticeable decrease in the number of CGRP-IR fibers surrounding explants and 100μM capsaicin produced an obvious decrease in the number of CGRP-IR neurites (Table 1, Fig. 4 E,F). It should be noticed, however, that there were many more CGRP-IR fibers than SP-IR fibers exposed to corresponding concentrations of capsaicin. The effect of RTX on CGRP-IR fibers was quite similar to the effect of capsaicin.

Figure 4. A. A low power bright field micrograph of a 7 day old organotypic culture started from a two day old rat and immunostained for CGRP. The culture is characterized by numerous CGRP-IR cell bodies in the DRG explant and CGRP-IR fibers radially oriented in the growing zone of the culture. B. A higher power micrograph of CGRP-IR cell bodies and fibers within the DRG explant. C. A bright field micrograph of CGRP-IR neurites in a 7 day old culture. D. In concentration of 10μM for 6 days capsaicin decreased number of CGRP-IR DRG neurons and fibers. E and F . 100μM capsaicin for 6 days produced an obvious decrease in the number of CGRP-IR fibers. G, H and I. I concentration of 100nM for 6 days RTX produced an obvious decrease in the number of CGRP-IR cell bodies and neurites in DRG culture. Bars A and G, 500μm; B,D,E and H, 160μm, C,F and I, 100μm.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10nM RTX produced a significant decrease in the CGRP immunoreactivity in neurites (Table 1), but 100nM RTX produced a decrease in the number of CGRP-IR fibers of such magnitude that it could be obvious in a photography (Fig. 4G,H and I). It is important to notice that in no instance there was complete elimination of CGRP-IR fibers. The effect of capsaicin and RTX on the number of CGRP-IR cell bodies in DRG explant proper was similar to that on SP-IR cell bodies. After counting we established that there was significantly less CGRP-IR cell bodies in cultures exposed to 100μM capsaicin and 10nM RTX in comparison to controls (Table 2).

The time-dependency of capsaicin and RTX toxic action was examined on CGRP-IR neurons and is similar to the effect of these toxins on SP-IR neurons and fibers. The capsaicin and RTX effect on CGRP-IR structures was dependent on time of exposure as well as on concentration of neurotoxins. In exposures of the cultures to 10μM capsaicin or 100nM RTX for 90 min no obvious effects on the CGRP-IR in neurites or cell bodies were recorded several days later when the culture was fixed and ICC procedure performed. The effects on CGRP was not quantified by using machine because there was no noticeable difference and the effect on more sensitive SP-IR neurites was absent (Table 1).

On the basis of the data that unique sensory neuron-specific actions of capsaicin result in an influx of a number of cations including sodium, potassium and calcium which is specifically antagonized with ruthenium red (RR), we tested the effect of RR on 22 cultures. In 30 to 60 min exposures to 0.5 μM RR we were unable to demonstrate convincing antagonistic effect on capsaicin or RTX (n=8). However, culture exposed to 0.5μM RR for 6 hours or longer (n=8) failed to show any immunoreactivity to SP or CGRP 4 to 5 days after exposure.

In these cultures we were not able to see any immunoreactivity to two peptides in cell bodies or neurites. In higher concentration (1μM) RR resulted in disintegration of the cultures (n=6). This "depleting" effect of RR prevented its use in studying the mechanism of action of capsaicin and RTX.

Discussion

The present series of experiments has examined the effects of capsaicin and RTX on cultured peptidergic rat sensory neurons. Using immunocytochemistry for labeling SP- and CGRP-IR we have been able to study the long lasting effect of these two irritants on growth and survival of peptidergic neurons in organotypic cultures.

It is clear that capsaicin and RTX, in a concentration dependent manner, eliminate SP -IR fibers and reduce, but do not eliminate, CGRP-IR fibers when presented to DRG cultures for period of 48 hours or longer. Exposure of the cultures to the irritants for 90 min did not produce a noticeable effect . It is also clear that neither capsaicin nor RTX eliminated all of the soma of SP-IR sensory neurons. These findings are in agreement with previous studies suggesting that processes of sensory neurons are much more sensitive to capsaicin than cell body [4, 24]. In addition, our findings that SP-IR fibers were much more sensitive to capsaicin are in agreement with results demonstrating that SP is present in small DRG neurons and small C-fibers only while CGRP is localized in both small and large sensory neurons [15, 28]. While it seems clear that only small fibers are affected by capsaicin and RTX in culture, the question of the toxicity of capsaicin and RTX towards all sensory neurons remains unanswered. Our findings that SP-IR cell bodies were present in cultures exposed to 100μM capsaicin or 100nM

RTX for 6 days convincingly demonstrates that these neurons are not killed by these irritants. Concentration-dependent effect towards peptidergic fibers and the greater effect on SP-IR fibers is further support that our procedure is sensitive enough and that these findings are not the result of some nonspecific factors. These results provide additional support for the data demonstrating that capsaicin and RTX act with great cellular specificity. Experimental approach, however, does not allow us to convincingly demonstrate that loss of peptide immunoreactivity is due to cell death.

It is important to establish that the effect of capsaicin and RTX on cultured neurons is of long duration and is therefore different from depletion of peptides from neurites. In cultures exposed to these irritants for two days after which the cultures were returned to growing media for 4 or 5 days, we were not able to notice an increase in SP-IR fibers. These cultures were the same as cultures that were exposed to the irritants for 2 days just prior to fixation and ICC. In addition, it is important to establish that the sensitivity of fibers is concentration dependent. In cultures exposed to 10μM capsaicin or 10nM RTX we were not able to produce complete elimination of SP-IR fibers, even though the cultures were exposed to neurotoxins for 7 days. However, 100μM capsaicin and 100nM RTX produced complete elimination of SP-IR fibers in two days. Single exposure to high concentrations of capsaicin produced a depletion of neuropeptides and excitatory amino acids from sensory neurons which may account for long lasting impairment of sensory functions (7,14). A strict correlation was found between the time-related recovery of neuropeptide levels in sensory terminals and functional recovery of sensory fibers (9). Present findings demonstrate important

role played by the time of exposure. While singe exposure to capsaicin produced a reversible impairment of sensory function prolonged exposure in our experiments resulted in irreversible changes. This may be very important element in proposed use of capsaicin in chronic pain treatment. Due to strong effect of ruthenium red on the peptidergic neurons we were not able to study the mechanism of prolonged capsaicin effect on sensory neurons. However, the capsaicin-induced excessive Ca accumulation in primary afferents accounts for "sensory neuron blocking" action produced by high capsaicin and might account for "irreversible" depletion of SP and CGRP from capsaicin sensitive neurites [30, 36]. Our inability to maintain the cultures for longer than 6 days following exposure to capsaicin makes it hard to be certain that effects are irreversible.

Previous studies have provided evidence that sensitivity to capsaicin and content of SP are properties of nociceptive primary sensory neurons but it is not known whether these markers are expressed in all nociceptive sensory neurons. It has been documented that in dissociated cultures the fraction of cells sensitive to capsaicin and fraction of cells that contain SP-IR are larger than in freshly dissected DRG [1]. In our experiments, the fraction of the cells in the culture that is stained with antiserum to SP is larger than the fraction of that stained with CGRP. This result suggests that SP sensory cells survive preferentially in our culture conditions. Together with finding that capsaicin and RTX eliminate all SP-IR neurites and only a certain proportion of CGRP-IR neurites, these results suggest that relatively early in development a large fraction of cultured sensory neurons are able to express the functional properties of differentiated nociceptive sensory neurons, and are likely to be useful tool in studying the neuronal mechanisms of nociception.

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CHAPTER 2. NEUROLIGAND-EVOKED CALCIUM-DEPENDENT RELEASE OF EXCITATORY AMINO ACIDS FROM SCHWANN CELLS

A paper submitted to The Journal of Neuroscience

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Abstract

Bradykinin caused a receptor-mediated increase in release of the excitatory amino acids (EAAs) glutamate and aspartate from Schwann cell cultures obtained from dorsal root ganglia (DRG) together with an increase in the cytoplasmic level of 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. Therefore, bradykinin causes calcium dependent-release of EAAs from DRG Schwann cells. Bradykinin did not cause cell swelling and p-chloromercuriphenylsulphonic acid, an inhibitor of the electrogenic glutamate transporter, did not reduce bradyknin-induced EAA release. Therefore, bradykinin stimulates EAA release from Schwann cells through a mechanism that is neither the previously described volume regulated release mechanism nor due to the reversal of the glutamate transporter.

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* Contributed equally

Introduction

It has been well established that glia have multiple roles in the nervous system which include the regulation of external potassium and the uptake of the excitatory amino acids glutamate and aspartate which are released from neurons into the extracellular space (Nicholls and Atwell, 1990; Barres, 1991; Kanai et al., 1993). Several lines of evidence suggest that glial cells may also play important roles in releasing neurotransmitters. Schwann cells can release acetylcholine following axotomy of motoneurons (Dennis and Miledi, 1974). Retinal Müller cell depolarization stimulates the release of glutamate through a reversal of an electrogenic glutamate uptake carrier (Szwatkovski et al., 1990). Depolarization causes the calcium-dependent release of the amino acid taurine from hippocampal glia (Philibert et al., 1988). Hyposmotic shock of cortical astrocytes stimulates cell swelling, an increase in internal calcium and a correlated release of the amino acids aspartate and taurine (Kimelberg et al., 1990; O'Connor and Kimelberg, 1993). Since glial cell calcium levels are regulated by neurotransmitters (Reisner et al., 1989; Cornell-Bell et al., 1990; Glaum et al., 1990; Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; Inagaki et al., 1991; Jensen and Chiu, 1991; McCarthy and Salm, 1991) this has prompted us to ask whether the neuroligand bradykinin causes a calcium-dependent release of excitatory amino acids (EAAs) from glial cells.

In the peripheral nervous system, the nonapeptide bradykinin is released from its precursors, the kininogens, by the action of the enzyme kallikrein in response to trauma (Dray and Perkins, 1993). Bradykinin is known to mobilize calcium from inositol 1,4,5-trisphosphate (IP_3)-sensitive calcium stores in several

cell types (for ref see Miller, 1987; Higashida and Ogura, 1991). Since bradykinin can mobilize internal calcium, we have asked whether this agent can cause the release of excitatory amino acids from glial cells derived from the dorsal root ganglia. This study demonstrates that the neuroligand, bradykinin, mobilizes internal calcium from Schwann cells which stimulates a calcium-dependent release of the excitatory amino acids, glutamate and aspartate.

Methods

Cell Culture

Glial cultures from DRG were obtained by modification of the organotypic procedure (Gähwiler, 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 form the DRG to minimize fibroblast contamination. DRG were than embedded onto a glass coverslip inlayed in a 35 mm dish (for release studies) or into a glass bottomed dish (for calcium 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 all experiments were performed. Neurites degenerated 24 to 48 hours following the removal of the central portion

of explants leaving cultures containing only non-neuronal cells. The absence of neuronal elements was confirmed by immunocytochemistry.

In some experiments we enriched the number of Schwann cells in cultures using two methods (see Kleitman et al, 1992). In the Wood method, DRG were cultured for 48-96 hours in the presence of cytosine arabinoside (Ara-C; 5 mg/mL). They were then transplanted to a new coverslip coated with chicken plasma. DRG explants were then cultured as described above to obtain DRG glia. In the Brockes method, DRG glia were incubated with Thy 1.1 antibody (hybridoma supernatant, American Type Culture, No. TIB103) and addition of rabbit complement (Sigma). Since Thy 1.1 is expressed on fibroblasts, but not on Schwann cells, this procedure leads to a complement mediated lysis of fibroblasts. In both culture methods, immunocytochemistry revealed that Schwann cells were enriched to >95%.

Immunocytochemistry

Antibody visualization was accomplished by using a Vectastain ABC kit (Vector) and the nickel-enchanced DAB method (Hsu et al., 1981). Immunocytochemistry was performed using antibodies directed against the synaptic proteins synaptophysin (1:1000; clone 7.4a, provided by R. Jahn) and synaptotagmin (1:5000; clone 41.1, provided by R. Jahn), against glial fibrillary acidic protein (GFAP; 1:5000, ICN Immunobiologicals) and against the low-affinity NGF receptor (1:2000; 192-IgG, provided by E.M. Johnson, Jr.). Anti-synaptophysin, synaptotagmin and anti-GFAP were found to be negative on DRG glia. Positive controls were performed with these antibodies using

hippocampal neurons and glia and using DRG explants in which axons were maintained in culture.

EAA release

The coverslips with glial cultures were mounted into 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 ml/min and 35-37°C. After an equilibration period of 40 to 60 minutes, samples of perfusate for amino acid determination were collected every minute. The amino acid content in samples was determined by high-performance liquid chromatography (HPLC) with fluorescence detection (Lindroth and Mopper, 1979). 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 (10 nM; Sigma) and its antagonist (D-Arg^0, Hyp^3, ß-Thi^{5,8}, D-Phe^7)-bradykinin (5 µM; Bachem Bioscience Inc.) were added to a perfusion solution. In control experiments we co-administered hydroxyproline (180 nM) together with bradykinin and determined that there is a 1 minute lag time from initiation of the addition of an agent until it is collected for HPLC analysis due to dead-volume in the chamber. All figures for HPLC have been correspondingly corrected. Elevated K^+ (50 mM) solution for HPLC was prepared by modification of a Ringer's solution where NaCl was replaced by KCl.

Calcium imaging

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 1ml of 4μM Fura-2 AM ester solution. After washing, cells were kept for 30-60 minutes at 37°C to permit dye de-esterification . All experiments took place at 22-24°C. All image processing and analysis were performed using ratiometric software (QFM, Quantex Corp. or Image-1/Fl, v 1.63g, Universal Imaging Corp.). Background subtracted, ratio images (340/380 nm or 350/380 nm) were used to calculate the $[Ca^{2+}]_i$ according to Equation 5 of Grynkiewicz et al. (1985). Calibration was performed *in situ* (Thomas and Delaville, 1991) using the Ca^{2+}-ionophore 4-bromo-A23187 (10 μM, Molecular Probes). An estimate of autofluorescence at each wavelength was 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 that was greater than 50% of the resting calcium level.

During calcium imaging, bradykinin (10 nM) and elevated K^+ (50 mM) were 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 spaced at 10 minute intervals. In some experiments the second bradykinin application followed the first application after 30 minutes. Ryanodine (10 μM), caffeine (10 mM) and furosemide (5 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). Elevated K^+ saline was prepared by modification of normal saline where NaCl was replaced by KCl.

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

Cell volume measurement

To determine whether bradykinin caused a shrinkage of cells in our studies we monitored cell volume using BCECF as described by Eriksson et al. (1992). Cells were loaded by incubation in membrane permeable BCECF AM (10 μM) for 40-60 minutes, followed by 40 minutes for de-esterification. BCECF was excited at the pH-insensitive wavelength of 440 nm. The emission intensity (510 DF 40nm bandpass) was monitored with a SIT camera. When cells swell, BCECF is diluted, reducing the fluorescent intensity detected. We confirmed that this approach effectively detected volume changes in our studies by applying hyposmotic saline that was made by removing 50 mM of NaCl from our standard saline (see above).

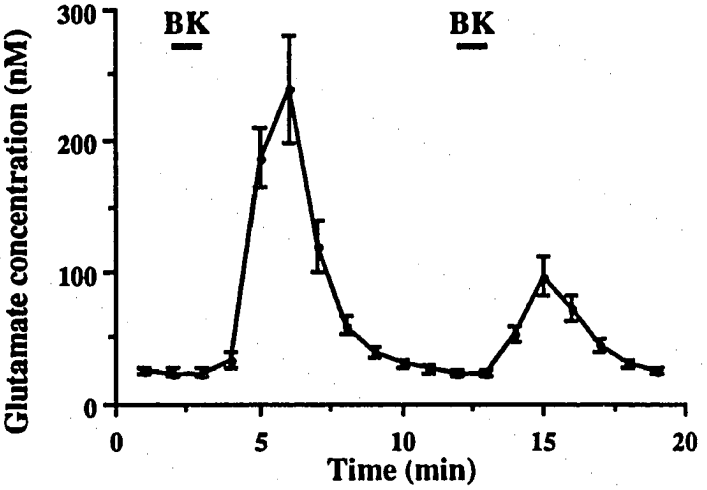
Results

Bradykinin stimulates excitatory amino acid release from dorsal root ganglia Schwann cells

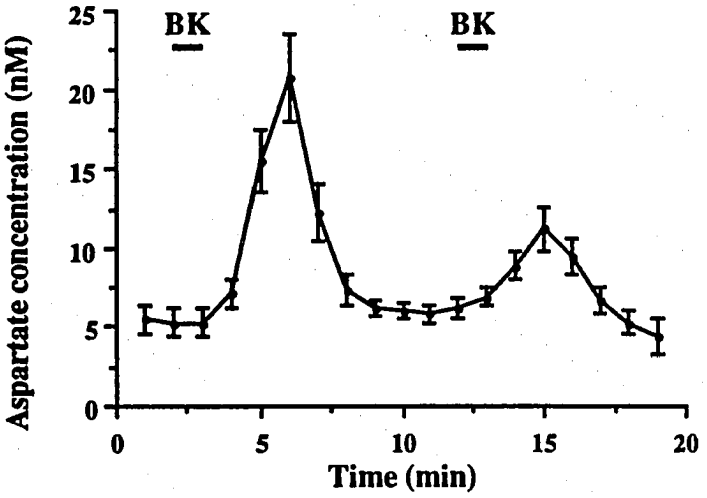
Primary glial cultures of dorsal root ganglia (DRG) were obtained using a modification of the organotypic procedure (Gähwiler, 1984). Dorsal root ganglia were cultured on coverslips to permit neurite extension and migration of glial cells. After cultures were established, the central portion of the ganglionic explant, which contains neuronal cell bodies, was removed from the culture. During the next 48 hours the residual neurites degenerated leaving a neuron-free glial cell culture. The release of EAAs from these cultures was assayed using HPLC on the superfusate. 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 EAA 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, ß-Thi^{5,8}, D-Phe^7)-bradykinin (5 µM), reversibly blocked the stimulatory action of bradykinin (n=6; Fig. 1C). While bradykinin stimulated the release of glutamate and aspartate from glial cultures it did not significantly affect the release of serine. The basal level of serine was 14 $\pm$ 2 nM compared to 16 $\pm$ 2 nM in the presence of

Figure 1. Bradykinin causes a receptor-mediated release of the EAAs, glutamate and aspartate, from DRG glia. Using HPLC, the amounts of glutamate and aspartate were determined in superfusate from rat DRG glia cultures. Addition of bradykinin (BK; 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.

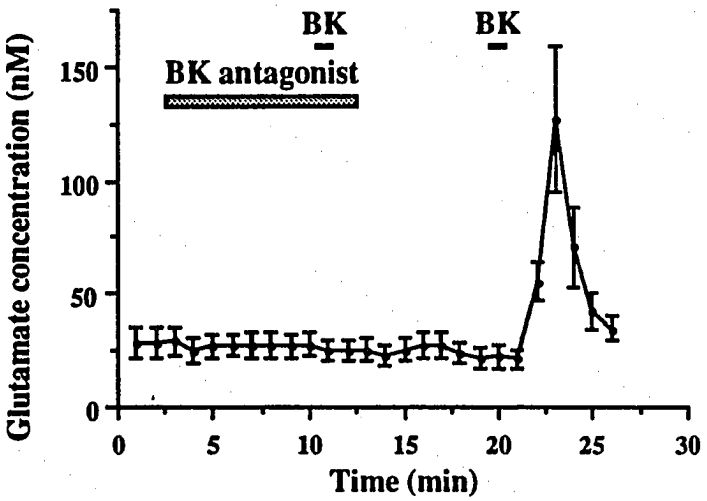
A


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)

bradykinin (n=13; p>0.5). These data demonstrate that the nonapeptide bradykinin potently causes the selective release of the EAAs glutamate and aspartate from DRG glia.

The cell types present in the glial cultures derived from DRG was examined using immunocytochemistry. We confirmed that glial cultures were neuron-free by using antibodies against the synaptic proteins synaptophysin and synaptotagmin. In these cultures immunoreactivity for these two synaptic proteins was absent while neurons were immunopositive in separate cultures. An antibody against glial fibrillary acidic protein was negative in DRG glial cultures. However, using an antibody against the low-affinity NGF receptor, characteristically expressed in Schwann cells localized distal to the site of axotomy, but not in surrounding fibroblasts (Taniuchi et al., 1988), we found that about 74% of the cultured glial cells possess NGF receptors (Fig. 2) . Therefore the predominant glial cell type in these DRG cultures is Schwann cells.

Since fibroblasts are likely to be a contaminating cell type in these cultures, we modified our techniques to enrich specifically in Schwann cells. Purified populations of Schwann cells (>95% of cells) were obtained by the Wood (n=6) and Brockes (n=6) methods (see Kleitman et al., 1992). These cultures showed similar increases of the EAAs glutamate and aspartate in the superfusate after application of bradykinin. Bradykinin increased the glutamate levels in the superfusate from a basal level of 127 ± 9 and 35 ± 7 to 465 ± 23 and 123 ± 16 using the Brockes and Wood methods, respectively. We therefore conclude that the nonapeptide, bradykinin causes the release of the the amino acids glutamate and aspartate from DRG Schwann cells.

Figure 2. DRG glia are immunopositive for the low-affinity NGF receptor. **A)** shows a phase-contrast micrograph of a field of DRG glia. Immunocytochemistry was performed on cultures using an antibody specific for the low-affinity NGF-receptor which is expressed in Schwann cells after axotomy. **B)** shows a bright-field image of the same cells. Most of the glia are immunopositive indicating that they are Schwann cells.

A

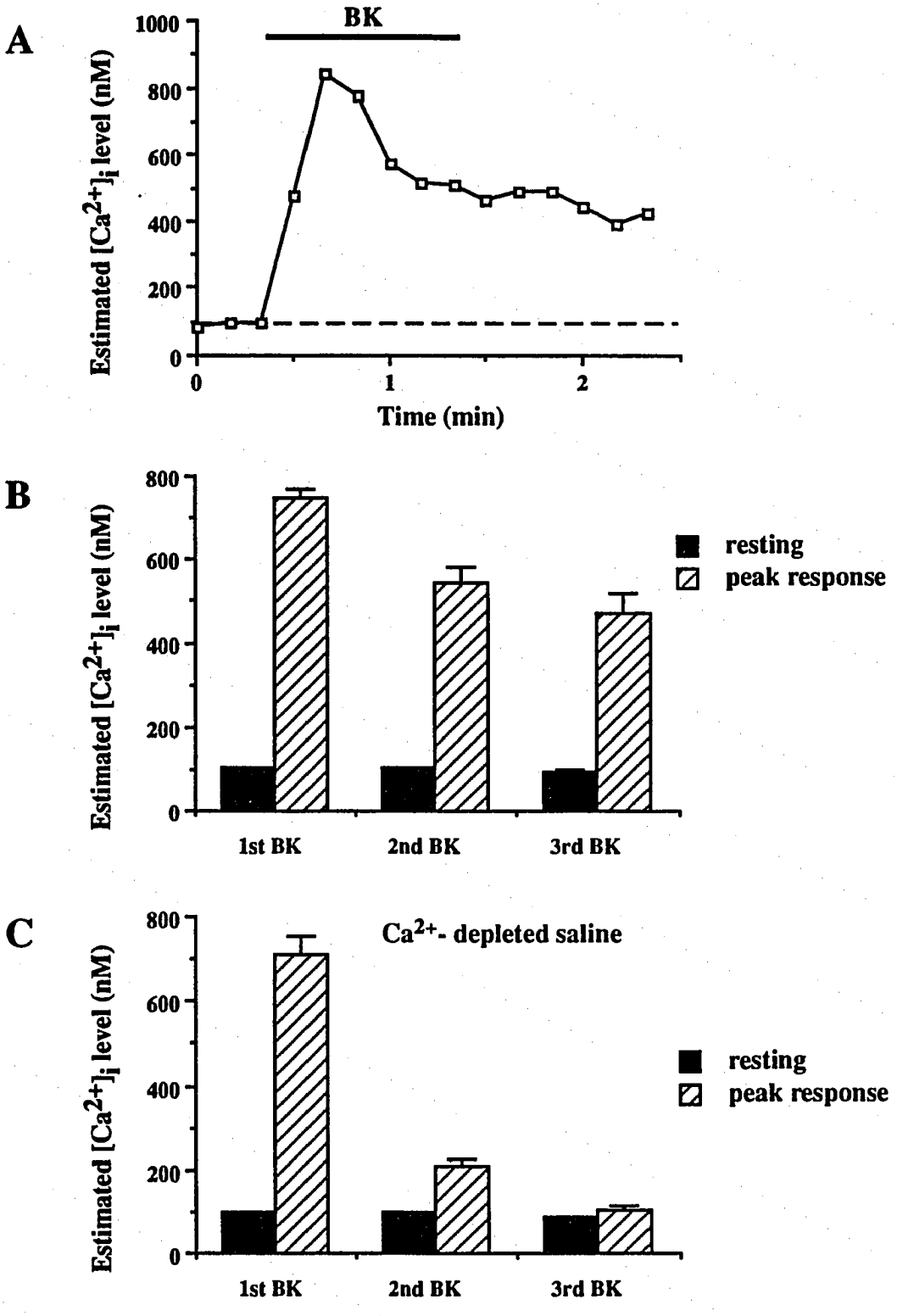
B

Bradykinin mobilizes calcium in dorsal root ganglion Schwann cells.

The release of neurotransmitter at neuronal synapses is calcium-dependent. To ask whether calcium might play a role in bradykinin-induced EAA release from DRG Schwann cells, we used ratiometric imaging techniques to monitor glial calcium levels. Cells were loaded with the membrane permeant calcium indicator fura-2 AM. Glia 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. 3A) about 20 seconds after the onset of bradykinin application and was sustained for several minutes. In all subsequent experiments, we have reported the calcium levels for only those cells that responded to bradykinin. These bradykinin responsive cells had the morphology of Schwann cells. In some experiments we performed immunocytochemistry following calcium imaging using the antibody directed against the low-affinity NGF receptor which is present in Schwann cell cultures. Immuncytochemistry demonstrated that those cells that responded to bradykinin with an elevation of internal calcium were immunopositive with the antibody directed against the low-affinity NGF-receptor (n=22 of 22 cells). Therefore, bradykinin elevates internal calcium levels in Schwann cells in a manner temporally coincident with the timing of release of glutamate and aspartate.

Repeated applications of bradykinin reliably elevated calcium levels in Schwann cells (Fig. 3B). The B_2 receptor antagonist, (D-Arg^0, Hyp^3, ß-Thi^{5,8}, D-Phe^7)-bradykinin (5 µM), reversibly attenuated the stimulatory action of

Figure 3. Bradykinin mobilizes Ca^{2+} from internal stores in DRG glia. In (**A**) a focal application of bradykinin (BK; 10nM) elevates DRG glial calcium level to a peak 20 seconds after the onset of application. During sustained application of bradykinin, glial calcium is maintained at a stable elevated plateau from basal calcium level (dashed line). In (**B**) repeated applications of bradykinin (10 nM) caused repeated elevations of glial cytoplasmic calcium levels. In (**C**) 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 **C** with **B**). Bars represent means $\pm$ SEM.


A
BK
1000
800
600
400
200
0
Estimated $[Ca^{2+}]_i$ level (nM)
0
1
2
Time (min)
B
800
600
400
200
0
Estimated $[Ca^{2+}]_i$ level (nM)
resting
peak response
1st BK
2nd BK
3rd BK
C
Ca^{2+}- depleted saline
800
600
400
200
0
Estimated $[Ca^{2+}]_i$ level (nM)
resting
peak response
1st BK
2nd BK
3rd BK

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. 3C). 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. 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. 3C). These data suggest that bradykinin mobilizes calcium from internal stores and that these stores must refill with calcium from the extracellular medium.

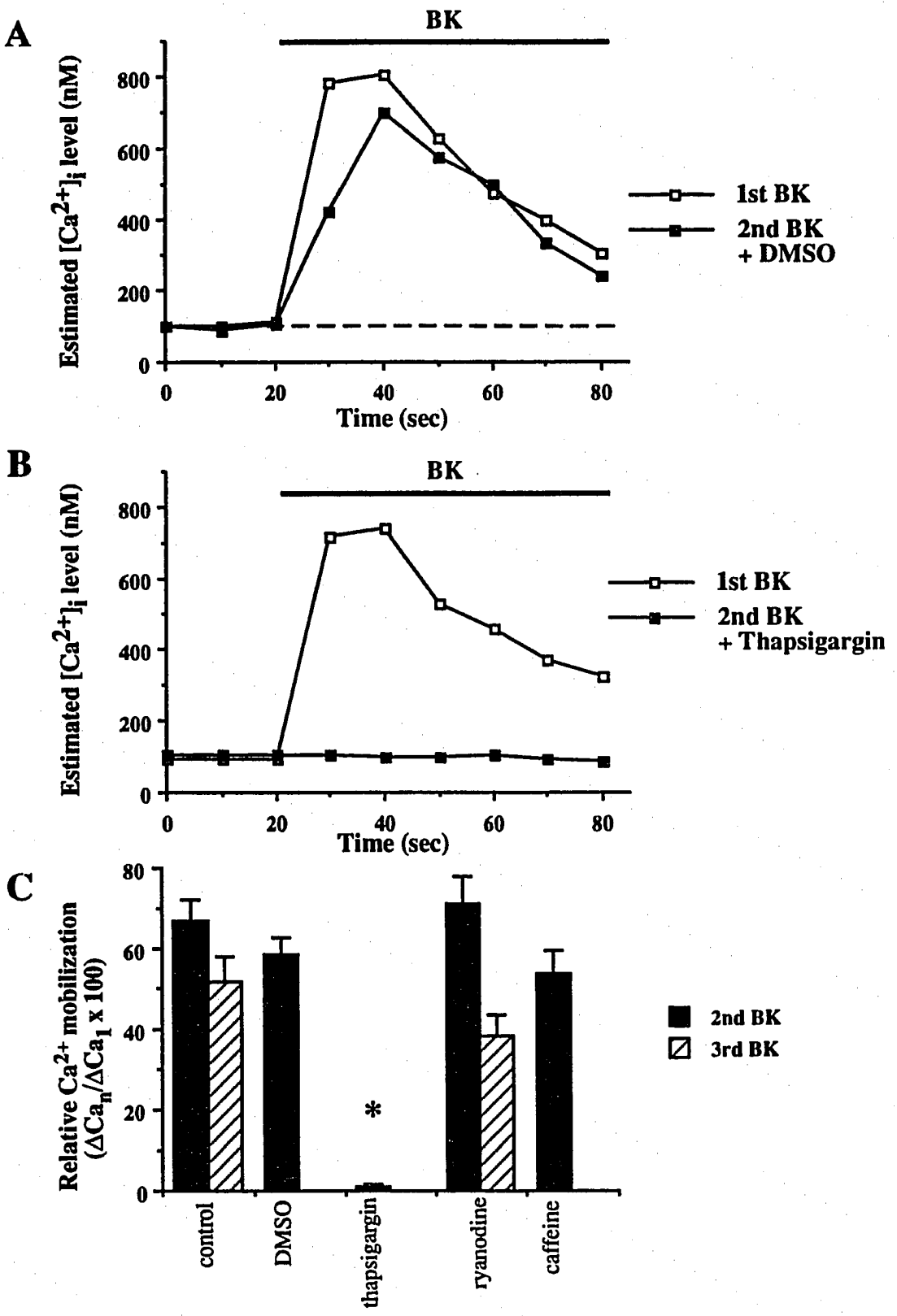
Calcium mobilization from an internal store is further supported by observations using thapsigargin, an inhibitor of the Ca^{2+}-ATPase of internal calcium stores. Following the first addition of bradykinin, thapsigargin (1 mM) was added to the culture to prevent reloading of internal stores with calcium. 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 which were incubated in the DMSO carrier saline (n=26, Scheffeé's test, $p<0.001$; Fig. 4) 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. 4C). 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. 4C). 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 (Ritchie et al., 1987).

Excitatory amino acid release from Schwann cells is calcium-dependent

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. 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. 5A) in calcium-depleted saline. However, a second application of bradykinin in calcium-depleted saline did not cause significant glutamate release

Figure 4. Bradykinin mobilizes calcium from internal calcium stores. **A)** Two applications of bradykinin (BK, 10 nM) reliably elevate internal Schwann cell calcium. Addition of DMSO (0.1 % v/v) did not attenuate the bradykinin induced calcium response. **B)** Addition of thapsigragin (1μM) in DMSO following the first addition of bradykinin, prevented subsequent applications of bradykinin from mobilizing internal calcium. **C)** Relative Ca^{2+} mobilization is expressed as percentage values of $[Ca^{2+}]_i$ accumulation (peak value subtracted from resting; DCa) 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. Points (in **A** and **B**) and bars (in **C**) 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
BK
Estimated $[Ca^{2+}]_i$ level (nM)
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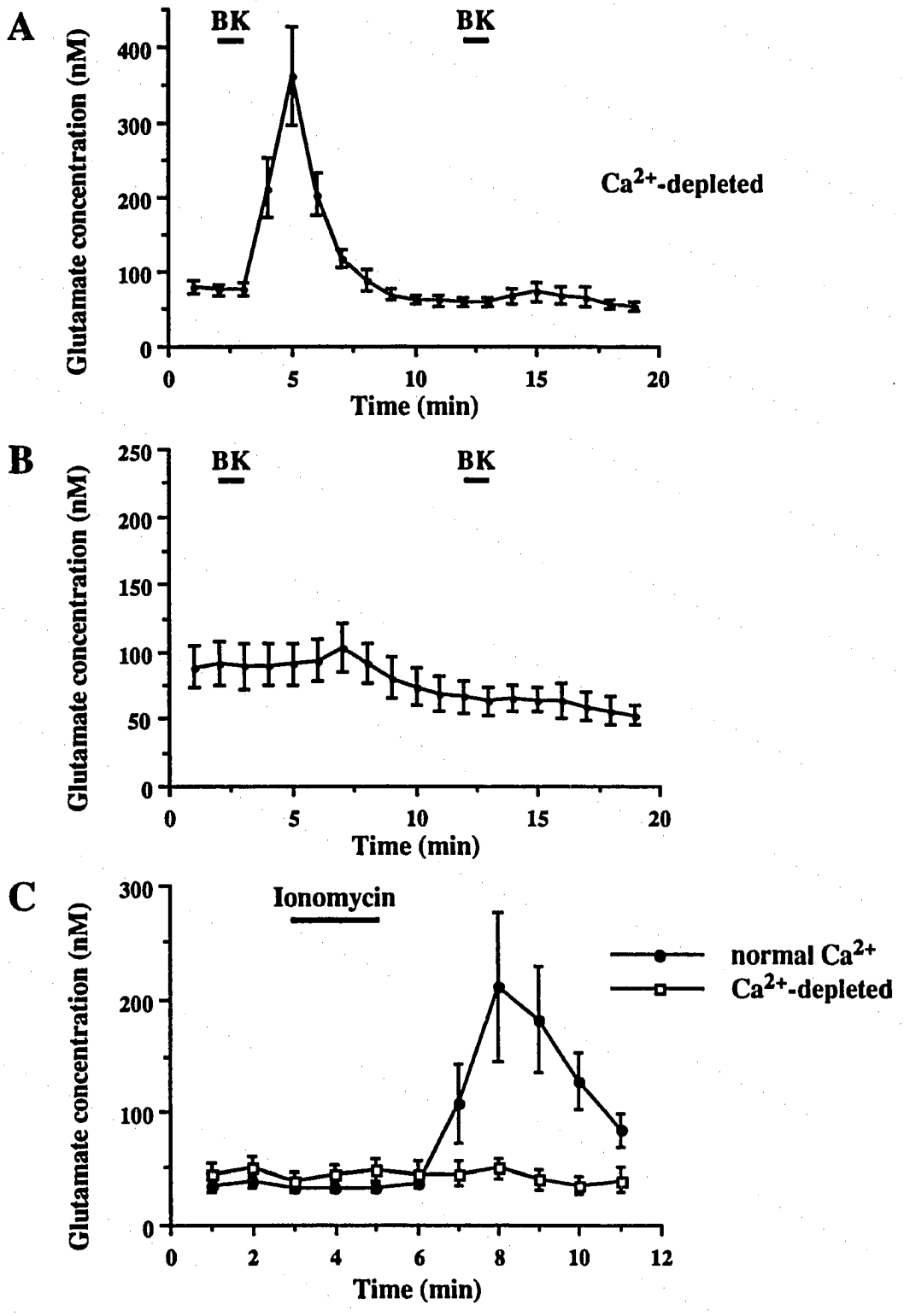
600
400
200
0
0
20
40
60
80
Time (sec)
1st BK
2nd BK
+ DMSO
B
BK
Estimated $[Ca^{2+}]_i$ level (nM)
800
600
400
200
0
0
20
40
60
80
Time (sec)
1st BK
2nd BK
+ Thapsigargin
C
Relative Ca^{2+} mobilization
($\Delta Ca_n/\Delta Ca_1$ x 100)
80
60
40
20
0
*
control
DMSO
thapsigargin
ryanodine
caffeine
2nd BK
3rd BK

Figure 5. Calcium is necessary and sufficient for glutamate release from DRG glia. The first application of bradykinin (BK) caused glutamate release from glial cells bathed in calcium-depleted saline. However, a second application of bradykinin 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 was a slight augmentation of basal glutamate levels in the supernatant. Points represent means ± SEM.


A
BK
BK
Ca2+-depleted
Glutamate concentration (nM)
400
300
200
100
0
0
5
10
15
20
Time (min)
B
BK
BK
Glutamate concentration (nM)
250
200
150
100
50
0
0
5
10
15
20
Time (min)
C
Ionomycin
normal Ca2+
Ca2+-depleted
Glutamate concentration (nM)
300
200
100
0
0
2
4
6
8
10
12
Time (min)

(n=6; Student t-test, $p>0.5$; Fig. 5A). 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. 5B).

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) stimulated the release of both glutamate (n=6; Student t-test, $p<0.025$, Fig. 5C) and aspartate (data not shown). Ionomycin was without stimulatory effects when glia were bathed in calcium-depleted saline (n=6; Student t-test, $p>0.9$; Fig. 5C). Taken together, these data demonstrate that calcium is mobilized in Schwann cells coincident with the timing of EAA release. This calcium mobilization is both necessary and sufficient for stimulating the release of EAAs from Schwann cells.

<u>p-chloromercuriphenylsulphonic acid does not inhibit EAA release from Schwann cells</u>

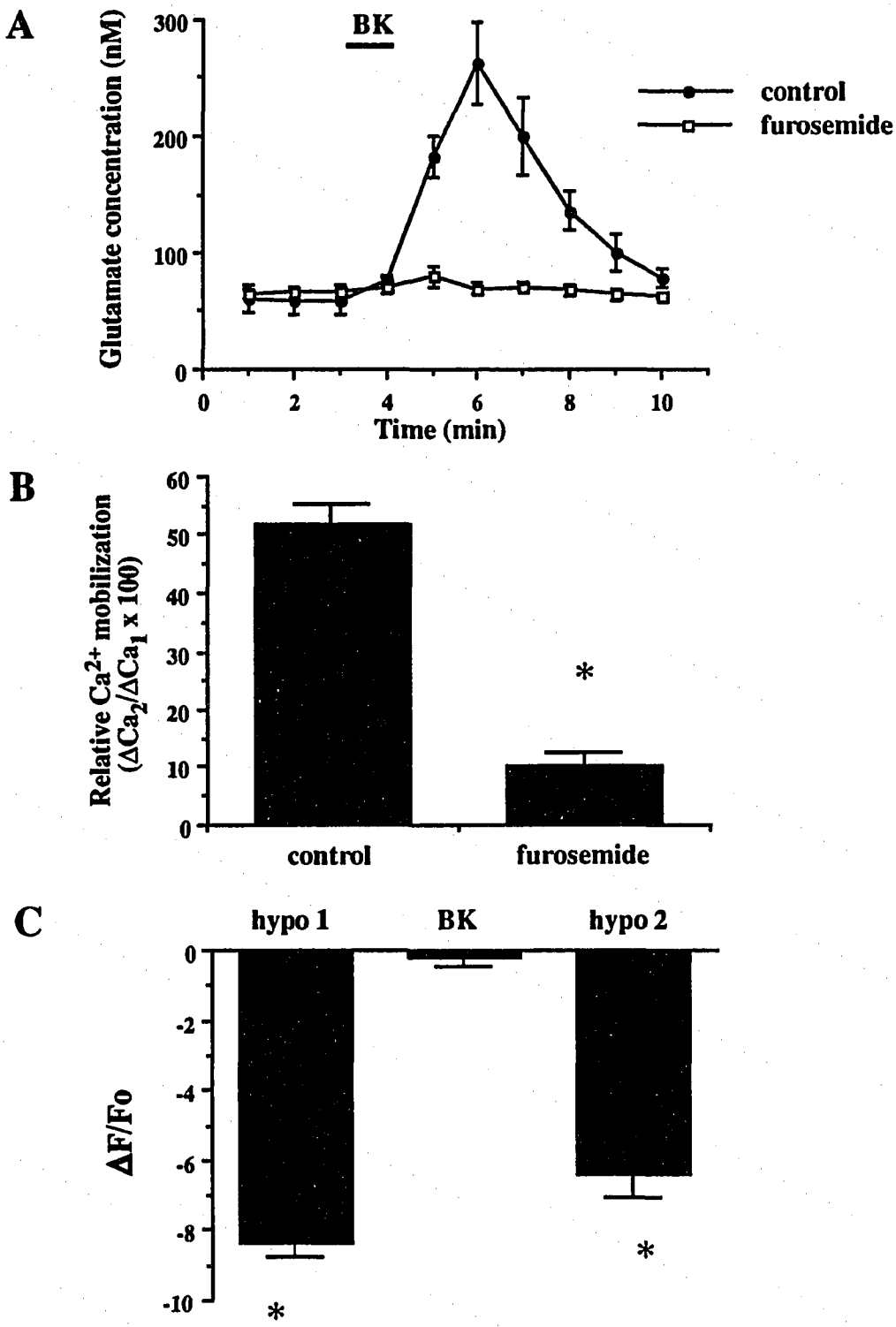
Glutamate can be released from cells during depolarization through the reversal of an electrogenic glutamate uptake carrier (Szwatkowski et al., 1990). To determine whether this mechanism might regulate glutamate release from DRG Schwann cells, we depolarized them using elevated potassium saline. Application of elevated K^+ (50 mM) did not cause a change in Schwann cells calcium levels (n=27; Student t-test, $p>0.1$) nor stimulated EAA release from DRG glia. In control conditions we detected 48 ± 7 nM glutamate in the superfusate. Following addition of 50 mM K^+ saline we detected 46 ± 6 nM glutamate (n=6,

Student t-test, $p>0.7$). The lack of glutamate release from DRG glia during the application of elevated K^+ saline is not consistent with a role for the reversal of a glutamate uptake carrier in mediating the bradykinin-induced release of EAAs from DRG Schwann cells. Futhermore, addition of the glutamate transporter inhibitor p-chloromercuriphenylsulphonic acid (p-CMPS; 50 mM) did not reduce the bradykinin stimulation of glutamate and aspartate release. However consistent with previous observations using glutamate transporter inhibitors (Balcar and Johnston, 1971; Isaacson and Nicoll, 1993), p-CMPS did elevate the basal level of glutamate in the bathing medium.

EAA release from Schwann cells is not mediated by a cell swelling-dependent mechanism.

Hyposmotic media cause a swelling-dependent release of aspartate and taurine from cortical astrocytes (Kimelberg et al, 1990). This release is sensitive to anion transport inhibitors. We determined whether Schwann cells have a similar sensitivity by incubating them in furosemide (5 mM). Bradykinin-induced release of glutamate was reduced by 98% ($n = 5$; $p < 0.0001$, Student t-test) in the presence of furosemide (Fig. 6A). To ask whether furosemide inhibits glutamate release by acting at the level of the calcium signal we determined the effect of furosemide on bradykinin-induced calcium mobilization. Using repeated applications of bradykinin (see Fig. 4 for protocol) we found that addition of furosemide (5mM) significantly attenuated ($n = 43$; $p < 0.0001$, Student t-test) the ability of the second application of bradykinin to mobilize calcium (Fig. 6B).

Figure 6. Furosemide, an anion transporter blocker, inhibits bradykinin-induced glutamate release from Schwann cells. In **A)** an application of bradykinin in presence of furosemide (5 mM, Sigma) did not cause glutamate release from Schwann cells. In parallel, bradykinin-induced calcium mobilization is greatly attenuated by the presence of furosemide in bathing medium (**B**). Relative Ca^{2+} mobilization is expressed as percentage values of $[Ca^{2+}]_i$ accumulation (peak value subtracted from resting; DCa) during the 2nd application of bradykinin (ΔCa_2) compared to the 1st (ΔCa_1) (relative calcium mobilization $=\Delta Ca_2/\Delta Ca_1 \times 100$). (C) BCECF-loaded Schwann cells were used to determine the effect of bradykinin on cell volume. BCECF emission intensity, in response to excitation at 440 nm, was used to monitor cell volume. Addition of hyposmotic medium significantly reduced the fluorescence intensity. Subsequent addition of bradykinin at a concentration of 10 nM, which stimulates calcium elevations and EAA release, did not change the volume of Schwann cells. A second addition of hyposmotic medium demonstrated, however, that these cells were still able to respond with volume changes. Points (in **A**) and bars (in **B** and **C**) represent means $\pm$ SEM. Student t-test was used to determine the significance of furosemide effect on bradykinin-induced calcium mobilization while in (C) a paired t-test was used. Significance was found to be $p<0.0001$ (*).


A
BK
Glutamate concentration (nM)
300
200
100
0
0
2
4
6
8
10
Time (min)
control
furosemide
B
Relative Ca^{2+} mobilization
($\Delta Ca_2/\Delta Ca_1$ x 100)
60
50
40
30
20
10
0
*
control
furosemide
C
hypo 1
BK
hypo 2
0
-2
-4
-6
-8
-10
$\Delta F/F_0$
*
*

Since furosemide reduces bradykinin-induced calcium mobilization, it is not clear whether the release mechanism *per se* is sensitive to this anion transport inhibitor as has been demonstrated in astrocytes in response to hyposmotic media (Kimelberg et al, 1990). Therefore, to further test whether bradykinin stimulates glutamate release from Schwann cells through a cell swelling mechanism we monitored cell volume during bradykinin application. Schwann cells were loaded with BCECF and the fluorescence emission was monitored in response to excitation at the pH-insensitive wavelength of 440 nm. Sequential additions of hyposmotic media repeatedly and reversibly reduced the intensity of BCECF fluorescence demonstrating that this method can detect swelling of Schwann cells (Fig. 6C; $n = 36$, $p < 0.0001$; paired t-test) . However, application of bradykinin (10 nM, 1 minute), a stimulus which mobilizes calcium and evokes EAA release, did not change Schwann cell volume (Fig. 6C; $n = 36$, $p > 0.25$; paired t-test). Since bradykinin does not regulate cell volume, it does not liberate EAAs from Schwann cells through a previously described cell swelling-dependent release mechanism.

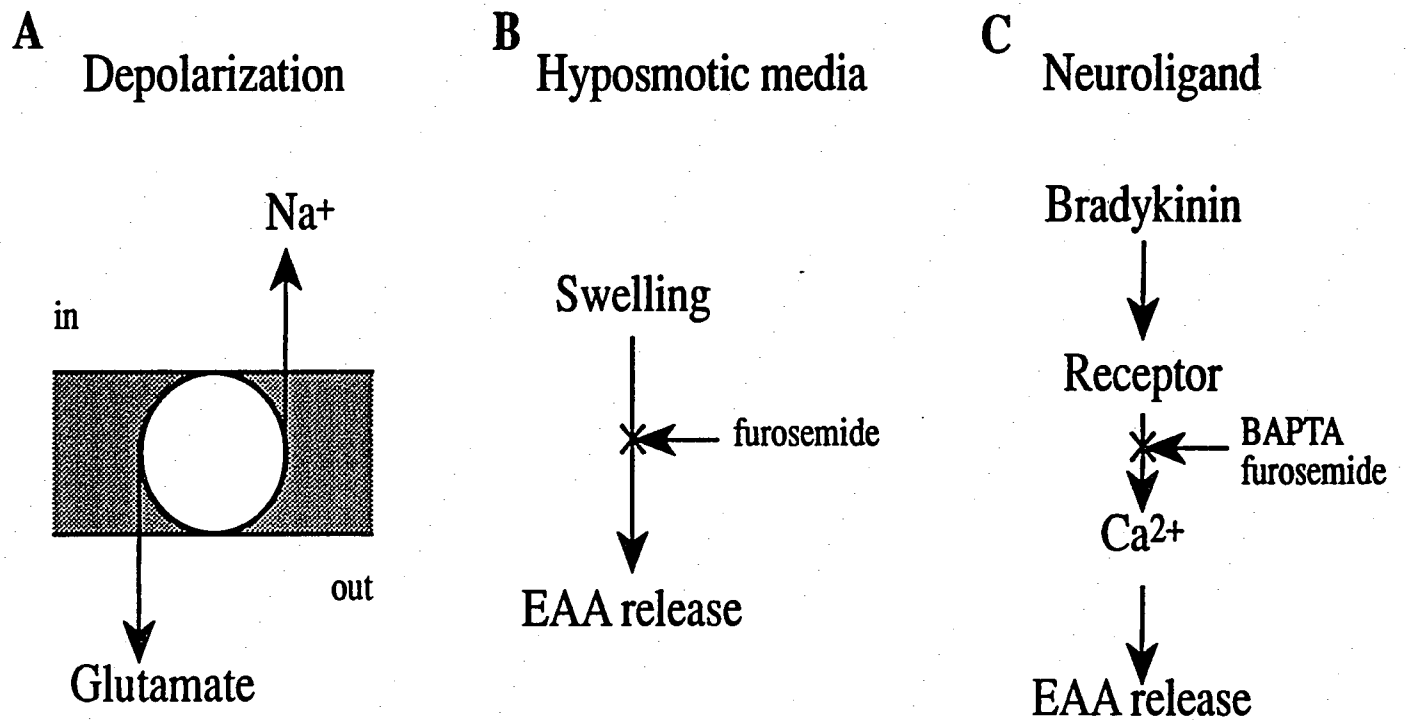
Discussion

Our data demonstrate that the release from Schwann cells of the excitatory amino acids, glutamate and aspartate, is regulated by the neuroligand bradykinin. Bradykinin causes the mobilization of calcium from internal stores in Schwann cells within seconds of addition to cultures. This calcium elevation is sustained for several minutes. In parallel cultures, bradykinin releases glutamate and aspartate within one minute of delivery of bradykinin to the superfusion

chamber. Thus, the bradykinin-induced calcium elevation is temporally coincident with the release of EAAs raising the possibility that it is required for EAA release. To test this hypothesis we asked whether calcium elevations are both necessary and sufficient for EAA release. When we by-passed the bradykinin receptor and directly elevated internal calcium using calcium ionophore EAA release was stimulated as long as a source of calcium was present. The calcium-elevation stimulated by bradykinin was also shown to be necessary for the release of EAAs. When calcium mobilization was blocked using BAPTA-AM or by removing external calcium during repeated bradykinin applications, bradykinin no longer stimulated the release of EAAs from Schwann cells. Taken together these data demonstrate that bradykinin mobilizes internal calcium in Schwann cells which is both necessary and sufficient to stimulate the release of glutamate and aspartate.

A key issue to address is the nature of the release mechanism that is responsible for EAA liberation from Schwann cells (Attwell, 1994; Smith 1994). This release mechanism may take at least three forms (Fig. 7). Glutamate can be released through the reversal of a glutamate transporter (Fig. 7A). Retinal Müller cells, for example, release glutamate in a calcium-independent fashion in response to elevated $[K^+]_o$ due to the reversal of the normal glutamate uptake mechanism (Szwatkovski et al., 1990). This mechanism probably does not account for the release of EAAs in response to bradykinin in DRG Schwann cells since the action of bradykinin is calcium-dependent, glutamate release is not stimulated by depolarizing stimuli which would reverse the glutamate uptake carrier and p-CMPS does not block bradykinin-induced release of EAAs.

Figure 7. Summary of EAA release mechanisms. Previous studies have demonstrated that EAAs can be released from cells due to (**A**) the reversal of an electrogenic transporter during depolarization (Szatkowski et al., 1990). Alternatively hyposmotic medium causes cell swelling which stimulates EAA release that is sensitive to the anion transport inhibitor, furosemide (**B**) (Kimelberg et al., 1990). Since Schwann cell depolarization does not induce EAA release, the glutamate transport inhibitor p-CMPS does not inhibit bradykinin stimulated EAA release and since bradykinin does not induce swelling of Schwann cells, neither of these release mechanisms is likely to be responsible for neuroligand-induced EAA release. The properties of the bradykinin induced EAA release mechanism of Schwann cells are summarized in (**C**). Bradykinin acts on a B_2 receptor which leads to a furosemide-sensitive elevation of calcium. Calcium ions, liberated from internal stores, are both necessary and sufficient to stimulate EAA release from Schwann cells. Further studies are required to determine whether this calcium-dependent release mechanism is similar to the neuronal vesicular release mechanism of synaptic transmission.


A
Depolarization
Na+
in
out
Glutamate
B
Hyposmotic media
Swelling
furosemide
EAA release
C
Neuroligand
Bradykinin
Receptor
BAPTA
furosemide
Ca2+
EAA release

In cortical astrocytes hyposmotic medium causes cell swelling and the consequent release of glutamate, taurine and aspartate (Kimelberg et al., 1990; O'Connor and Kimelberg, 1993; Fig. 7B). An anion transport blocker, furosemide, reduces cell swelling dependent release from cortical astrocytes (Kimelberg et al., 1990). This raises the hypothesis that bradykinin causes cell swelling which in turn stimulates EAA release from Schwann cells. We therefore tested the action of furosemide on EAA release. In Schwann cells furosemide reduced bradykinin-stimulated calcium mobilization and EAA release. Since furosemide significantly modified bradykinin-induced calcium signaling this experiment did not allow us to determine whether the cell swelling dependent release mechanism is utilized. Therefore, we directly measured cell volume and asked whether bradykinin induces cell swelling. Using BCECF our data clearly demonstrated that while hyposmotic media induced swelling of Schwann cells, bradykinin did not. These data are not consistent with bradykinin inducing the release of EAAs through the previously described volume regulated mechanism (Kimelberg et al., 1990; O'Connor and Kimelberg, 1993). Furthermore, these previous studies have shown that cell swelling induced EAA release is calcium-independent, while calcium is both necessary and sufficient for bradykinin-induced EAA release from Schwann cells.

A third potential mechanism of bradykinin-stimulated EAA release is through a membrane/vesicle release mechanism similar to that of neurons. 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 Schwann

cells. However, these observations do not rule out quantal membrane release mechanisms since muscle (Dan and Poo, 1992) and fibroblasts (Adler et al., 1993) have been demonstrated competent to release neurotransmitters in a quantal fashion.

Figure 7C summarizes our current understanding of the bradykinin-induced EAA release mechanism. Bradykinin, acting through a receptor, mobilizes internal calcium which is necessary and sufficient to stimulate EAA release from Schwann cells independent of cell volume changes. The nature of this calcium-dependent release apparatus remains to be defined.

The regulated release of neurotransmitters from glia may be a widespread property. Stimulation of motoneurons leads to the elevation of calcium in the Schwann cells which surround the presynaptic terminal (Jahromi et al., 1992). After denervation, Schwann cells have been shown capable of releasing acetylcholine (Dennis and Miledi, 1974). Depolarization of hippocampal astrocytes causes the calcium-dependent release of the amino acid taurine, but not glutamate (Philibert et al., 1988). Recently, we demonstrated that glial cells from the cerebral cortex release aspartate and glutamate in response to bradykinin (Parpura et al., 1994). Since many neurotransmitters utilized by the nervous system can mobilize calcium in glia (Reisner et al., 1989; Cornell-Bell et al., 1990; Glaum et al., 1990; Charles et al., 1991; Cornell-Bell and Finkbeiner, 1991; Inagaki et al., 1991; Jensen and Chiu, 1991; McCarthy and Salm, 1991), it is possible that neurons have in addition to their fast neuron-neuron synaptic actions, a calcium mobilizing action on neighboring glia (Dani et al., 1992) which in turn leads to the release of other neurotransmitters from glia that may serve secondary roles in glia-neuronal transmission. In support of this possibility

recent studies have demonstrated that glia can signal to neurons (Parpura, 1993; Nedergaard, 1994; Parpura et al., 1994).

The specific roles for release of excitatory amino acids from glial cells remains to be elucidated. In the peripheral nervous system, bradykinin is released from its precursors, the kininogens, by the action of the enzyme kallikrein in response to trauma (Dray and Perkins, 1993). Thus, during trauma and tissue damage, bradykinin is produced where it can initiate the transmission of nociceptive information. Perhaps the bradykinin induced-release of glutamate and aspartate from Schwann cells is involved in mediating certain aspects of immediate responses to injury. In the central nervous system, 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 release from glia also contributes to neurodegenerative disorders.

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CHAPTER 3. ATP-EVOKED Ca^{2+}-DEPENDENT RELEASE OF EXCITATORY AMINO ACIDS FROM CULTURED SCHWANN CELLS

A paper to be submitted to Brain Research

Fang Liu, Ksenija Jeftinija, Srdija Jeftinija

Abstract

The specific objective of this study was to determine the mechanism by which ATP evokes release of EAAs from cultured Schwann cells. Basal concentrations of aspartate and glutamate in Schwann cell cultures were 7.64±0.74 nM (mean±SEM) and 30.52±3.89 nM, respectively. Perfusion application of 100μM ATP for 2 minutes to cultured Schwann glia resulted in an 201±28 % increase of aspartate and 203±15 % increase of glutamate. A second application of ATP 10 minutes after the first application similarly caused EAA release, although the magnitude of this response was attenuated. Application of ATP to glia for 10 minutes resulted in a peak increase in the release followed by a decline to a plateau significantly higher than baseline release. Bath application of adenosine (100μM) was without effect on release of EAAs suggesting involvement of P_2 receptors. The release of EAAs evoked by ATP was not abolished in low Ca^{2+}-EGTA saline. Pretreatment of the glial cultures with 50μM BAPTA-AM abolished the effect of ATP. Thapsigargin (1μM), an inhibitor of Ca^{2+}-ATPase or the Ca^{2+} pump of internal stores, significantly reduced the release of EAAs evoked by ATP. Our results show that

ATP evokes the release of EAAs from cultured Schwann cells by activating intracellular calcium stores.

Introduction

Although the functional importance of intracellular adenosine 5'-triphosphate (ATP) levels in cellular metabolism has been recognized for many years, the observation that extracellular ATP can influence neurotransmission, cardiac function, muscle contraction and other biological processes is more recent (reviewed by Gordon, 1986; Burnstock, 1990). ATP acting as a neurotransmitter or neuromodulator performs important functions in the central and peripheral nervous system.

It has been shown that ATP can be released or co-released with acetylcholine or norepinephrine from nerve endings (Burnstock, 1976; 1986a; 1986b; Fyffe and Perl, 1984). Extracellular ATP excites a suppopulation of rat dorsal horn neurons (Jahr and Jessel, 1983) and rat sensory neurons (Krishtal et al., 1988). It was also reported ATP depolarized cultured hippocampal neurons and induced release of glutamate from the neurons (Inoue et al., 1992).

The actions of ATP are mediated by P_2-purinoceptors which are present on many cell types including neurons and astrocytes (Phillis and Wu, 1981; Salter and Henry, 1985; Gebicke-Haerter et al, 1988; Pearce et al., 1989). There is evidence that activation of P_2-purinoceptors leads to an increase in intracellular calcium in a variety of excitable and unexcitable cells (O'Connor et al., 1991; El-Moatassim et al., 1992). Two mechanisms are involved in ATP-evoked intracellular calcium increase. First, ATP can activate nonselective cation

channels, resulting in depolarization and subsequent opening of voltage-dependent Ca^{2+} channels (Nakazawa and Matsuki, 1987; Friel, 1988). The increased calcium influx is also the result of direct activation of ATP-gated cation channels (Benham and Tsien 1987; Charest et al., 1987). Second, the extracellular ATP can stimulate the breakdown of inositol phospholipids and the resulting increase in inositol 1,4,5,-triphosphate (IP_3) is responsible in part for the elevation of cytosolic Ca^{2+} (Boeynaenis et al., 1988; Danziger et al., 1988).

There is an increasing body of evidence showing that neuroglia may be more actively involved in brain function than has been previously thought. It has been demonstrated that neuroglia have multiple roles in the nervous system which include the regulation of extracellular potassium, the uptake of excitatory amino acids (EAAs) and the release of neurotransmitter such as glutamate (Glu), aspartate (Asp) and acetylcholine (ACh) (Nicholls and Atwell, 1990; Barres, 1991; Kanai et al., 1993; Smith, 1994). Our recent study shows that the release of the excitatory neurotransmitter glutamate from glial cells plays a key role in glia-neuron signalling (Parpura et al., 1994a).

Although it has been determined that extracellular ATP can stimulate Ca^{2+} influx and increase intracellular calcium in cultured astrocytes (Neary et al., 1991; Salter and Hicks, 1994), little is known about the effects of extracellular ATP on Schwann cells, which are the predominate glial cells in the peripheral nervous system. Our previous study shows that the neuroligand bradykinin can induce the release of EAAs from cultured Schwann cells in a Ca^{2+}-dependent manner (Parpura et al.; 1994b). This evidence prompted us to propose that extracellular ATP might influence the functions of Schwann cells. In this study, we first

demonstrate that ATP can evoke release of EAAs from cultured Schwann and the intracellular Ca^{2+} plays a role in the release of EAAs.

Materials and Methods

Cell culture Organotypic DRG cultures will be prepared according to modification of methods described by Gähwiler (1984), and previously reported (Jeftinija et al., 1991). Following decapitation, DRG from one to four day old Sprague-Dawley rats will be rapidly removed and washed in cold (4°C) oxygenated Gey's balanced salt solution (GBSS, Gibco) modified by the addition of 2.0% glucose. Culturing will be carried out on glass coverslips which are specially prepared by soaking 24 hours in xylene, 4 hours in acetone, 24 hours in absolute alcohol, and dried for 30 min at 80° C. Isolated explants will be placed on a glass coverslip completely covered with chicken plasma (Sigma) and coagulated by adding thrombin solution (Sigma). The coverslips bearing the explants will be placed into 35 mm Petri dishes. Cultures will be incubated in a humid 5% CO_2 atmosphere at 37° C. The culture medium contained 25% horse serum, 25% Earls Balanced Salt Solution, 50% Basal Medium Eagles with added glucose (6.4 mg/ml). When the culture is incubated for 3-4 days, it has a lot of processes arising from DRG explant and also Schwann cells in growing zone. In order to get DRG glial cells, we'll remove the central part of explant which contains neuronal cell bodies from established cultures (4 to 5 days old culture). The residual glia cells left behind will be maintained for additional 72 hours. Fibroblasts will be removed by incubating the culture with thy1.1 antibody (hybridoma supernatant, American Type Culture, No. TIB103) and addition of rabbit serum complement (Sigma). This procedure is used to lysogenize the fibroblasts and

purified Schwann cells. The DRG glial cells and fibroblasts were identified by immunocytochemistry using antibodies against low affinity NGF receptor and fibronectin. Immunocytochemistry revealed that at least 95% of the cell population were Schwann cells.

Release method and HPLC This procedure has been developed in our laboratory. The coverslips with cell cultures are mounted in a 50 ml perfusion chamber. The modified Ringer's perfusion solution (in mM: NaCl 127, KCl 1.9, $KH_2PO_4$1.2, $CaCl_2$ 2.4, $MgSO_4$ 1.3, $NaHCO_3$ 26, glucose 10, pH7.4) bubbled with 95% oxygen and 5% carbon dioxygen is pumped into the perfusing system by a minipuls pump at a rate of 200 ml/min. After an equilibration period of half an hour, samples of perfusate are collected every minute for amino acids determinations. The amino acid content in the samples is determined by high-performance liquid chromatography (HPLC) with fluorescence detection using a Shimadzu system. Prior to injection, aliquots of the samples are derivatized with o-phthalaldehyde (OPA) 2-mercaptoethanol reagent (Pierce). Chromatography is performed on a 15 cm Rainin column using a pH 5.9 sodium acetate methanol gradient.

The concentrations of Glu and Asp are expressed in nMols. Basal concentrations of EAAs were determined as the mean of the EAA content of three samples collected just prior to stimulation with ATP, adenosine or bradykinin. All data are presented as the mean values ± S.E.M.. The data were analyzed by Student's t-test.

Results

ATP evokes EAA release from dorsal root ganglion Schwann cells

The basal concentration of glutamate and aspartate was 30.52±3.89 nM (mean±SEM) and 7.64±0.74 nM, respectively. Application of 100μM ATP for two min produced a significant increase in the concentrations of Glu and Asp. The peak concentrations of Glu and Asp were 203±15 % (n=8, $p<0.0005$) and 201±28 % (n=8, $P<0.005$) of the basal concentration, respectively. These data show that extracellular ATP can stimulate the release of EAAs from cultured Schwann cells.

A second application of ATP similarly caused EAA release, although the magnitude of this response was attenuated. The second peak concentrations of glutamate and aspartate were 163± 11% (n=8, $P<0.0005$) and 176±11% (n=8, $P<0.0005$) of the control concentration, respectively (Figure 1a and Figure 1b).

Application of ATP to glial cultures for 10 minutes resulted in a biphasic response, which involved a increased glutamate peak that was 232±10 % (n=4, $P<0.005$) of the basal concentration followed by a declining release to a sustained release plateau significantly higher than the control release. The increased release returned to the basal concentration when ATP was removed (Figure 1c). This data illustrate that prolonged exposure of ATP to Schwann cells could induce long lasting release of EAAs.

P_1-purinoceptors are not involved in ATP-evoked release of EAAs

To test receptor specificity of ATP-evoked release of EAAs, we used adenosine to stimulate cultures. Application of adenosine (100 μM) for two minutes failed to induce release of EAAs from cultured Schwann cells

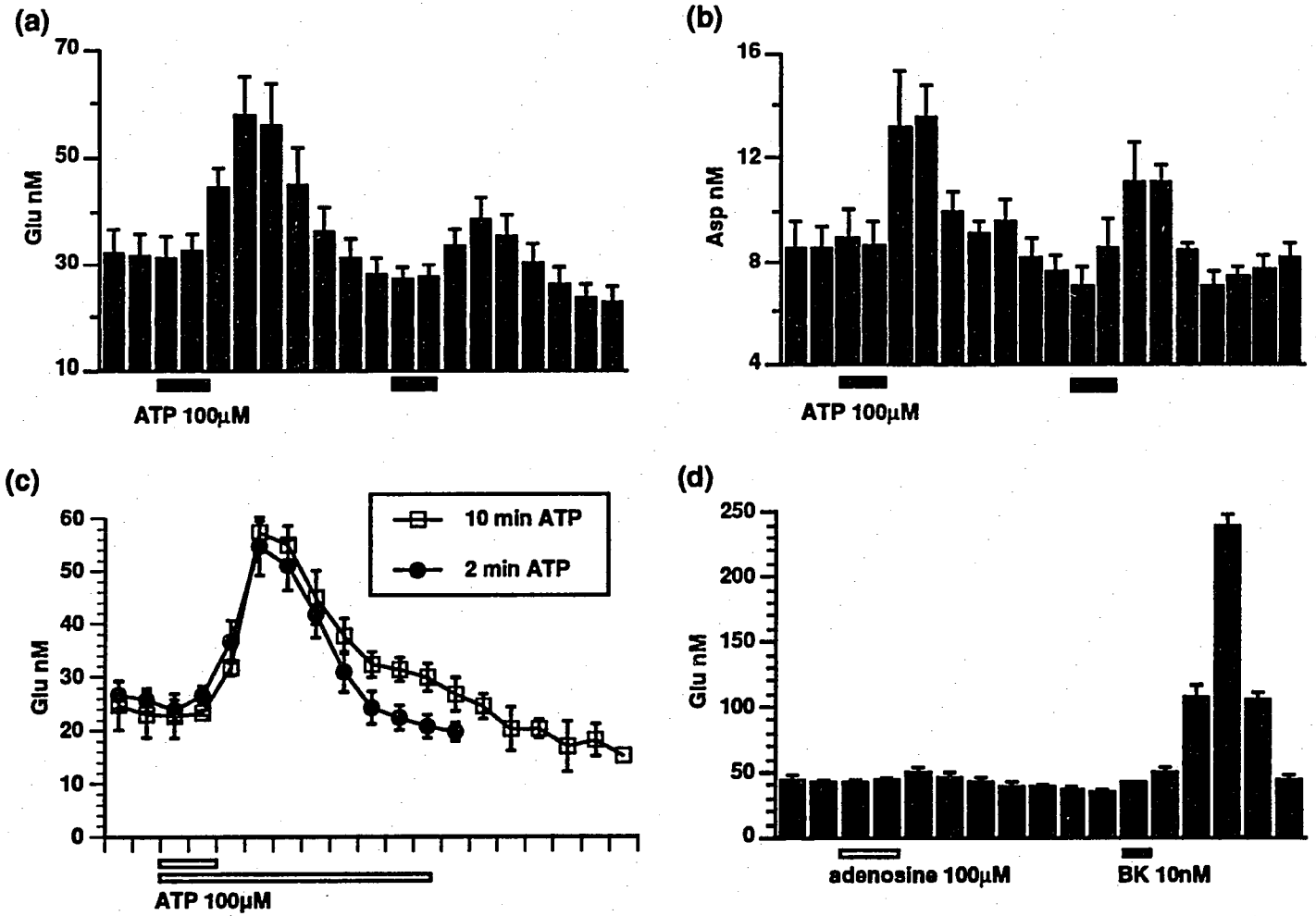
Figure 1. Time course of changes in the concentration of glutamate and aspartate following exposure of the cultures to ATP or adenosine.

(a) Application of 100μM ATP for two minutes significantly increased concentration of glutamate.

(b) Application of 100μM ATP for two minutes significantly increased concentration of aspartate.

(c) The effects of exposure of 100 μM ATP for different time periods. Application of ATP to the cultures for ten minute resulted in a biphasic response with a initial peak followed by a decline to a sustained plateau which is higher than that in control group which had a two minute application of ATP.

(d) Adenosine (100 μM) failed to induce release of glutamate. This suggests that P_1-purinoceptors are not involved in this release. Furthermore, it implies that the ATP-evoked release of glutamate might be mediated by P_2-purinoceptors.


(a)
Glu nM
70
50
30
10
ATP 100μM
(b)
Asp nM
16
12
8
4
ATP 100μM
(c)
Glu nM
60
50
40
30
20
10
0
10 min ATP
2 min ATP
ATP 100μM
(d)
Glu nM
250
200
150
100
50
0
adenosine 100μM
BK 10nM

(n=4, P>0.3). Exposure of 10 nM bradykinin to the cultures ten minutes after the application of adenosine evoked a six fold increase of glutamate release. This result indicates that these cultures responded to the stimulation of bradykinin very well, although they were not sensitive to adenosine (Figure 1d).

It has been proposed that there are two classes of purinergic receptors (Burnstock, 1978). One is the adenosine-sensitive P_1-purinoceptor, and the other one is the ATP-sensitive P_2-purinoceptor. The latter is insensitive to adenosine. Because adenosine did not induce the release of EAAs from cultured Schwann cells, the possibility that the P_1-purinoceptor is involved in this process is eliminated. Furthermore, it implies that ATP might evoke release of EAAs by activating P_2 -purinoceptors.

ATP-induced release of EAAs is calcium-dependent

ATP has been found to increase intracellular Ca^{2+} concentration in many cells (reviewed by Burnstock, 1990). On the basis of this evidence, we have investigated whether Ca^{2+} plays a role in ATP-induced release of EAAs.

Cultures were incubated with low Ca^{2+} (0.2mM), high Mg^{2+} (2.5 mM), EGTA (1 mM) saline for 30 minutes. We found that, after removing the extracellular calcium, ATP still caused a increased release of EAAs similar to that seen in the control group. The peak concentration of glutamate was 162±25 % of the basal concentration (n=6, P<0.05). This result demonstrated that extracellular calcium is not involved in ATP-induced release of EAAs from Schwann cells (Figure 2a).

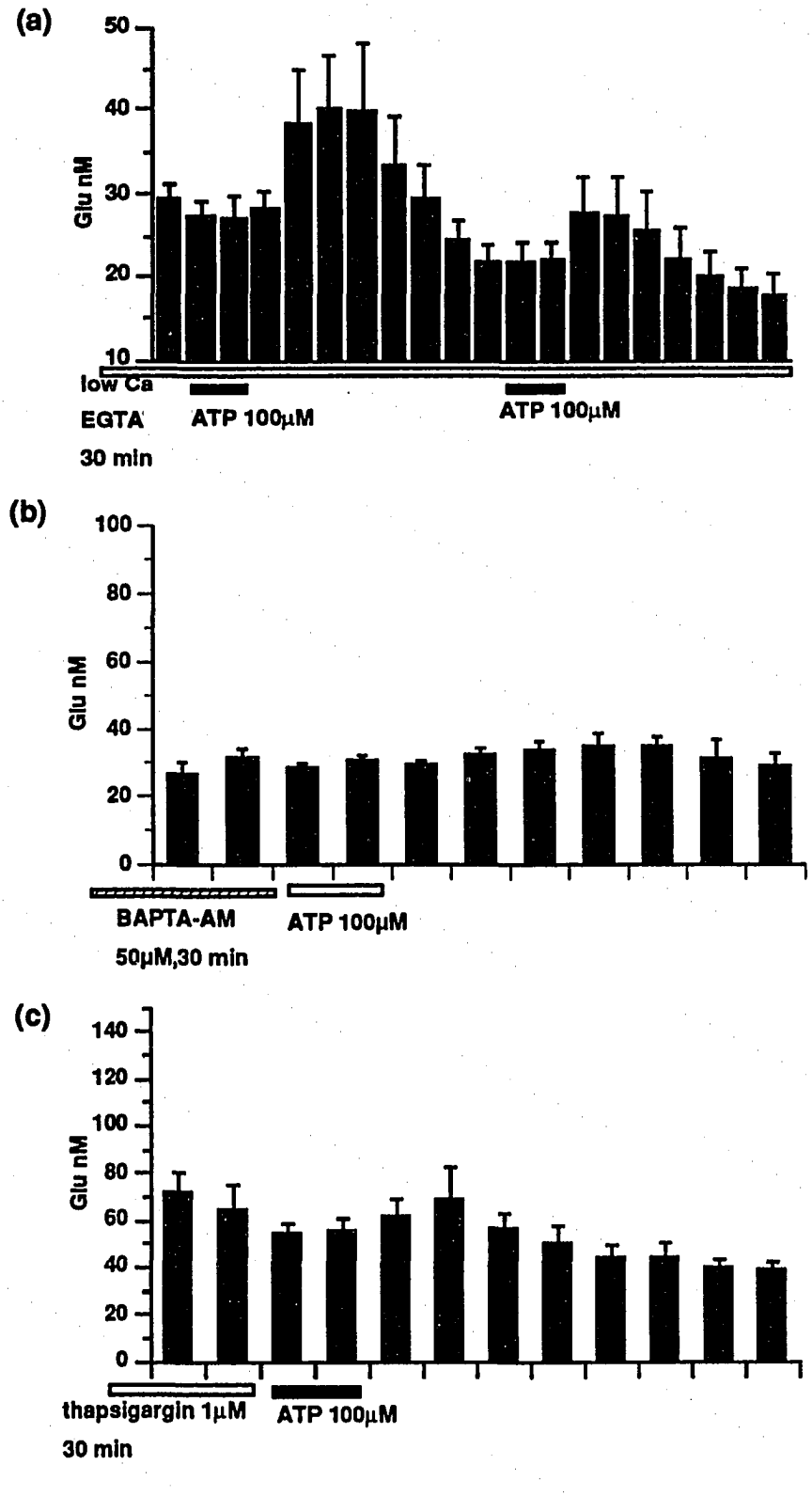
In order to determine whether Ca^{2+} from internal stores is involved in glutamate release, we pretreated cultures with 50 μM BAPTA-AM [1,2bis(2-aminophenoxy) ethane-N,N,N',N'-tetraceticacid, acetoxymethyl ester] saline for

Figure 2. ATP-evoked release of glutamate is Ca^{2+}-dependent.

(a) Low Ca^{2+}-EGTA saline didn't significantly affect the ATP-evoked release of glutamate.

(b) BAPTA-AM (50 μM), the membrane permeant calcium chelator, blocked the ATP-evoked glutamate release.

(c) Thapsigargin (1 μM), the inhibitor of the Ca^{2+}-ATPase of internal calcium stores, abolished the stimulatory effect of ATP on release of glutamate.


(a)
50
40
30
20
10
Glu nM
low Ca
EGTA
30 min
ATP 100μM
ATP 100μM
(b)
100
80
60
40
20
0
Glu nM
BAPTA-AM
50μM,30 min
ATP 100μM
(c)
140
120
100
80
60
40
20
0
Glu nM
thapsigargin 1μM
30 min
ATP 100μM

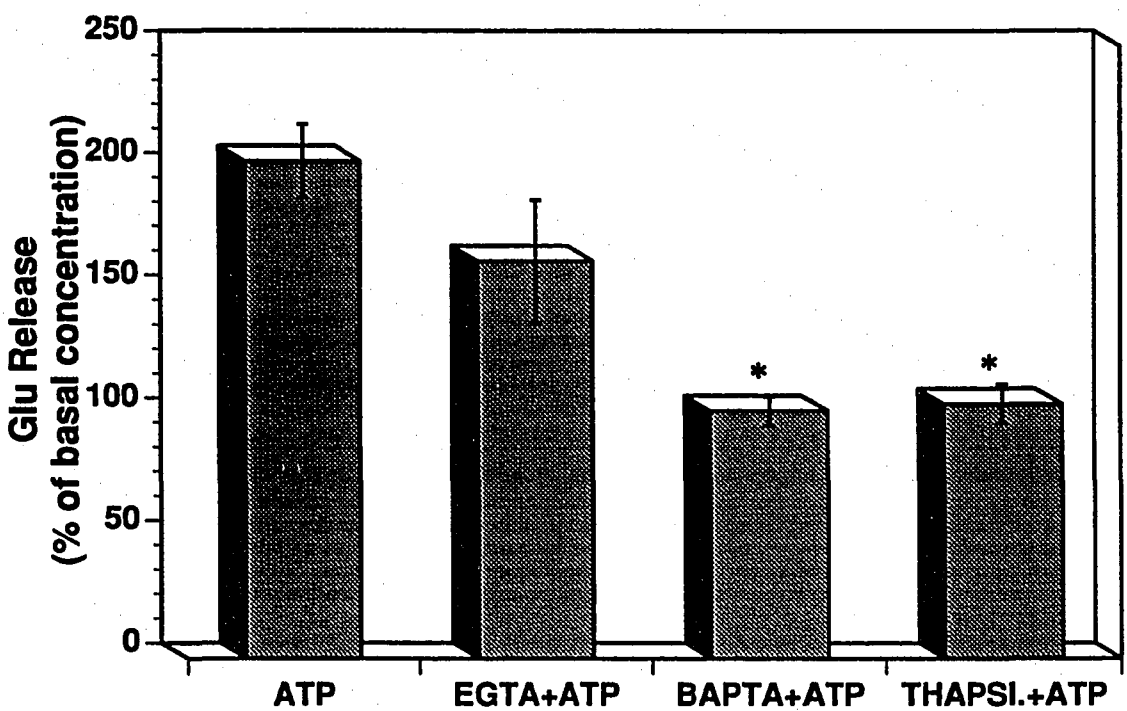
30 minutes. The ATP-induced maximal release of glutamate was only 101±6 % (n=4, P>0.4) of the control concentration. BAPTA-AM, the membrane permeant calcium chelator, blocked the effect of ATP on glutamate release. This result suggests that intracellular Ca^{2+} might be responsible for ATP-induced release of glutamate (Figure 2b).

To further test the involvement of intracellular Ca^{2+} in glutamate release, thapsigargin (1 μM), an inhibitor of the Ca^{2+}-ATPase of internal calcium stores (Thastrup, 1990), was applied to the cultures for 30 minutes before the application of 100 μM ATP. The maximal release of glutamate evoked by ATP was 104±7 % (n=4, P>0.3) of the control concentration in the presence of thapsigargin. This result confirms that ATP-induced release of EAAs from cultured Schwann cells depends on intracellular calcium (Figure 2c).

Taken together, the experiments above showed that application of 100 μM ATP for two minutes induced a two-fold increases in the concentration of glutamate released from Schwann cells. This effect of ATP on glutamate release was not significantly affected by low Ca^{2+}-EGTA saline (P>0.4). However, both 50 μM BAPTA (P<0.005) and 1 μM thapsigargin (P<0.005) significantly blocked ATP-evoked release of glutamate (Figure 3). These data demonstrate that extracellular ATP evokes the release of EAAs from cultured Schwann cells by activating intracellular calcium stores.

Figure 3. Summary of effects of different pretreatment on ATP-evoked release of glutamate.

Application of 100 μM ATP for two minutes evoked a two-fold increases in concentration of glutamate (marked as ATP). Pretreatment of low Ca^{2+}-EGTA saline didn't significantly affect this release (marked as EGTA+ATP). However, both BAPTA-AM and thapsigargin significantly blocked ATP-evoked glutamate release (marked as BAPTA+ATP and THAPSI+ATP, respectively). These data suggest that Ca^{2+} from internal stores is involved in ATP-evoked release of glutamate from cultured Schwann cells. (* =$P<0.005$)


250
200
150
100
50
0
Glu Release
(% of basal concentration)
*
*
ATP
EGTA+ATP
BAPTA+ATP
THAPSI.+ATP

Discussion

In this study, we present data demonstrating that extracellular ATP can evoke the release of the excitatory amino acids glutamate and aspartate from cultured Schwann cells.

It has been shown that neuroglia, including Schwann cells, release neurotransmitter. Dennis and Miledi (1974) reported that Schwann cells can release acetylcholine following axotomy of motoneurons. Szwatkouski et al. (1990) found that depolarization of retinal Müller cells stimulates the release of glutamate. Our previous studies show that the nonapeptide bradykinin can evoke the release of glutamate and aspartate from cultured astrocytes (Parpura et al., 1994a) and Schwann cells (Parpura et al., 1994b) in a calcium-dependent manner. The present study provides additional evidence that Schwann cells can release glutamate and aspartate in response to stimulation of ATP.

Extracellular ATP can activate specific cell surface receptors, P_2-purinoceptors (Burnstock, 1978). This purinergic receptor is insensitive to adenosine, which activates P_1-purinoceptors. We demonstrates that ATP significantly induced release of EAAs from Schwann cells, whereas adenosine doesn't stimulate the release. Although neither P_2-purinoceptors nor P_1-purinoceptors has ever been identified on the surface of Schwann cells, our data suggests that purinergic P_2-receptors might be involved ATP-induced release of EAAs.

ATP can cause an increase in intracellular calcium in many cell types. This process involves calcium entry across the plasma membrane via receptor-operated channels as well as calcium mobilization from internal stores via

receptor coupling to phosphatidylinositol hydrolysis (reviewed by Burnstock, 1990; Neary and Norenberg,1992). We found that ATP-evoked release of EAAs from cultured Schwann cells might result as a functional consequence of increases in intracellular calcium. Removing extracellular calcium by using low Ca^{2+}-EGTA saline did not significantly affect the release of EAAs from Schwann cells. This result indicated that at least Ca^{2+} influx is not involved in the release. In contrast, BAPTA-AM, which can penetrate cell membrane and chelate intracellular Ca^{2+}, abolished the ATP-evoked release of EAAs. This suggests that calcium from internal stores has to be responsible for this release. Thapsigargin, which depletes internal calcium stores, also blocked ATP-evoked release of EAAs. This result provided more evidence that ATP-induced release of EAAs from cultured Schwann cells is dependent upon intracellular calcium.

It has been shown that extracellular ATP confines its effects to glial cells. ATP activating its receptors evoked increases in intracellular calcium in astrocytes (Salter and Hicks, 1994). Furthermore, ATP not only evoked changes in astrocytic calcium signal, but also in protein phosphorylation and morphology (Neary and Norenberg, 1992). Our present study that ATP can induce release of EAAs from cultured Schwann cells first demonstrated that extracellular ATP has effects on Schwann cells, the glial cells in the peripheral nervous system. There is evidence that axon-to-Schwann cell signalling was mediated by neurotransmitter glutamate via quisqualate/kainate receptors in the squids (Liebernman et al., 1989). Our data provides new evidence that extracellular ATP might also serve as a mediator in neuron-glia signalling.

The functions of ATP-evoked release of EAAs from Schwann cells are not clear. Our recent study demonstrated that release of glutamate from astrocytes

mediates the process of astrocyte-neuron signalling (Parpura et al., 1994a). This prompts us to propose that the release of EAAs from Schwann cells might play a role in glia-neuron signalling in peripheral nervous system. Since ATP can be released under physiological and pathological conditions (Neary and Norenberg, 1992), ATP-evoked release of EAAs from Schwann cells might be involved in certain functions of Schwann cells in both situations.

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CHAPTER 4. GLUTAMATE-MEDIATED ASTROCYTE-NEURON SIGNALING

A paper published in Nature

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Srdija Jeftinija and Philip G. Haydon

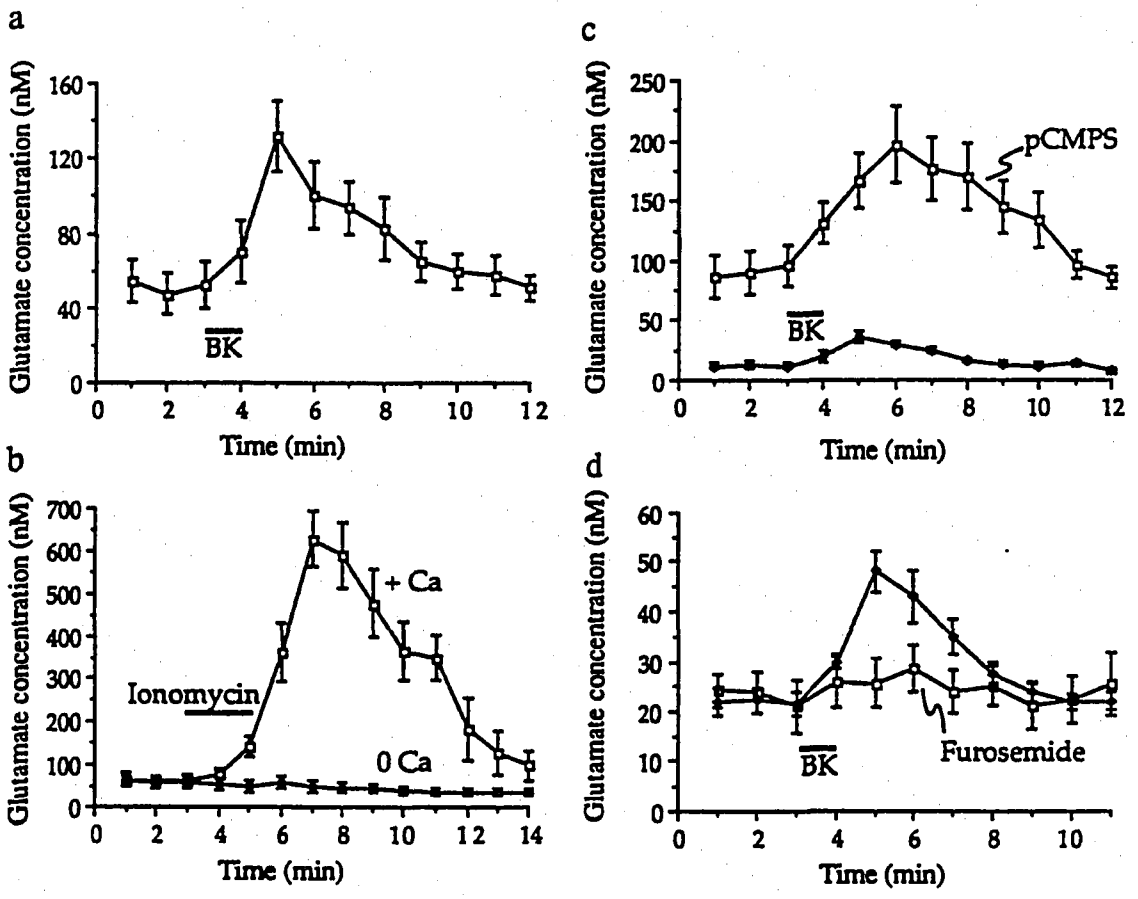
Neurotransmitter released from neurons is known to signal to neighboring neurons and glia [1-3]. Here we demonstrate an additional signaling pathway in which glutamate is released from astrocytes and causes an NMDA (N-methyl-D-aspartate) receptor-mediated increase in neuronal calcium . Internal calcium was elevated and glutamate release stimulated by application of the neuroligand bradykinin to cultured astrocytes. Elevation of astrocyte internal calcium was also sufficient to induce glutamate release. To determine whether this released glutamate signals to neurons, we studied astrocyte-neuron co-cultures. Bradykinin significantly increased calcium levels in neurons co-cultured with astrocytes, but not in solitary neurons. The glutamate receptor antagonists D-2-amino-5-phosphonopentanoic acid and D-glutamylglycine prevented bradykinin-induced neuronal calcium elevations. When single astrocytes were directly stimulated to increase internal calcium and release glutamate, calcium levels of adjacent neurons were increased; this increase could be blocked by D-glutamylglycine. Thus, astrocytes regulate neuronal calcium levels through the calcium-dependent release of glutamate.

The release of glutamate from neuron-free cultures of neocortical astrocytes was monitored using high-performance liquid chromatography (HPLC). The neuroligand bradykinin (100 nM) elevated glutamate levels in the superfusate ($n = 16$; $p < 0.02$, paired t-test; Fig. 1a). To ask whether internal calcium might play a role in glutamate release from astrocytes, we monitored internal calcium levels using fura-2. Bradykinin stimulated an elevation of astrocyte calcium levels from 99 ± 10 to 568 ± 89 nM (mean $\pm$ SEM, $n = 20$; Fig. 2). To determine whether elevated internal calcium is sufficient to stimulate glutamate release, we used the calcium ionophore ionomycin. Addition of ionomycin (5μM) in the presence of external calcium, but not in its absence, stimulated the release of glutamate from astrocytes (Fig. 1b). Furthermore, bradykinin did not cause significant glutamate release when calcium was removed from the external saline ($p > 0.1$, paired t-test). These data demonstrate that bradykinin induces the release of glutamate from neocortical astrocytes and that elevated internal calcium can induce glutamate release.

The role of glutamate transporters as mediators of bradykinin-induced glutamate release was investigated by using glutamate transport inhibitors. Consistent with previous observations [4,5], p-chloromercuriphenylsulfonic acid (p-CMPS, 50 μM) and L-trans-pyrrolidine-2,4-dicarboxylate (PDC, 100 μM - 1 mM) raised the basal level of glutamate in the astrocyte superfusate ($p < 0.02$, Mann-Whitney U-test [6]). Furthermore, neither p-CMPS (Fig. 1c, $n = 6$) nor PDC ($n = 6$) impaired the ability of bradykinin to stimulate glutamate release from astrocytes. While glutamate transporter inhibitors block glutamate uptake it is not known whether they also block reversed uptake. Furosemide, an anion transport inhibitor,

Figure 1. Bradykinin causes calcium-dependent release of glutamate from astrocytes. The superfusate from astrocyte cultures was collected at one minute intervals and levels of glutamate were measured using HPLC. a) Bradykinin (100 nM) elevates glutamate release from astrocytes (n = 4). b). Addition of ionomycin (5μM) to cultures stimulated glutamate release in calcium containing saline (+ Ca, n = 6), but not in its absence (0 Ca; n = 7). c) The glutamate transport inhibitor p-CMPS (50 μM) raised the basal level of glutamate and enhanced the bradykinin-induced elevation of glutamate in superfusate (n = 6). These enhancements are presumably due to the inhibition of glutamate uptake. d) Furosemide (5 mM) blocks bradykinin-induced release of glutamate (n = 4) without significantly affecting bradykinin-induced astrocyte calcium mobilization.

METHODS. Cultures from 1-4 day-old Sprague-Dawely rat cortices were enriched in type-1 astrocytes according to the methods of Levison and McCarthy [25] and were maintained in a-MEM. These cultures were neuron-free as revealed by immunocytochemistry using an antibody directed against the synaptic protein synaptotagmin (1:250; clone 41.1, provided by R. Jahn). The amino acid content of samples was determined by HPLC with fluorescence detection. 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 (pH 5.9) methanol gradient. Points represent mean ± SEM. *Solutions.* A modified Ringer's solution used for perfusion contained (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). In 0 Ca solution calcium was replaced by 2 mM EGTA.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120
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Furosemide
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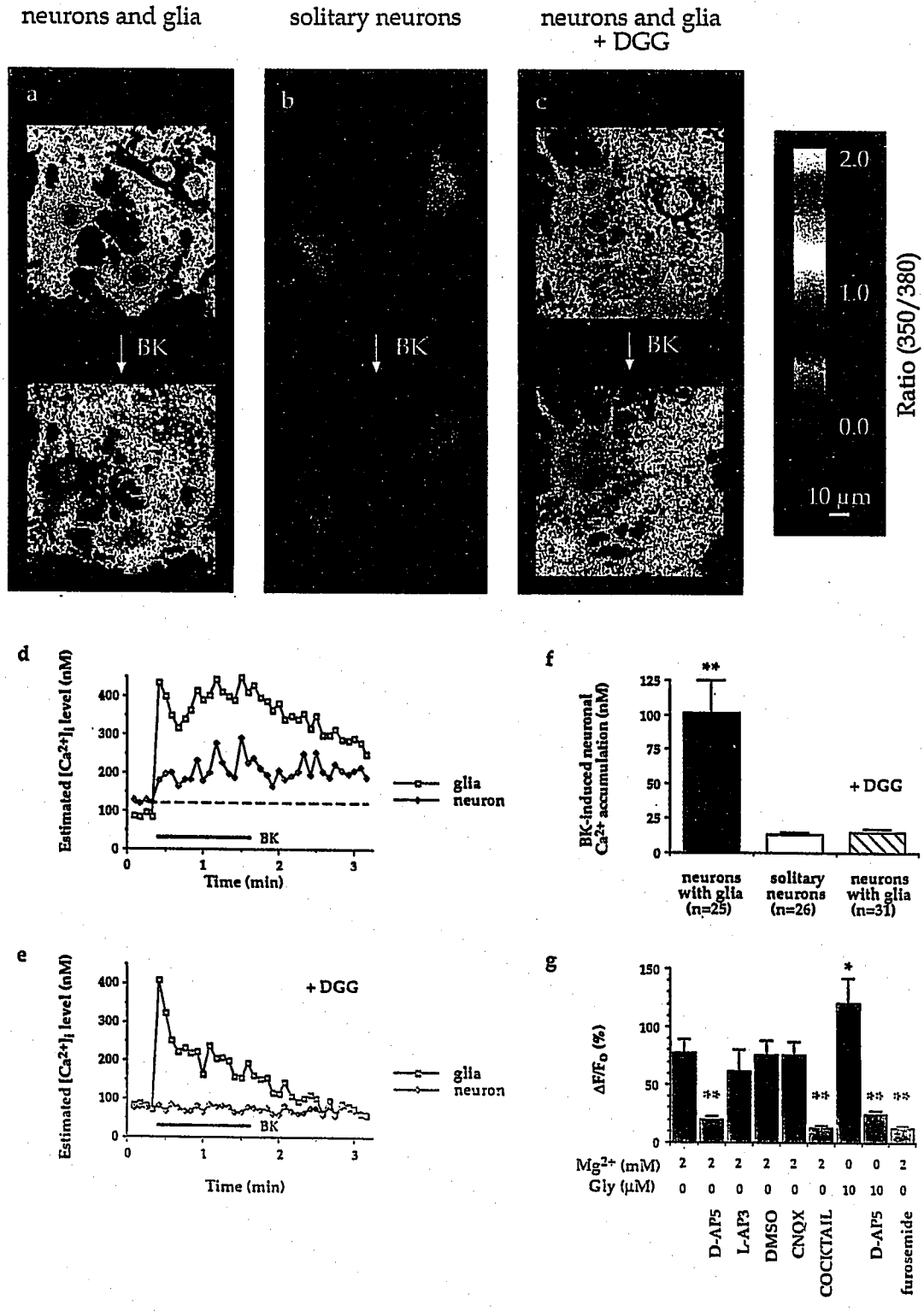
Time (min)

Figure 2. Bradykinin causes a glutamate-mediated elevation of neuronal calcium level in neurons co-cultured with astrocytes. Calcium levels in neocortical neurons and astrocytes were monitored using fura-2. a) Puffer application of bradykinin (1 μM, 75s) elevated calcium levels of neurons (circled) and astrocytes (A). When neurons were cultured alone (b), bradykinin did not significantly alter their calcium levels. In (c) three neurons are shown on top of astrocytes in the presence of DGG (1mM). Bradykinin elevated the astrocyte calcium levels, but not the level of calcium in adjacent neurons. Color scale is a linear pseudocolor representation of $[Ca^{2+}]_i$ shown in the form of the ratio of fura-2 emission at 510 nm due to sequential excitation at 350 and 380 nm. Scale bar, 10 μm. d-e) Time course of astrocyte and neuronal calcium responses after addition of bradykinin (BK) in control conditions (d) and in the presence of DGG (1 mM) (e). The astrocyte change in calcium (peak value subtracted from resting) induced by bradykinin (469 ± 86 nM, n=20; control) was not significantly affected by DGG (424 ± 45 nM, n=22; Student's t-test, $p > 0.6$). In f) the mean bradykinin-evoked calcium accumulations from experiments reported in (a-e) are shown. g) Calcium elevations in neurons co-cultured with astrocytes were monitored using fluo-3 and confocal microscopy. Changes in calcium are represented as ΔF/Fo. Bradykinin-induced elevations of neuronal calcium were reduced by D-AP5 (50 μM). Mg^{2+}-deficient medium containing glycine (10 μM) augmented the neuronal calcium response to bradykinin, which was significantly reduced by D-AP5 (50 μM). Furosemide (5 mM) reduced neuronal calcium elevations in response to bradykinin. Bars represent means ± SEM (n=18 for each group in g).

METHODS. Dissociated cultures from visual cortices were prepared using a modification of the Heuttner & Baughman procedure [26-28] and were used after 10-15 days in culture. A monoclonal antibody directed against glial fibrillary acidic protein (GFAP, 1:500; ICN Immunobiologicals), showed that glial cells in this culture were astrocytes (n=159 of 159 tested). *Calcium Imaging.* (a-f) Cells were loaded with fura-2 AM (2 μM) and calcium levels were estimated according to Basarsky *et al.* [28] In (g) cells were loaded with fluo-3AM (20 μg/mL). [29] Images were acquired using a Noran Odyssey real-time confocal microscope (Noran). Imaging saline contained (in mM); NaCl 140, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2 and HEPES 10 (pH 7.35). In magnesium deficient saline Mg^{2+} was replaced by calcium and glycine (10 μM) was added. Pharmacological agents were bath applied 10 minutes prior to application of bradykinin. Cocktail contained D-AP5 (50 μM), CNQX (10 μM) and L-AP3 (1 mM). *Statistical analysis.* One-way ANOVA followed by Scheffeé's (f) or Tukey's (g) *post hoc* comparisons was used. Significance was established at $p < 0.05$ (*) and $p < 0.01$ (**).


neurons and glia
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neurons and glia
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Estimated [Ca2+]i level (nM)
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200
100
0
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BK
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125
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L-AP3
DMSO
CNQX
COCKTAIL
D-AP5
furosemide

reduces the hypo-osmotic induced release of glutamate from astrocytes [7]. Addition of furosemide (5mM) to cortical astrocytes blocked bradykinin-induced release of glutamate (Fig. 1d) compared to parallel controls ($p < 0.05$, Mann-Whitney U-test) without significantly affecting bradykinin's calcium mobilizing action (bradykinin-induced calcium mobilization in furosemide was 714 $\pm$ 56 nM, n = 29 compared to 609 $\pm$ 107 in control, n=28 nM; $p>0.3$ Student's t-test). Thus, bradykinin mobilizes calcium to stimulate glutamate release, through a furosemide-sensitive release mechanism.

To ask whether glutamate released from astrocytes acts on adjacent neurons we determined the effect of bradykinin on $[Ca^{2+}]_i$ in neurons co-cultured with astrocytes. Bradykinin elevated neuronal calcium levels only when neurons were in contact with astrocytes (Fig. 2). Bradykinin caused a neuronal calcium accumulation of 101 $\pm$ 25 nM (n=25; Fig. 2a, d, f). Following the onset of bradykinin application calcium levels were significantly elevated in astrocytes (n = 20) and neurons (n = 25) after 15 and 50 seconds respectively (Tukey's *post hoc*, $p < 0.05$). When neurons were cultured alone, they did not significantly respond to bradykinin (calcium accumulation of 13 $\pm$ 2 nM, n=26; Fig. 2b, f). To determine whether bradykinin acts directly on neurons to elevate neuronal calcium levels or acts indirectly through glutamate released from astrocytes, we added a broad spectrum glutamate receptor antagonist D-glutamylglycine [8] (DGG). DGG (1 mM) significantly reduced the bradykinin-induced calcium accumulation in neurons to 15 $\pm$ 2 nM (n=31) as compared to 101 $\pm$ 25 nM in parallel cultures (Fig. 2c, e, f) without altering the astrocyte response to bradykinin (Fig. 2e). Subsequently DGG was washed out of the bath and glutamate was applied to neurons to determine

whether glutamate receptors were present. Direct application of glutamate (100 μM) elevated internal calcium in each category of neurons shown in figure 2a, f (neurons and glia, solitary neurons and neurons and glia that had previously been exposed to DGG). These responses were not significantly different from one another (Scheffeé's *post hoc* comparison [9]). These data suggest that bradykinin elevates neuronal calcium by the action of glutamate, which is released from astrocytes in response to bradykinin.

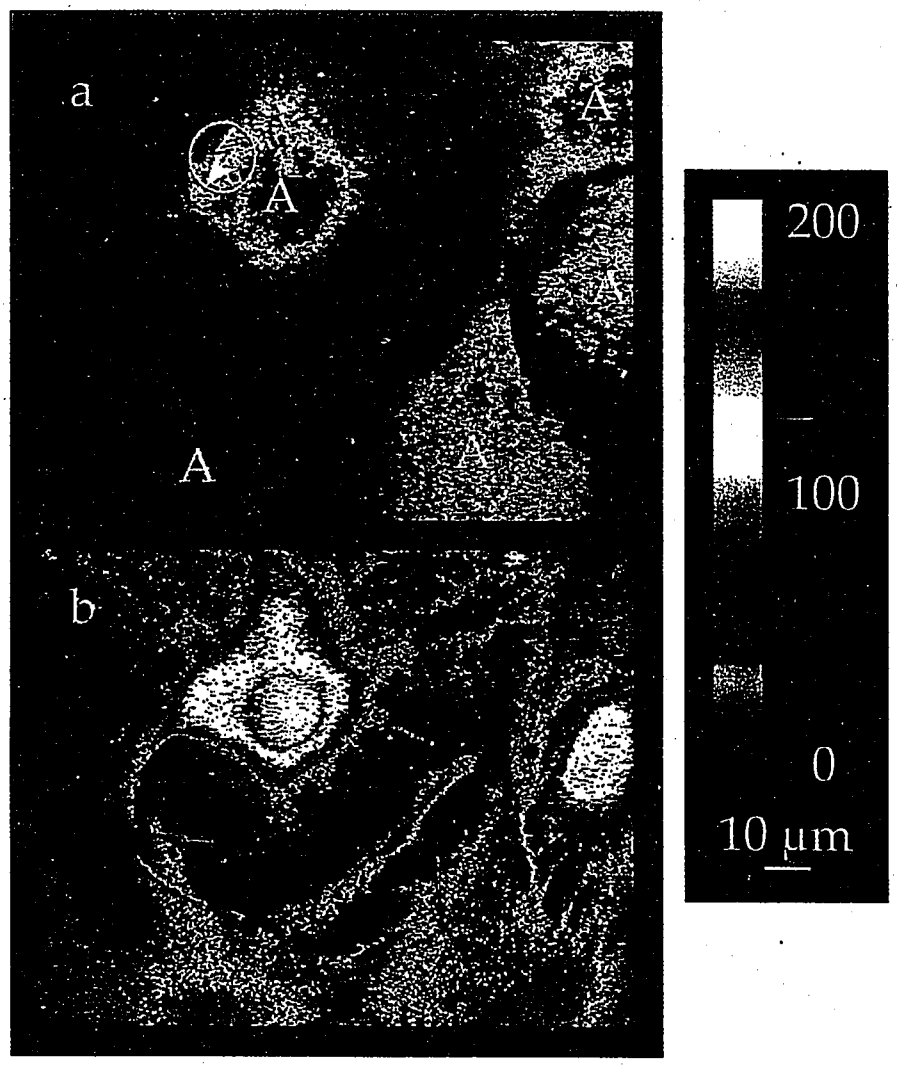
To critically determine whether bradykinin causes a neuronal change in calcium level and to further study receptor pharmacology we used confocal microscopy. Co-cultures of astrocytes and neurons were loaded with fluo-3 and the fluorescent emission at the plane of neuronal somata was monitored. Bradykinin reliably elevated neuronal calcium in astrocyte-neuron co-cultures (Fig. 2g). Addition of the N-methyl-D-aspartate (NMDA) receptor antagonist D-2-amino-5-phosphonopentanoic acid (D-AP5, 50 μM), but not 6-cyano-7-nitroquinoxaline-2,3 dione (CNQX, 10 μM) or L-2-amino-3-phosphonopropionic acid (L-AP3, 1mM), significantly reduced bradykinin-induced neuronal calcium elevation (Fig. 2g). Consistent with a role for NMDA receptors in mediating the action of released glutamate, removal of external Mg^{2+} and addition of glycine (10 μM) significantly augmented the bradykinin-induced neuronal calcium elevation (Fig. 2g).

HPLC data demonstrated that furosemide blocks bradykinin-induced glutamate release from astrocytes (Fig. 1). If glutamate released from astrocytes signals to adjacent neurons then furosemide should block this intercellular signaling pathway. Furosemide (5mM) significantly reduced the ability of bradykinin to

elevate neuronal calcium levels by 84 % (Fig. 2g) and reduced the neuronal calcium elevation in response to focally applied glutamate (100 mM) by 43% (n = 25; p< 0.001, Student's t-test). Since furosemide blocks bradykinin-induced glutamate release from astrocytes and reduces glutamate-induced neuronal calcium mobilization, the sensitivity of the bradykinin-induced neuronal calcium elevation to furosemide is consistent with glutamate mediating astrocyte-neuron signaling.

Since calcium elevations in astrocytes stimulate glutamate release we used two methods which directly raise calcium in astrocytes and asked whether there was a consequent elevation in neuronal calcium. First, tapping astrocytes [10] with micropipettes elevated astrocyte calcium levels and significantly raised neuronal calcium levels ($\Delta F/Fo = 62 \pm 25$ %, n = 6; $p < 0.005$, Mann-Whitney U-test). Second, we photo-stimulated astrocytes to elevate their calcium levels [11]. A portion of a single astrocyte was exposed to focal UV light. Photo-stimulation reliably elevated astrocyte calcium levels ($\Delta F/Fo = 202 \pm 19$ %, n=7), which then spread to adjacent astrocytes (Fig. 3). When astrocytes were photo-stimulated, the calcium levels of neurons in contact with astrocytes also increased (Fig. 4). Photo-stimulation of astrocytes 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; p>0.25, Scheffeé's test) to the medium. However, addition of the glutamate receptor antagonist DGG significantly reduced the astrocyte-induced elevation of calcium in adjacent neurons ($\Delta F/Fo = 14 \pm 4$ %, n=15; p<0.01, Scheffeé's test; Fig. 4).

Figure 3. Photo-stimulation of a single astrocyte causes an increase in $[Ca^{2+}]_i$ in an astrocyte (A) network. An astrocyte in (a) was exposed to UV light (2 min) from a Xenon arc lamp 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. Photo-stimulation elevated calcium for several minutes in all astrocytes 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. Summary of data is shown in (c). Color scale indicates linear 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.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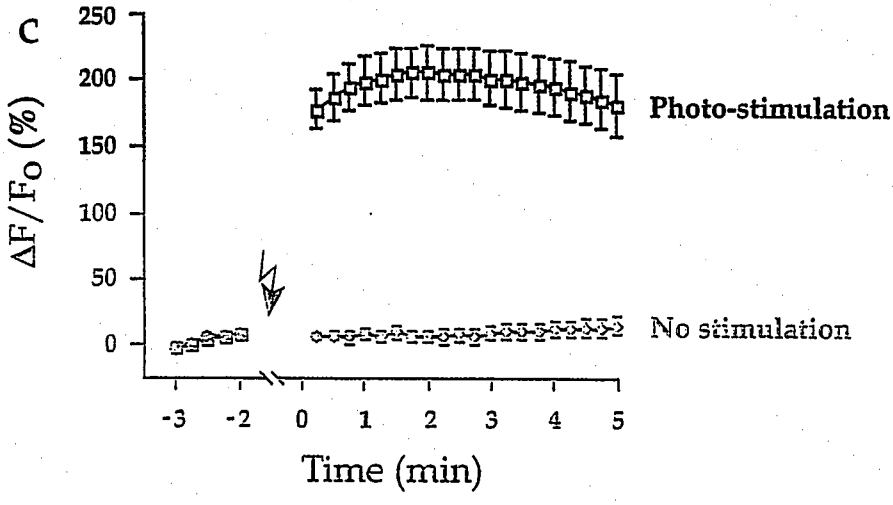
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ΔF/Fo (%)
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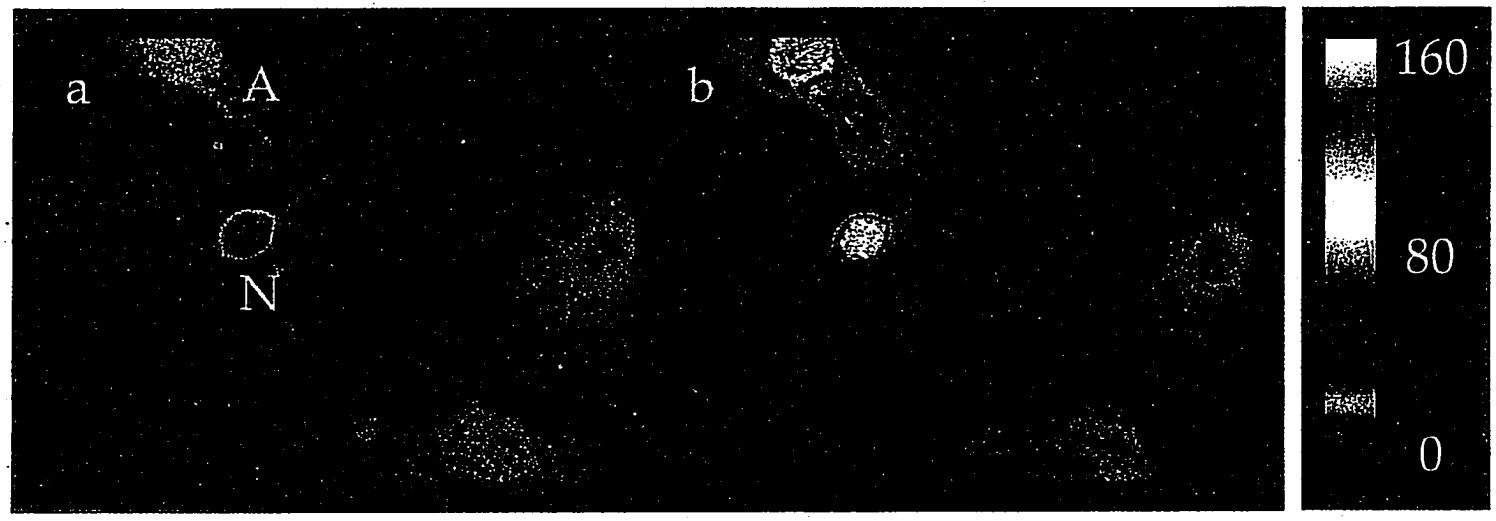
Time (min)

Figure 4. Photo-stimulation of astrocytes 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 astrocyte. After stimulation, $[Ca^{2+}]_i$ increased both in astrocytes (A) 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) Microinjection of sulforhodamine 101 (10 mM) into individual neurons (n = 8) confirmed that fluo-3 revealed the full extent of neuronal processes. In control experiments (n=6) we gradually moved the photo-stimulus closer to an isolated cell and determined that calcium levels were only elevated when the stimulus was within less than 5 μm from the cell border. Neurites were always greater than 11 μm from the edge of the photo-stimulus and thus were not directly activated by this stimulus. The image in (c) was acquired at the end of the experiment at the level of astrocytes and neuronal processes. The time course of $[Ca^{2+}]_i$ changes in neurons due to photo-stimulation (PS) of astrocytes 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 linear 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) and DGG (1 mM) were uniformly applied to the bath by saline exchange 10-20 minutes prior to UV stimulation.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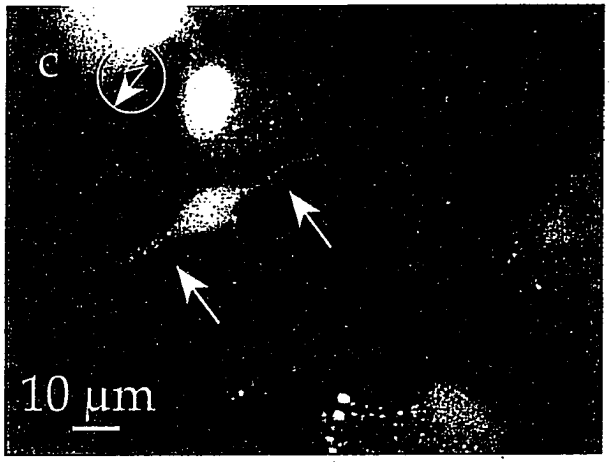
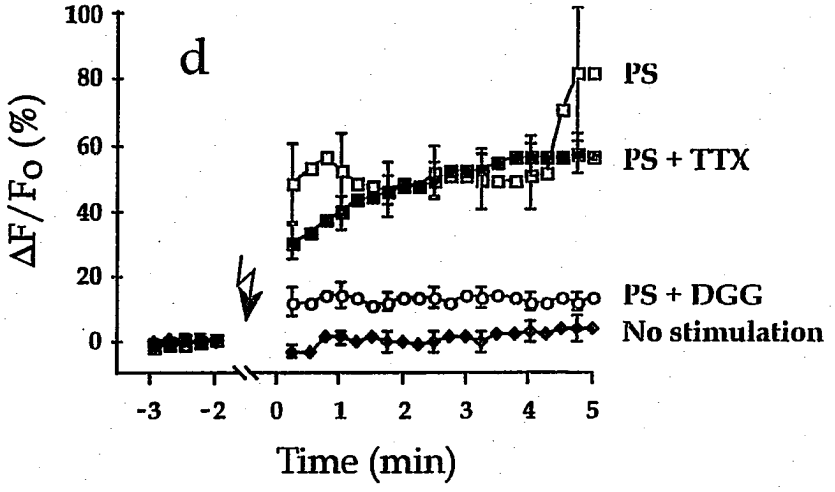

c
10 µm


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

This study demonstrates that a neuroligand, bradykinin, causes the calcium-dependent release of the neurotransmitter glutamate from neocortical astrocytes. The calcium-dependent glutamate release mechanism of astrocytes is likely to be distinct from neuronal release mechanisms since we found that astrocytes are not immunoreactive for the calcium-sensitive vesicle protein synaptotagmin. Using three distinct stimuli which each elevate astrocyte calcium levels (bradykinin, tapping astrocytes with micropipettes and direct astrocyte photo-stimulation) we demonstrate that astrocytes can cause a glutamate-dependent elevation of neuronal calcium. These data point to a novel form of transmitter signaling in the nervous system in which stimuli that mobilize calcium in astrocytes cause a calcium-dependent release of the excitatory amino acid glutamate, that can then act on adjacent neurons. It is possible that other pathways, such as gap junctions [12], also contribute to astrocyte-neuron signaling. However, since gap-junction uncoupling agents such as octanol have many additional actions [13-15] which include a reduction of calcium influx through the NMDA receptor [13] we have not tested this possibility. Glutamate released at synaptic terminals can trigger calcium waves in an astrocyte network [1]. Since many neurotransmitters can mobilize glial calcium [10, 16-22], it is possible that neurons stimulate neighboring astrocytes to release glutamate, which in turn signals back to the neuron. In this case astrocytes 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 [23], and under conditions of excessive release, can cause neuronal damage and degeneration [24]. This raises the possibility that astrocyte-

mediated glutamate release plays an important role in regulating neuronal physiology and pathology.

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GENERAL CONCLUSION

It has become clear that the excitatory amino acids glutamate and aspartate do not confine their excitatory effects to nerve cells, but also interact with glial cells. The objectives of this dissertation are to elucidate effects of neuroligand capsaicin on cultured peptidergic sensory neurons and to determine whether neuroligands such as bradykinin (BK) and adenosine 5'-triphosphate (ATP) can stimulate release of EAAs from cultured glial cells as well as to identify the possible signal transduction pathways for the neuroligand-induced release of EAAs.

Summary

Effects of capsaicin and resiniferatoxin on peptidergic neurons in cultured dorsal root ganglion

The purpose of the first study was to provide evidence of the long lasting effect of capsaicin and its ultrapotent analog resiniferatoxin (RTX) on sensory peptidergic neurons maintained in organotypic cultures. The effects of the two irritants were examined on neurons that contained substance P (SP) and calcitonin gene-related peptide (CGRP). Exposure of the cultures to 10μM capsaicin and 100nM RTX for periods of 2 days or longer resulted in almost complete elimination of SP-immunoreactive (IR) neurites and reduction, but not elimination, of CGRP-IR neurites. In addition, both 10μM capsaicin and 100nM RTX significantly reduced the number of SP- and CGRP-IR cell bodies within

DRG explants. Capsaicin in 100μM concentration produced complete elimination of SP-IR fibers and a great decrease in the number of CGRP-IR fibers, but failed to completely eliminate IR cell bodies. Exposure of the cultures to the irritants in the same concentrations for 90 min did not produce a measurable effect on SP- or CGRP-IR in neurites or cell bodies. It is important to establish that the effect of capsaicin and RTX on cultured neurons was of long duration (longer than 4 days) and is therefore different from depletion of peptides.

The neurotoxic effect of capsaicin has been shown to be selective on a subpopulation of small dorsal root ganglion neurons in newborn animals. Our findings demonstrate that processes of cultured sensory neurons are much more sensitive to capsaicin and RTX than cell bodies. Furthermore, these results show that SP-IR neuronal elements are more sensitive to capsaicin than CGRP-IR elements. Our data suggest that cultured sensory neurons express the functional properties of differentiated sensory neurons in vivo.

Neuroligand-evoked calcium-dependent release of excitatory amino acids from Schwann cells

This study deals with release of neurotransmitter from Schwann cells obtained from cultured rat DRGs. We found that neither capsaicin nor high potassium can stimulate the release of EAAs from Schwann cells. However, BK (10nM) caused significant release of glutamate and aspartate from cultured Schwann cells together with an increase in the cytoplasmic level of free calcium. BK-induced release of glutamate and the increase of the cytoplasmic calcium are receptor-mediated. Pretreatment with the BK receptor antagonist,

(D-Arg^0, Hyp^3, b-Thi^{5,8}, D-Phe^7)-bradykinin (5μM), blocked glutamate release and calcium increase evoked by BK. Although BK still stimulated release of glutamate without extracellular calcium, this effect was abolished by pretreatment with the membrane permeant calcium chelator BAPTA-AM [1,2bis(2-aminophenoxy) ethane-N,N,N',N'-tetraceticacid, acetoxymethyl ester, 50μM]. Perturbations which inhibited BK-induced calcium mobilization prevented the release of EAAs from Schwann cells. The calcium ionophore ionomycin (5μM) significantly increased the concentration of glutamate in glial cultures. The increase of glutamate induced by ionomycin was abolished by low Ca^{2+}-EGTA saline. These results prove that calcium mobilization is both necessary and sufficient for stimulating the release of EAAs from Schwann cells. To answer the question whether BK-induced increase of glutamate is due to the reversal uptake of glutamate, we used the glutamate transport blocker p-chloromercuriphenylsulphonic acid (p-CMPS, 50μM). Although p-CMPS elevated the basal concentration of glutamate, it didn't block the release of glutamate evoked by BK. The anion inhibitor furosemide (5mM) significantly attenuated the ability of the second application of BK to mobilize calcium. Furthermore, furosemide abolished the BK-induced release of glutamate from Schwann cells. To test whether the release of EAAs is through the mechanism of BK-induced cell swelling, we measured the volume of the Schwann cell and found that BK didn't cause cell swelling. Taken together, these data prove that the neuroligand BK can induce release of EAAs from Schwann cells. The mechanism of BK-induced release of EAAs is neither due to the reversal of the glutamate transporter nor volume regulated release mechanism. BK evokes EAA release by mobilizing calcium from intracellular calcium stores.

ATP-evoked Ca^{2+}-dependent release of excitatory amino acids from cultured Schwann cells

The specific objective of this study was to determine the mechanism by which ATP evokes release of EAAs from cultured Schwann cells. Basal concentrations of aspartate and glutamate in Schwann cell cultures were 7.64±0.74 nM (mean±SEM) and 30.52±3.89 nM, respectively. Perfusion application of 100μM ATP for 2 minutes to cultured Schwann glia resulted in an 201±28 % increase of aspartate and 203±15 % increase of glutamate. A second applications of ATP 10 minutes after the first application similarly caused EAA release, although the magnitude of this response was attenuated. Application of ATP to glia for ten minutes resulted in a peak increase in the release followed by a decline to a plateau significantly higher than baseline release. Bath application of adenosine (100μM) was without effect on the release of EAAs suggesting involvement of P_2 receptors. The release of EAAs evoked by ATP was not abolished in low Ca^{2+}-EGTA saline. Pretreatment of the glial cultures with 50μM BAPTA-AM abolished the effect of ATP. Thapsigargin (1μM), an inhibitor of Ca^{2+}-ATPase or the Ca^{2+} pump of internal stores, significantly reduced the release of EAAs evoked by ATP. Our results show that ATP evokes the release of EAAs from cultured Schwann cells by activating intracellular calcium stores.

Glutamate-mediated astrocyte-neuron signalling

The last study focused on glutamate-mediated astrocyte-neuron signaling. Neurotransmitter released from neurons is known to signal to neighboring neurons and glia. We demonstrate an additional signaling pathway in which

glutamate is released from astrocytes and causes an NMDA receptor-mediated neuronal calcium elevation. Application of the neuroligand bradykinin to cultured astrocytes elevated internal calcium and stimulated glutamate release. Elevation of astrocytic internal calcium was also sufficient to induce glutamate release. To determine whether this released glutamate signals to neurons, we studied astrocyte-neuron co-cultures. Bradykinin significantly elevated calcium levels in the neurons co-cultured with astrocytes, but not in solitary neurons. The glutamate receptor antagonists, D-2-amino-5-phosphonopentanoic acid and D-glutamylglycine, prevented bradykinin-induced neuronal calcium elevations. When single astrocytes were directly stimulated to increase internal calcium, and release glutamate, calcium levels of adjacent neurons were increased, this increase could be blocked by D-glutamylglycine. Thus, astrocytes regulate neuronal calcium levels through the calcium-dependent release of glutamate.

Discussion

The studies in this dissertation demonstrate that the neuroligands can induce release of EAAs from cultured glial cells.

There are three different mechanisms proposed for increase of extracellular glutamate. One of them is the calcium dependency of glutamate release (Nicholls and Sihra, 1986), which appears to be due to the exocytosis of synaptic vesicles; The second one is reversal of uptake of glutamate (Miller and Schwartz, 1983); Furosemide-sensitive release of glutamate from swelling cells is the third proposed

mechanism (Kimelberg and Ransom, 1986). According to our studies, calcium plays an important role in the neuroligand-induced release of EAAs.

Release of EAAs evoked by BK or ATP from Schwann cells depends on intracellular Ca^{2+}

Internal calcium appears to be responsible for BK- or ATP-induced release of EAAs from cultured glial cells. As we know, there are two intracellular Ca^{2+} stores (Hardie, 1991). One is IP_3-sensitive calcium store and the other one is caffeine-sensitive store. Which Ca^{2+} store is involved in release of EAAs? On the basis of the fact that caffeine doesn't induce any release of EAAs at all and ryanodine fails to block BK-induced release, we infer that IP_3-sensitive Ca^{2+} stores must be responsible for the EAA release. Furthermore, the involvement of the IP_3-sensitive Ca^{2+} store is confirmed by the result that pretreatment of phospholipase C inhibitor (U73122, 8μM) significantly blocks the BK-induced release of EAAs (our unpublished data).

There is no evidence yet that glial cells contain synaptic vesicles. It has been suggested that glia might release neurotransmitters by Ca^{2+} independent carrier mechanisms. A recurrent suggestion is that the release might be mediated by the reverse operation of electrogenic neurotransmitter carrier protein, which normally function to take up neurotransmitters (reviewed by Barres, 1991). Although there is evidence that glia release neurotransmitters by "reversed uptakes" in special experimental conditions (Schwartz, 1987) and under physiological conditions (Barbour, 1987; Szatkowski et al., 1990), this "Ca^{2+}-independent release" theory is challenged now. We demonstrated that elevated intracellular Ca^{2+} is necessary and sufficient for release of EAAs from glial cells. Our data suggest that there might be

a novel neurotransmitter release mechanism for glial cells. The release of EAAs from glia probably reflects a Ca^{2+}-dependent vesicle exocytosis process. However, this process has to be different from the exocytosis which occurs at nerve terminals, since glial lack typical structure of synapses - the focal active zone (reviewed by Smith, 1994) and also lack immunoreactivity to synaptic vesicle proteins such as synaptotagmin, synaptophysin and synaptobrevin (our unpublished data).

Release of glutamate from astrocytes plays a key role in glia-neuron signalling

Although a glia-neural signalling theory of brain function was proposed thirty years ago (Galambos, 1961), it has only recently received much attention. Our studies give new evidence that glial cells (astrocytes) indeed signal to neurons and the neurotransmitter glutamate released from the glial cells plays a key role in this process.

Our data show that neuroligand BK induced elevated free Ca^{2+} in purified astrocyte cultures, but not in neuron cultures. However, BK caused an intracellular Ca^{2+} increase in both astrocytes and neurons when it was applied to co-cultures of the neurons and the astrocytes. This result strongly suggests signalling from astrocyte to neuron. The next question is how a astrocyte could signal to a neuron. We found that BK greatly evoked release of glutamate from cultured neuron-free astrocytes. Furthermore, D-2-amino-5-phosphonopentanoic acid, the glutamate receptor antagonist, effectively blocked the astrocyte-neuron signalling. Taken these evidence together, we conclude that neurotransmitter glutamate plays a key role in glia-neuron signalling.

Nedergaard (1994) provides similar evidence for astrocyte-neuron signalling. However, he indicates that gap-junctions might mediate the astrocyte-neuron signalling. The reasons that there are two different mechanisms for astrocyte-neuron signalling may be due to gap-junctions functioning under some specific co-culture conditions or with some combination of neuronal or astrocytic sub-types, but the glutamate-mediated mechanism predominating in other circumstances (Smith, 1994).

The function of glia-neuron signalling is not clear yet. On the basis of the available information, one would expect that glia-neuron signalling might participate modulation of brain functions such as mood, motivational state, arousal and learning and memory.

Is neuroligand-evoked release of EAAs involved in neurodegenerative disorders?

Excessive glutamate is toxic to neurons. Exposed to glutamate for even a few minutes, neurons undergo a degenerative process leading to cell death (Teichberg, 1991). The toxic effect of glutamate may be involved in acute and chronic neurodegenerative disorders. It has been shown that considerate endogenous glutamate can be released into the extracellular space after cerebral ischemia and trauma (Benveniste et al., 1987, Katayama et al., 1988). NMDA antagonist can protect against hypoxic/ischemic brain damage in vivo (Gill et al., 1987; Park et al., 1987; Kochhar et al., 1988). There is also some evidence which implicates that EAA neurotoxity may be a factor in chronic degenerative diseases such as Huntington's disease, Alzheimer's disease and Parkinsonism (reviewed by Olney, 1990 and Choi, 1988).

The studies in this dissertation deal with neuroligand-induced release of EAAs. It has been shown that the neuroligand BK is released from its precursors in response to trauma, anoxia and inflammation (Dray and Perkins, 1993) and higher extracellular levels of ATP may be released following tissue injury (Neary and Norenbery, 1992). Although it is not clear what effects that BK- or ATP-induced EAA release from glia would have on CNS under centain pathological condition, the present information hints that neuroligand-induced release of EAAs might potentiate a pathological state associated with brain damage. Furthermore, utilizing certain neuroligand antagonists, which block the release of EAAs, might attenuate the pathological state.

Conclusion

This dissertation focuses on elucidating the mechanism of neuroligand-evoked release of EAAs. Our studies have demonstrated that the EAA release appears to be in a Ca^{2+}-dependent manner. We have showed, at least in part, the signal pathways of release of EAAs. However, there is still a gap left. Further investigation is needed to explore how Ca^{2+} triggers EAA release from glia, which lack synaptic vesicles.

As we know, release of EAAs plays an important role in neuron-neuron signalling. However, there is increasing evidence that the release of EAAs is actively involved in the signalling processes between neuron and glia. Although it is not clear what functions these signalling processes serve, they deserve further study.

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