J. exp. Biol. 167, 251–265 (1992)
Printed in Great Britain © *The Company of Biologists Limited 1992*

CALCIUM TRANSIENTS IN IDENTIFIED LEECH GLIAL CELLS *IN SITU* EVOKED BY HIGH POTASSIUM CONCENTRATIONS AND 5-HYDROXYTRYPTAMINE

BY THOMAS MUNSCH AND JOACHIM W. DEITMER
Abteilung für Allgemeine Zoologie, FB Biologie, Universität Kaiserslautern, D-6750 Kaiserslautern, Germany

Accepted 24 February 1992

Summary

We have recorded the fluorescence of Fura-2-loaded, identified glial cells in the neuropile of the central nervous system of the leech *Hirudo medicinalis* using the ratio of emission at 350 nm excitation to that at 380 nm excitation as an indicator of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The neuropile glial cells were exposed by mechanically removing the overlying ganglionic capsule and the neuronal cell bodies and were then impaled using a microelectrode under visual control to inject Fura-2 ionophoretically. The resting Ca^{2+} level was measured using digitonin or Triton to permeabilize the cell membrane at different external concentrations of Ca^{2+}; it was found to vary between 5 and 79 nmol l^{-1} and averaged 32 ± 23 nmol l^{-1} (±S.D., N=7). Raising the external K^+ concentration from 4 to 20 mmol l^{-1} or adding 50 μmol l^{-1} 5-hydroxytryptamine (5-HT) produced a rapid, reversible rise in $[Ca^{2+}]_i$. During prolonged exposure to high $[K^+]$ or 5-HT, $[Ca^{2+}]_i$ remained high. Upon restoring normal external $[K^+]$ or removing 5-HT, $[Ca^{2+}]_i$ returned to its initial resting value within 1–2 min. The responses of $[Ca^{2+}]_i$ to high $[K^+]$ and 5-HT were greatly reduced in nominally Ca^{2+}-free saline, suggesting that the $[Ca^{2+}]_i$ transients required an influx of Ca^{2+} into the cells. In the presence of 5-HT, the rise in $[Ca^{2+}]_i$ was accompanied by a decrease in the resistance and an increase in the responsiveness to K^+ of the glial cell membrane, indicating the existence of a Ca^{2+}-dependent K^+ conductance elicited by 5-HT.

Introduction

Intracellular Ca^{2+} transients are widespread and are important steps in signal transduction leading to activation of cellular activity. In the nervous system, many functions of nerve cells, such as regulation and modulation of ion conductances, electrical activity and synaptic transmitter release, are mediated by a rise in intracellular $[Ca^{2+}]$ (Miller, 1987; Meldolesi and Pozzan, 1987).

In glial cells, much less is known about similar second-messenger pathways involving intracellular Ca^{2+} transients. Earlier studies have reported a rise in

Key words: intracellular Ca^{2+} concentration, Fura-2 fluorescence, identified glial cells, 5-hydroxytryptamine, leech, *Hirudo medicinalis*, central nervous system.

$[Ca^{2+}]_i$ in a cultured glioma cell line following application of transmitter substances such as 5-hydroxytryptamine (5-HT; Sugino *et al.* 1984). In recent years, evidence has accumulated that different kinds of stimuli, such as amino acid transmitters, norepinephrine, endothelin and mechanical deformation, may induce a rise in $[Ca^{2+}]_i$ in glioma and glial cells (Ashley *et al.* 1989; Hertz *et al.* 1989; Cornell-Bell *et al.* 1990; Jensen and Chiu, 1990, 1991; MacVicar *et al.* 1990, 1991; Salm and McCarthy, 1990). These studies have been carried out, however, on isolated glial cells *in vitro*, where the cells are deprived of their normal glial and neuronal environment.

In the leech central nervous system, glial cells can easily be identified (Coggeshall and Fawcett, 1964; Kuffler and Nicholls, 1966). Microelectrodes can be impaled into glial cells in the neuropile of leech ganglia to record membrane potentials and intracellular ion activities (Walz and Schlue, 1982; Deitmer and Schlue, 1987, 1989). We have now developed a method of removing the ganglionic capsule and the neuronal cell bodies to expose the neuropile glial cells *in situ*, allowing direct measurement of intracellular ion activities using fluorescent dyes injected into a glial cell within the ganglion.

We have injected the Ca^{2+}-sensitive fluorescent dye Fura-2 into the cells by passing current through a Fura-2-filled intracellular microelectrode, and have recorded intracellular Ca^{2+} transients following an increase in the external K^+ concentration or the addition of 5-HT, a hormone and neurotransmitter in the leech nervous system (Wallace, 1981; Lent and Dickinson, 1984). Our results suggest that the elevation of $[Ca^{2+}]_i$ is, at least in part, due to Ca^{2+} influx through membrane channels and may modulate the K^+ conductance of the glial membrane. A preliminary report was communicated at the joint meeting of the German and Dutch Physiological Societies (Munsch and Deitmer, 1992).

Materials and methods

Preparation

The experiments were performed on isolated ganglia of the leech *Hirudo medicinalis* L. Adult leeches were kept in freshwater tanks at 16–18 °C and acclimated for 3–7 days to room temperature (20–25 °C) before experimentation. Individual ganglia (Fig. 1A) were dissected from the leech and pinned ventral side uppermost in a Sylgard-coated Perspex chamber in modified Leibowitz (L-15) tissue culture medium. This Leibowitz-15 medium (obtained from Gibco) was modified by dilution (1:3) to reach iso-osmolality and to give Na^+ and K^+ concentrations comparable to the physiological leech saline with the following salt solution ($mmol\,l^{-1}$): $CaCl_2$, 6.87; $MgCl_2$, 2.51; KCl, 3.32; sodium malate, 20.1; sodium pyruvate, 12.5; Hepes, 15; glucose, 15; gentamycin ($10\,mg\,ml^{-1}$), 0.3 %; adjusted to pH 7.4 with NaOH. The ventral ganglionic capsule was removed mechanically with fine forceps. The ganglia were then incubated for 1–1.5 h in $2\,mg\,ml^{-1}$ collagenase/dispase (Boehringer-Mannheim, Germany) dissolved in modified L-15 medium at room temperature (20–25 °C). After enzyme treatment

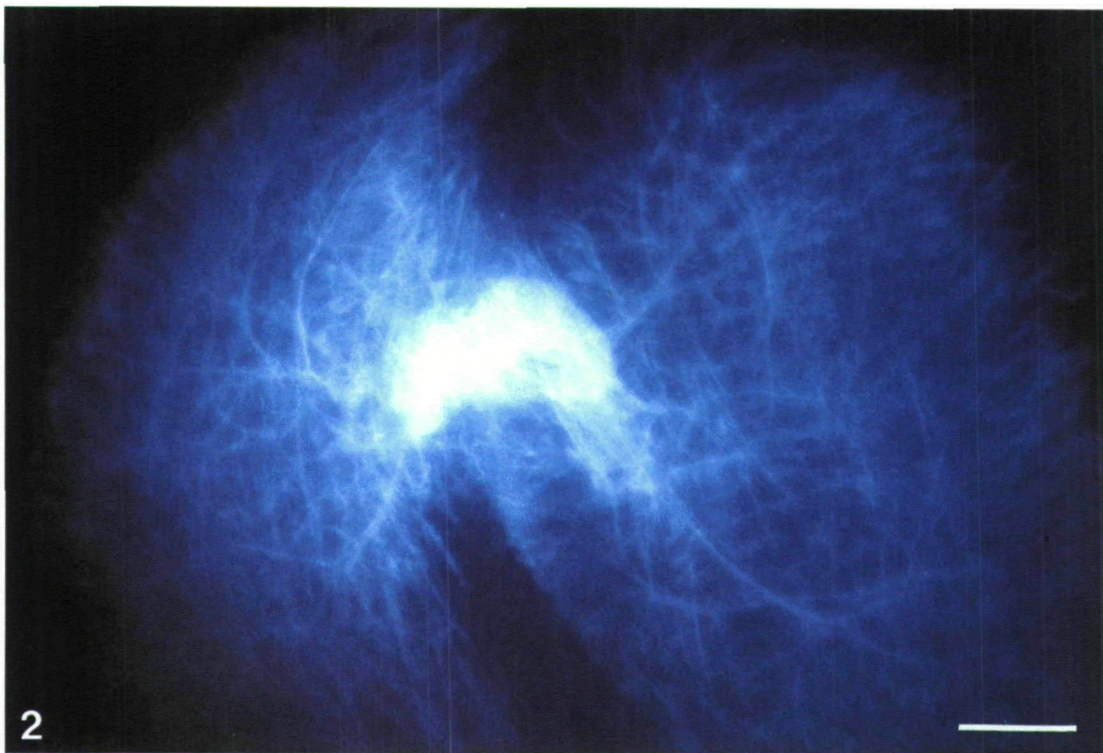

Fig. 2. Fluorescence photomicrograph of a Fura-2-loaded neuropile glial cell. Scale bar, 50 μm.

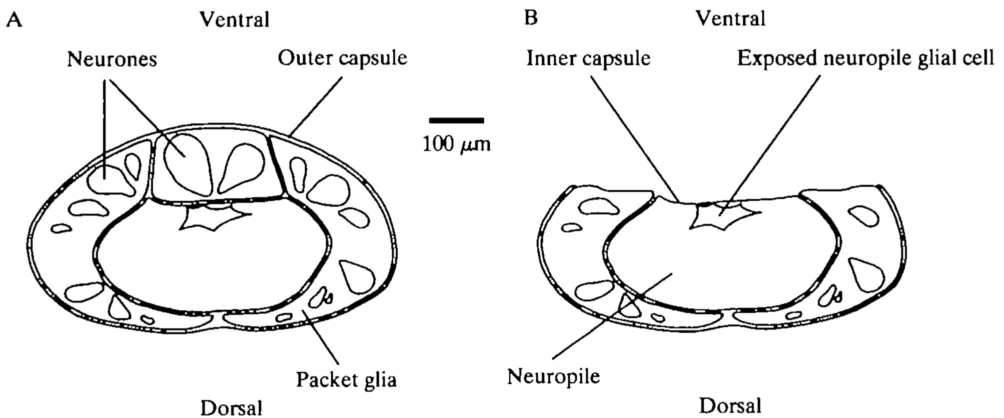

Fig. 1. Schematic cross section through the anterior part of a leech segmental ganglion, (A) left intact and (B) with the neuropile glial cell exposed after removal of the overlying outer ganglionic capsule and neurone cell bodies.

the ganglia were thoroughly washed with enzyme-free medium. Neurones overlying the two neuropile glial cells of each segmental ganglion were removed by suction into a glass micropipette (tip diameter 50–100 μm), thus exposing the neuropile and revealing the cell bodies of the neuropile glial cells covered only by the inner ganglionic capsule (Fig. 1B). Ganglia were then transferred into leech saline for experimentation. Microelectrodes filled with Fura-2 ($10\,mmol\,l^{-1}$ in $0.1\,mol\,l^{-1}$ KCl) were inserted into the neuropile glial cells under direct visual control. The dye was injected ionophoretically with negative constant current of 2–5 nA for 10–15 min, after which the electrode was removed.

Measurement of intracellular $[Ca^{2+}]$

Fluorescence imaging of glial cells was begun 5–10 min after Fura-2 injection. Changes in intracellular Ca^{2+} concentration were measured by determining the ratio of Fura-2 fluorescence at 350 nm excitation to that at 380 nm excitation. Experiments were performed using a Deltascan dual-excitation spectrofluorimeter (LTI, Tornesch, Germany) in which shutters, monochromator settings and data aquisition were controlled by computer software and by interfaces from LTI. The cells were excited alternately at 350 nm and 380 nm (bandwidth 4–8 nm) with a 75 W xenon arc lamp through the epifluorescence port of a Nikon Diaphot microscope with an oil-immersion CF-Fluor 20× objective. Fluorescence intensity was measured at 510 nm using a photon-counting photomultiplier tube, and measurements were limited to a field of view slightly larger than the injected glial cell (diameter 60–100 μm, Fig. 2) by a rectangular diaphragm.

$[Ca^{2+}]_i$ was computed from an *in situ* calibration method using the equation described by Grynkiewicz *et al.* (1985): $[Ca^{2+}]_i = K_d\ [(R-R_{min})/(R_{max}-R)]\ (F_0/F_s)$. Minimum and maximum fluorescence ratios at 350 nm and 380 nm (R_{min}, 0.385; R_{max}, 0.945) were determined by applying leech salines to the preparation

containing either 0.1 % Triton X-100 or 50 μmol l^{-1} digitonin, first in the presence of 10 mmol l^{-1} EGTA and then with 5 mmol l^{-1} Ca^{2+}. The fluorescence ratio measured in normal leech saline is R. F_0/F_s is the ratio of fluorescence at 380 nm excitation in the absence of Ca^{2+} to that at saturating Ca^{2+} levels and was determined automatically from the calibration. The apparent dissociation constant (K_d) between Fura-2 and Ca^{2+} was determined from a single measurement *in situ* to be 177 nmol l^{-1} using a Ca^{2+} buffer solution containing 100 nmol l^{-1} Ca^{2+} (pCa 7; after Tsien and Rink, 1980). It is possible that during the calibration procedures the cells lost dye after exposure to digitonin or Triton. However, relatively stable values were obtained for at least 5 min during these calibrations, indicating that, even if some dye had leaked out of the cell, this did not influence the *ratio* measurement. Large cells, such as the leech neuropile glial cells, may be difficult to buffer to stable $[Ca^{2+}]_i$ using ionophores (Cohan *et al.* 1987).

Once the dye had leaked out of the cell it would be expected to wash away rapidly, as was the case in intact ganglia. Dye leakage and/or bleaching was also observed during experiments and this could amount to 40–70 % of the fluorescence intensity loss (Fig. 3). However, this intensity loss occurred over a period of 30–60 min and should not alter the *ratio* of fluorescence.

Before calculating $[Ca^{2+}]_i$, the ratios were corrected for background fluorescence. The background fluorescence was obtained from an exposed glial cell that had not been injected with Fura-2, but with all other conditions identical to the

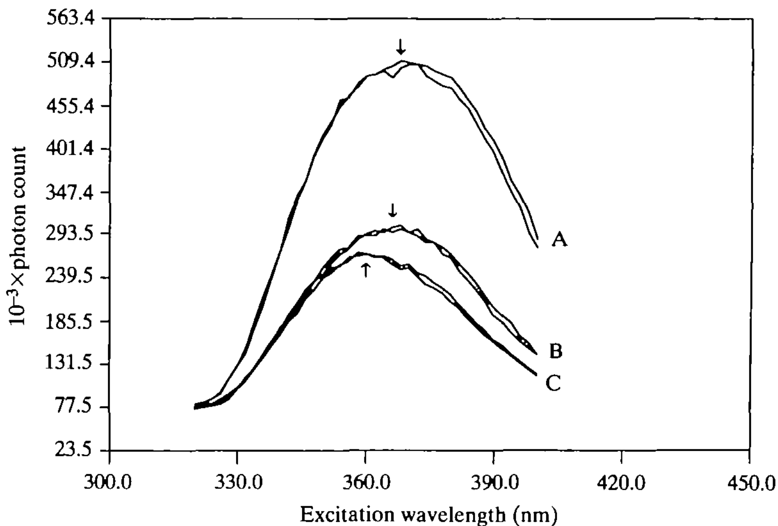

Fig. 3. Excitation spectra of a neuropile glial cell loaded with Fura-2. Excitation wavelengths of two independent monochromators were scanned from 320 to 400 nm (A) at the beginning of the experiment, (B) after 40 min and (C) after 58 min when the experiment ended. Peaks of the spectra are indicated by arrows. Emission was measured at 510 nm.

fluorescent dye measurements. The value was stored by the computer program and recalled for each calculation of $[Ca^{2+}]_i$.

In most experiments the responses of $[Ca^{2+}]_i$ to the same, repeatedly applied stimuli tended to decrease in amplitude during the course of the experiment. In some, but not all, of these experiments, this was accompanied by a slow upward movement of the fluorescence ratio, presumably indicating a slow rise in the resting $[Ca^{2+}]_i$. This phenomenon was concomitant with a large decrease in absolute fluorescence, and a shift in the spectra to lower wavelengths (Fig. 3).

One of the advantages of using a monochromator system is that it enables the excitation spectra of the dye-loaded cell to be measured (Fig. 3). From such spectra the shape and peak of the emission can be obtained, giving an indication of the relative dye quantity injected into the cell and the approximate resting $[Ca^{2+}]_i$.

All experiments were performed 5–10 times, except where otherwise indicated.

Electrophysiology

The preparation of the neuropile glial cells was the same as described above. Ganglia with exposed glial cells (Fig. 1B) were pinned in an experimental chamber (volume 0.2 ml) and superfused continuously at a flow rate of $1.5\,ml\,min^{-1}$. For determination of the membrane input resistance, two microelectrodes, filled with $4\,mol\,l^{-1}$ potassium acetate, were inserted into a neuropile glial cell, one for recording voltage and the other for injecting constant current pulses. In the experiments in which the relative K^+ conductance was to be determined, only one electrode was inserted into a neuropile glial cell. The extracellular $[K^+]$ was repeatedly raised from 4 to $10\,mmol\,l^{-1}$, for 1 min, and the amplitudes of the resulting membrane depolarizations were used as a measurement of the relative K^+ conductance.

Microelectrodes were connected to the input of a high-impedance voltage follower and to the bridge amplifier input of a Neurohel V87 (Meyer, Munich, Germany) by chlorided silver wires. The indifferent electrode was a Ag/AgCl wire embedded in 2 % agar. The signals were displayed on an oscilloscope and recorded with a pen recorder.

Solutions

The normal bathing fluid (physiological, isotonic leech saline) had the following composition ($mmol\,l^{-1}$): NaCl, 85; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; Hepes, 10; adjusted to pH 7.4 with NaOH. In solutions with a raised K^+ concentration, equimolar amounts of Na^+ were replaced by K^+ to maintain iso-osmolality. In nominally Ca^{2+}-free solutions (no EGTA added), Mg^{2+} was substituted for Ca^{2+}. 5-Hydroxytryptamine (5-HT) was dissolved in the appropriate saline to give a final concentration of 50 or $100\,\mu mol\,l^{-1}$.

5-HT and digitonin were purchased from Fluka (Neu-Ulm, Germany). Fura-2 pentapotassium salt was purchased from Molecular Probes (Eugene, OR) and Triton X-100 (*t*-octylphenoxypolyethoxyethanol) was obtained from Sigma (St

Louis, MO). Tissue culture medium and antibiotics were obtained from Gibco (Eggenstein, Germany).

Results

Resting $[Ca^{2+}]_i$ in neuropile glial cells

A neuropile glial cell loaded with Fura-2 is shown in Fig. 2. Fura-2 was distributed relatively evenly throughout the cell soma and within the cell processes. The neuropile glial cell body was 60–100 μm in diameter, and the numerous branching processes of the glial cell extended over 350–400 μm to fill half the neuropile space. Cells filled with Lucifer Yellow exhibited a very similar shape and branching pattern.

The resting $[Ca^{2+}]_i$ was 32 ± 23 nmol l^{-1} (mean$\pm$S.D.), ranging from 5 to 79 nmol l^{-1} for seven individually calibrated cells. Changes in $[Ca^{2+}]_i$ induced by elevation of extracellular $[K^+]$ or by addition of 5-HT are presented in terms of fluorescence ratio changes rather than absolute values of $[Ca^{2+}]_i$, since many cells lost the dye within 5–15 min of addition of Triton or digitonin.

Responses of $[Ca^{2+}]_i$ to increased external $[K^+]$

The rise in $[Ca^{2+}]_i$ in response to an increase in the external K^+ concentration from 4 to 20 mmol l^{-1} is shown in Fig. 4. During a 60 s exposure to 20 mmol l^{-1} K^+ (Fig. 4A) there was a sharp increase in $[Ca^{2+}]_i$ after a delay of about 20–25 s. This delay was partly due to the wash-in of saline with the increased K^+ concentration. When the external K^+ concentration was restored to normal (4 mmol l^{-1}), $[Ca^{2+}]_i$ rapidly decreased. The return of $[Ca^{2+}]_i$ to the control level appeared to occur with at least two time constants, as indicated by the changes in the fluorescence ratio, with half-times of 15 and 70 s. Membrane potential measurements of these glial cells have shown that in 20 mmol l^{-1} K^+ the glial membrane depolarizes by about 30 mV, from -70 to -40 mV (inset of Fig. 4A; see also Deitmer and Szatkowski, 1990).

Comparison of a brief and a prolonged exposure (for 7 min) to the high-K^+ saline (Fig. 4B) revealed that $[Ca^{2+}]_i$ remained elevated throughout the exposure period and decreased only very slowly with a half-time of approximately 8 min at high external $[K^+]$. Return to the control K^+ concentration initiated a decrease in $[Ca^{2+}]_i$, apparently with a single time course having a half-time of 24 s. This shows that $[Ca^{2+}]_i$ would remain high for at least 15–20 min in a high-K^+ saline.

The fast rise of $[Ca^{2+}]_i$ during a K^+-induced membrane depolarization suggests that the rise might be due to Ca^{2+} influx through voltage-gated Ca^{2+} channels in the glial cell membrane. This was tested in an experiment where external Ca^{2+} was removed prior to an increase in external K^+ concentration (Fig. 5). In the presence of external Ca^{2+}, high external $[K^+]$ again evoked a large, fast rise in $[Ca^{2+}]_i$, but the intracellular Ca^{2+} transient produced in the nominal absence of external Ca^{2+} was relatively slow and much smaller. Since the removal of external Ca^{2+} occurred only 1 min before the increase in external $[K^+]$ elicited the small

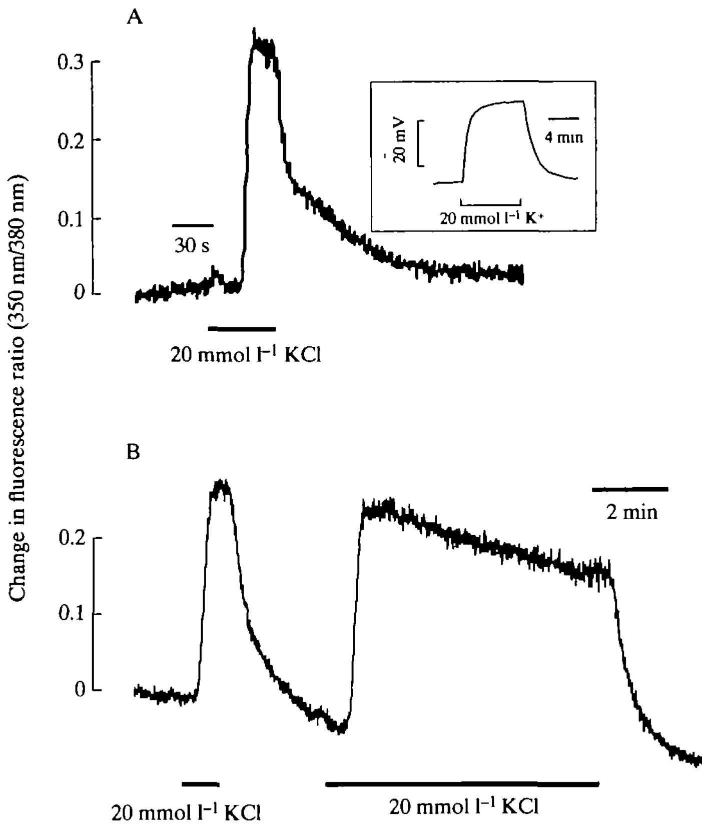

Fig. 4. (A) Fluorescence measurements from a neuropile glial cell loaded with Fura-2: an intracellular Ca^{2+} transient induced by 20 mmol l^{-1} K^+. $[Ca^{2+}]_i$ appears to return to the control level with two time constants. Inset: membrane potential response to 20 mmol l^{-1} K^+. (B) Responses of $[Ca^{2+}]_i$ following a brief (1 min) and a prolonged (7 min) elevation of external $[K^+]$. This is representative of 22 experiments.

rise in $[Ca^{2+}]_i$, it is unlikely that stored intracellular Ca^{2+} had been affected. The resting $[Ca^{2+}]_i$ remained virtually unchanged during the 10 min absence of external Ca^{2+}.

In all experiments of this type there was a small, but significant, rise in $[Ca^{2+}]_i$ following an increase in the external K^+ concentration. Since no Ca^{2+} buffer was used in these experiments, it is possible that the remaining Ca^{2+} in the saline, presumably between 1 and 10 μmol l^{-1}, could provide enough Ca^{2+} influx into the cells to produce this residual intracellular Ca^{2+} response.

In other experiments, 1 mmol l^{-1} Co^{2+} or 0.5 mmol l^{-1} Cd^{2+} was used to block Ca^{2+} channels in the glial membrane. These Ca^{2+} channel blockers considerably reduced the rise in $[Ca^{2+}]_i$ in the presence of 20 mmol l^{-1} K^+, though a small, slow intracellular Ca^{2+} transient remained even in the presence of these Ca^{2+} antagonists. Both Co^{2+} and Cd^{2+} also produced an apparent upward movement of the baseline of the fluorescence ratio. This might be due to the decreasing viability

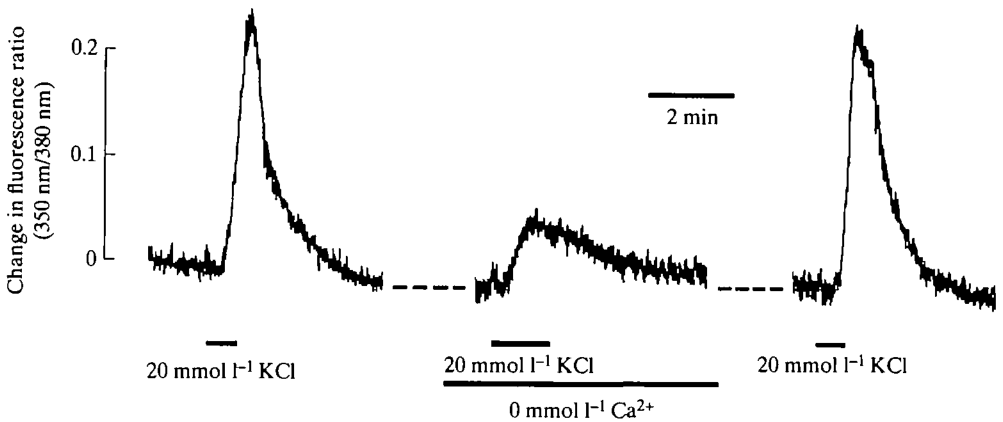

Fig. 5. K^+-induced intracellular Ca^{2+} transients in a neuropile glial cell in the presence ($2\,mmol\,l^{-1}$) and nominal absence ($0\,mmol\,l^{-1}$) of extracellular Ca^{2+}. In $0\,mmol\,l^{-1}$ Ca^{2+} there was only a slight increase in $[Ca^{2+}]_i$ compared to the increase in the control.

of the cells in solutions containing Co^{2+} and Cd^{2+} or to some interference of these divalent cations with the fluorescent dye.

Responses of $[Ca^{2+}]_i$ to 5-hydroxytryptamine

Responses of $[Ca^{2+}]_i$ to $50\,\mu mol\,l^{-1}$ 5-HT, a common transmitter and hormone in the nervous system of the leech (Lent and Dickinson, 1984), are shown in Fig. 6. Compared to the intracellular Ca^{2+} transient elicited by increasing the external K^+ concentration, the rise in $[Ca^{2+}]_i$ following addition of 5-HT was significantly slower and smaller (Fig. 6A). After wash-out of 5-HT, $[Ca^{2+}]_i$ returned to control levels at the same rate as when the external K^+ concentration was reduced from 20 to $4\,mmol\,l^{-1}$. In most experiments $[Ca^{2+}]_i$ also appeared to decrease with two time constants following 5-HT removal.

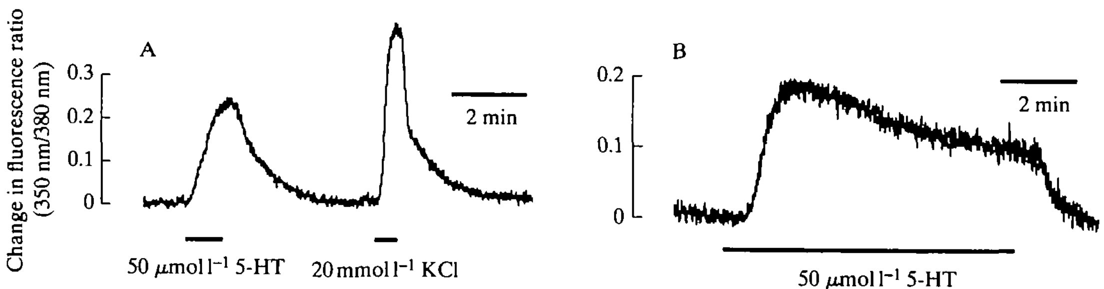

Fig. 6. (A) Rises in $[Ca^{2+}]_i$ in a neuropile glial cell in response to $50\,\mu mol\,l^{-1}$ 5-hydroxytryptamine (5-HT) and, for comparison, to $20\,mmol\,l^{-1}$ extracellular K^+. (B) $[Ca^{2+}]_i$ during prolonged exposure (8 min) of a neuropile glial cell to 5-HT.

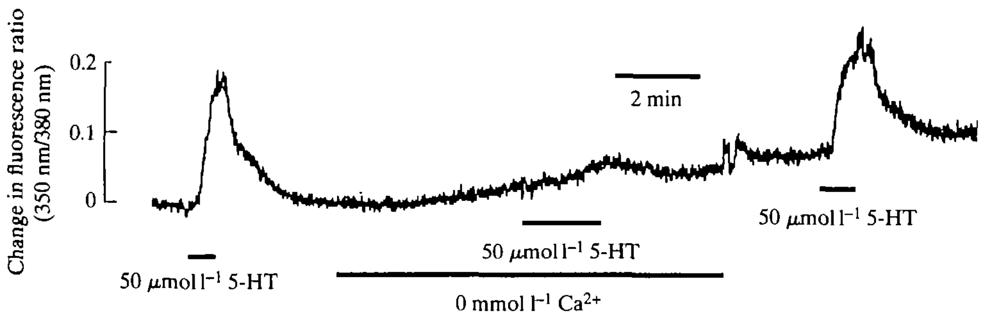

Fig. 7. 5-HT-induced intracellular Ca^{2+} transients in the presence ($2\,mmol\,l^{-1}$) and nominal absence ($0\,mmol\,l^{-1}$) of extracellular Ca^{2+}. In $0\,mmol\,l^{-1}$ Ca^{2+} there was only a very small, delayed increase in $[Ca^{2+}]_i$ compared to that in the controls.

In some experiments (fewer than 20 %) there appeared to be no, or only a very small, rise in $[Ca^{2+}]_i$ in the presence of 5-HT, although these cells did show a normal $[Ca^{2+}]_i$ response to increased $[K^+]$. The reason for the unresponsiveness of some cells to 5-HT is not yet known.

During prolonged exposure to 5-HT the $[Ca^{2+}]_i$ of responding glial cells remained high for many minutes (Fig. 6B) and only returned to the control level after the removal of 5-HT. After an exposure to 5-HT for 7.5 min, $[Ca^{2+}]_i$ returned to normal within 1–2 min, apparently with a single time course. Hence, the $[Ca^{2+}]_i$ responses to 5-HT were quite like those to increased $[K^+]$, except for their smaller amplitude and slower rate of rise.

We also looked for the possible source of Ca^{2+} leading to the $[Ca^{2+}]_i$ response to 5-HT. In nominally Ca^{2+}-free saline, the Ca^{2+} transient was much reduced, but never *completely* abolished (Fig. 7). After re-addition of external Ca^{2+}, 5-HT again elicited a typical intracellular Ca^{2+} transient.

Effect of 5-HT on glial membrane properties

It has been reported that 5-HT hyperpolarizes the membrane by increasing a K^+ conductance in these glial cells (Walz and Schlue, 1982). This was confirmed in the present study by measuring the effects of 5-HT on the membrane input resistance and the effects of brief exposures to $10\,mmol\,l^{-1}$ K^+ (Fig. 8). The membrane input resistance decreased by 44 % from 0.41 ± 0.07 MΩ (N=12) to 0.23 ± 0.07 MΩ (N=8) after the addition of $100\,\mu mol\,l^{-1}$ 5-HT (Fig. 8A). The membrane depolarization in response to $10\,mmol\,l^{-1}$ K^+ increased about twofold from 9.2 ± 2.8 mV (N=9) to 18.4 ± 1.2 mV (N=8) in the presence of $50\,\mu mol\,l^{-1}$ 5-HT (Fig. 8B). The increased responsiveness of the cell membrane to K^+, together with the reduced membrane input resistance, indicates that the K^+ conductance of the glial membrane was considerably enlarged by 5-HT. Both changes, that in membrane input resistance and that in the membrane responsiveness to K^+, were readily reversible after exposures to 5-HT lasting up to 3–4 min, but were longer lasting when 5-HT was applied for 5 min or more. The time courses of the 5-HT

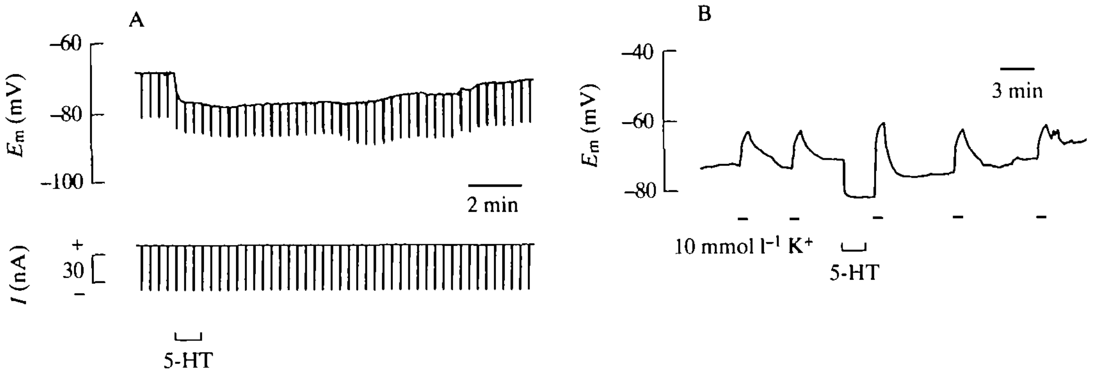

Fig. 8. Effect of 5-HT on (A) membrane resistance and (B) relative K^+ permeability of neuropile glial cells. 5-HT was added to the bathing solution for 1 and 2 min in A and B, respectively. 5-HT concentrations were 100 μmol l^{-1} (A) and 50 μmol l^{-1} (B).

effects on the glial membrane properties might be linked to the time courses of the Ca^{2+}-related intracellular second-messenger pathway.

Discussion

This study shows that $[Ca^{2+}]_i$ can be measured in identified glial cells *in situ* with the Fura-2 method. Rises in $[Ca^{2+}]_i$ induced by membrane depolarization and 5-hydroxytryptamine indicated the existence of voltage- and ligand-gated Ca^{2+} channels in the membrane of neuropile glial cells. Additional electrophysiological data suggested that the 5-HT-induced hyperpolarization of the glial membrane might be due to a Ca^{2+}-dependent K^+ conductance.

In situ *measurement of* $[Ca^{2+}]_i$

A single segmental ganglion of *Hirudo medicinalis* contains only a few large glial cells, easily identified by their position and electrophysiological properties (Kuffler and Potter, 1964; Schlue *et al.* 1980). Being located in the neuropile, where synaptic transmission takes place, the neuropile glial cells are well-positioned to participate in ion homeostasis. By removing the overlying ganglionic capsule and neuronal cell bodies, we were able to perform the Ca^{2+} measurements in identified glial cells *in situ*. In contrast, previous fluorescence measurements of $[Ca^{2+}]$ in glial cells have only been carried out in cell cultures (Jensen and Chiu, 1990, 1991; Cornell-Bell *et al.* 1990; Salm and McCarthy, 1990; Cornell-Bell and Finkbeiner, 1991; Charles *et al.* 1991; MacVicar *et al.* 1991; Dave *et al.* 1991), where glial cells were completely removed from their original environment.

We also determined the calibration parameters in the cells *in situ*, because there is increasing evidence that the chemical and optical properties of fluorescent dyes inside a cell may be different from those in an aqueous solution (Konishi *et al.* 1988; Owen, 1991). *In vitro* calibration parameters may yield different resting and

peak values of $[Ca^{2+}]_i$ from those determined *in situ* (Callewaert *et al.* 1991; Williams *et al.* 1990).

The resting value for $[Ca^{2+}]_i$ found in the present study (32 nmol l^{-1}, range 5–79 nmol l^{-1}) corresponds well with the resting $[Ca^{2+}]_i$ measured in cultured astrocytes (34±3.7 nmol l^{-1}, Jensen and Chiu, 1990) and in mixed glial cultures (40–75 nmol l^{-1}, Charles *et al.* 1991). Higher values have been reported for cultured rat cerebellar glial cells (79 nmol l^{-1}, Ashley *et al.* 1989), for murine cortical astrocytes (100–200 nmol l^{-1}, MacVicar *et al.* 1991) and for cultured rat cerebral type-1 astrocytes (101±9 nmol l^{-1}, Salm and McCarthy, 1990).

Membrane depolarization and 5-HT-induced rises in $[Ca^{2+}]_i$

A brief elevation of extracellular $[K^+]$, which depolarizes the glial membrane, leads to transient rises in $[Ca^{2+}]_i$ in leech neuropile glial cells. This suggests the presence of voltage-gated Ca^{2+} channels in the membrane of these cells. Ca^{2+} channels, known to be present in cultured cortical astrocytes (MacVicar, 1984; Barres *et al.* 1990; MacVicar *et al.* 1991) and Müller glial cells (Newman, 1985), would be activated by K^+-induced membrane depolarization. The idea that Ca^{2+} enters the cell *via* voltage-gated channels is supported by the observation that ions such as Co^{2+} and Cd^{2+}, which are known to block Ca^{2+} channels in glial cell membranes (MacVicar and Tse, 1988; Barres *et al.* 1988), considerably reduce rises in $[Ca^{2+}]_i$ in neuropile glial cells.

Ca^{2+} channels activated by neurotransmitters such as 5-HT also seem to be present in neuropile glial cells. The addition of 5-HT induced an increase in glial $[Ca^{2+}]_i$, which was dependent upon the presence of extracellular Ca^{2+}. In contrast to high extracellular $[K^+]$, which leads to a depolarization, 5-HT hyperpolarizes the glial cell membrane (Fig. 8). Therefore, Ca^{2+} influx through voltage-gated Ca^{2+} channels seems not to be involved in 5-HT-induced increases in $[Ca^{2+}]_i$.

5-HT-induced increases in $[Ca^{2+}]_i$ have been measured using fluorescence methods in a rat glioma cell line (Ogura *et al.* 1986). Recently Dave *et al.* (1991) have shown that only 9 % of identified cerebral type 2 astrocytes responded to 5-HT with a rise in $[Ca^{2+}]_i$. So far, direct activation of Ca^{2+} channels in the membrane of cultured astrocytes has only been shown for putative L-type channels coupled to glutamate receptors of type 2 astrocytes (Jensen and Chiu, 1990, 1991) and norepinephrine receptors of type 1 astrocytes (Salm and MacCarthy, 1990).

Source of the rises in $[Ca^{2+}]_i$

By comparing the depolarization- and agonist-evoked responses of $[Ca^{2+}]_i$ in the presence and absence of external Ca^{2+}, it appears that most, if not all, of the increase in $[Ca^{2+}]_i$ is due to Ca^{2+} influx across the cell membrane. In the case of the response to high-K^+ saline, the small, slow increase in $[Ca^{2+}]_i$ in nominally Ca^{2+}-free saline might be due to the small amounts of Ca^{2+} remaining in the non-buffered saline (presumably less than 10^{-5} mol l^{-1}).

Membrane depolarization could also lead to an influx of Na^+ through voltage-

gated Na^+ channels known to be present in the membrane of astrocytes and oligodendrocytes (Bevan *et al.* 1985; Barres *et al.* 1988), which could activate some influx of Ca^{2+} *via* the Na^+/Ca^{2+} exchanger. There is no evidence for the existence of a Na^+/Ca^{2+} exchange mechanism in glial membranes, and if Na^+/Ca^{2+} exchange were to be responsible, a much slower increase in $[Ca^{2+}]_i$ would be anticipated.

Another source of the intracellular Ca^{2+} transients might be Ca^{2+} release from internal stores, such as endoplasmic reticulum, by receptor-mediated generation of a second messenger. Ligand-induced inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3] is known to trigger release of Ca^{2+} from intracellular stores in cortical mammalian astrocytes and astrocytoma cell lines (Pearce and Murphy, 1988). In a rat glioma cell line, stimulation of phosphoinositide hydrolysis by activation of 5-HT receptors has been demonstrated (Ananth *et al.* 1987), and in a polyploid glioma cell line activation of 5-HT receptors induces a rise in cytosolic $[Ca^{2+}]$, possibly *via* Ins(1,4,5)P_3 hydrolysis (Reiser *et al.* 1989). Two observations, however, argue against 5-HT-induced intracellular Ca^{2+} release in leech neuropile glial cells: (1) 5-HT-evoked rises in $[Ca^{2+}]_i$ were greatly reduced in the absence of external Ca^{2+}, and (2) Ca^{2+} oscillations, which are characteristic of receptor-mediated intracellular Ca^{2+} transients in astrocytes (Jensen and Chiu, 1990, 1991; Cornell-Bell and Finkbeiner, 1991; Charles *et al.* 1991; Salm and McCarthy, 1990), were never observed even during long exposures to 5-HT.

Mechanism of the membrane responses to 5-HT

Addition of 5-HT hyperpolarizes the neuropile glial membrane, and it has been suggested that this results from the activation of a K^+ conductance (Walz and Schlue, 1982). Our experiments confirm this suggestion by showing that 5-HT induced an increase in the relative K^+ conductance and a decrease in the input resistance of neuropile glial cells. We have shown that 5-HT also evokes an increase in $[Ca^{2+}]_i$. These changes in membrane properties and $[Ca^{2+}]_i$ are only reversible when glial cells are given short exposures to 5-HT. Applications of 5-HT for 5 min or more led to sustained rises in $[Ca^{2+}]_i$, which might be responsible for the long-lasting effect (>20 min) of 5-HT on the K^+ conductance of the glial membrane.

These parallel effects of 5-HT on $[Ca^{2+}]_i$ and on the membrane properties indicate that the K^+ conductance induced by 5-HT might be elicited by the rise in $[Ca^{2+}]_i$, suggesting the presence of Ca^{2+}-dependent K^+ channels in the neuropile glial cell membrane. Indeed, Ca^{2+}-dependent K^+ channels have been reported in astrocytes (Quandt and MacVicar, 1986). In rat glioma cells, activation of 5-HT_2 receptors directly opened Ca^{2+} channels; the resulting increase in $[Ca^{2+}]_i$ caused a hyperpolarization by activating Ca^{2+}-dependent K^+ channels (Ogura *et al.* 1986). A similar mechanism might be involved when depolarization of the glial membrane by high external $[K^+]$ leads to an influx of Ca^{2+} through voltage-activated Ca^{2+} channels (MacVicar, 1984; MacVicar *et al.* 1991).

The rises in $[Ca^{2+}]_i$ elicited by membrane depolarization and 5-HT may play a

role in the K^+ clearance properties of glial cells by coupling a high K^+ permeability of glial membranes to neuronal activity and the concentration of a neurohormone, such as 5-HT, in the nervous tissue (Walz, 1988, 1989). Similarly, $[Ca^{2+}]_i$-induced membrane potential changes of the glial cell could influence pH regulation in the nervous system by affecting electrogenic Na^+/HCO_3^- cotransport (Deitmer and Szatkowski, 1990; Deitmer, 1991) and other voltage-dependent conductances and transport systems in the glial membrane.

We thank Fatima Pfeiffer for providing the photomicrograph of a Fura-2-filled glial cell and H.-P. Schneider for excellent technical assistance. We are grateful to the LTI company for providing some equipment. This study was supported by grants from the Deutsche Forschungsgemeinschaft (De 231/7,9).

References

ANANTH, U. S., LELI, U. AND HAUSER, G. (1987). Stimulation of phosphoinositide hydrolysis by serotonin in C6 glioma cells. *J. Neurochem.* **48**, 253–261.

ASHLEY, C. C., SIMPSON, A. W. M. AND SUPATTAPONE, S. (1989). Endothelin increases intracellular calcium levels in cultured rat cerebellar glial cells. *J. Physiol., Lond.* **417**, 72P.

BARRES, B. A., CHUN, L. L. Y. AND COREY, D. P. (1988). Ion channel expression by white matter glia. I. Type 2 astrocytes and oligodendrocytes. *Glia* **1**, 10–30.

BARRES, B. A., CHUN, L. L. Y. AND COREY, D. P. (1990). Ion channels in vertebrate glia. *A. Rev. Neurosci.* **13**, 441–474.

BEVAN, S., CHIU, S. Y., GRAY, P. T. A. AND RITCHIE, J. M. (1985). The presence of voltage-gated sodium, potassium and chloride channels in rat cultured astrocytes. *Proc. R. Soc. Lond. B* **225**, 299–313.

CALLEWAERT, G., LIPP, P., POTT, L. AND CARMELIET, E. (1991). High-resolution measurement and calibration of Ca^{2+}-transients using Indo-1 in guinea-pig atrial myocytes under voltage clamp. *Cell Calcium* **12**, 269–277.

CHARLES, A. C., MERRILL, J. E., DIRKSEN, E. T. AND SANDERSON, M. J. (1991). Intercellular signaling in glial cells: calcium waves and oscillations in response to mechanical stimulation and glutamate. *Neuron* **6**, 983–992.

COGGESHALL, R. E. AND FAWCETT, D. W. (1964). The fine structure of the central nervous system of the leech, *Hirudo medicinalis*. *J. Neurophysiol.* **27**, 229–289.

COHAN, C. S., CONNOR, J. A. AND KATER, S. B. (1987). Electrically and chemically mediated increases in intracellular calcium in neuronal growth cones. *J. Neurosci.* **7**, 3588–3599.

CORNELL-BELL, A. H. AND FINKBEINER, S. M. (1991). Ca^{2+} waves in astrocytes. *Cell Calcium* **12**, 185–204.

CORNELL-BELL, A. H., FINKBEINER, S. M., COOPER, M. S. AND SMITH, S. J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. *Science* **247**, 470–473.

DAVE, V., GORDON, G. W. AND MCCARTHY, K. D. (1991). Cerebral type 2 astroglia are heterogeneous with respect to their ability to respond to neuroligands linked to calcium mobilization. *Glia* **4**, 440–447.

DEITMER, J. W. (1991). Electrogenic sodium-dependent bicarbonate secretion by glial cells of the leech central nervous system. *J. gen. Physiol.* **98**, 637–655.

DEITMER, J. W. AND SCHLUE, W. R. (1987). The regulation of intracellular pH by identified glial cells and neurones in the central nervous system of the leech. *J. Physiol., Lond.* **388**, 261–283.

DEITMER, J. W. AND SCHLUE, W. R. (1989). An inwardly directed electrogenic sodium-bicarbonate co-transport in leech glial cells. *J. Physiol., Lond.* **411**, 179–194.

DEITMER, J. W. AND SZATKOWSKI, M. (1990). Membrane potential dependence of intracellular pH regulation by identified glial cells in the leech central nervous system. *J. Physiol., Lond.* **421**, 617–631.

GRYNKIEWICZ, G., POENIE, M. AND TSIEN, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. biol. Chem.* **260**, 3440–3450.

HERTZ, L., BENDER, A. S., WOODBURY, D. M. AND WHITE, H. S. (1989). Potassium-stimulated calcium uptake in astrocytes and its potent inhibition by nimodipine. *J. Neurosci. Res.* **22**, 209–215.

JENSEN, A. M. AND CHIU, S. Y. (1990). Fluorescence measurement of changes in intracellular calcium induced by excitatory amino acids in cultured cortical astrocytes. *J. Neurosci.* **10**, 1165–1175.

JENSEN, A. M. AND CHIU, S. Y. (1991). Differential intracellular calcium responses to glutamate in type 1 and type 2 cultured brain astrocytes. *J. Neurosci.* **11**, 1674–1684.

KONISHI, M., OLSON, A., HOLLINGWORTH, S. AND BAYLOR, S. M. (1988). Myoplasmic binding of Fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophys. J.* **54**, 1089–1104.

KUFFLER, S. W. AND NICHOLLS, J. G. (1966). The physiology of neuroglial cells. *Ergebn. Physiol.* **57**, 1–90.

KUFFLER, S. W. AND POTTER, D. D. (1964). Glia in the leech nervous system: Physiological properties and neuron–glia relationships. *J. Neurophysiol.* **27**, 290–320.

LENT, C. M. AND DICKINSON, M. H. (1984). Serotonin integrates the feeding behavior of the medicinal leech. *J. comp. Physiol.* A **154**, 457–471.

MACVICAR, B. A. (1984). Voltage-dependent calcium channels in glial cells. *Science* **226**, 1345–1347.

MACVICAR, B. A., HOCHMAN, D., DELAY, M. J. AND WEISS, S. (1991). Modulation of intracellular Ca^{++} in cultured astrocytes by influx through voltage-activated Ca^{++} channels. *Glia* **4**, 448–455.

MACVICAR, B. A. AND TSE, F. W. Y. (1988). Norepinephrine and cyclic adenosine 3′:5′-cyclic monophosphate enhance a nifedipine-sensitive calcium current in cultured rat astrocytes. *Glia* **1**, 359–365.

MACVICAR, B. A., TSE, F. W. Y. AND WEISS, S. (1990). Voltage- and transmitter-activated ion channels in glial cells. In *Differentiation and Function of Glial Cells*, vol. 55 (ed. G. Levi), pp. 191–201. New York: Wiley-Liss.

MELDOLESI, J. AND POZZAN, T. (1987). Pathways of Ca^{2+} influx at the plasma membrane: voltage-, receptor-, and second messenger-operated channels. *Expl Cell Res.* **171**, 271–283.

MILLER, R. J. (1987). Multiple calcium channels and neuronal function. *Science* **235**, 46–52.

MUNSCH, T. M. AND DEITMER, J. W. (1992). 5-Hydroxytryptamine and potassium induced increases in intracellular calcium in identified leech glial cells. *Pflügers Arch.* (Suppl.) **420**, R27.

NEWMAN, E. A. (1985). Voltage-dependent calcium and potassium channels in retinal glial cells. *Nature* **317**, 809–811.

OGURA, A., OZAKI, K., KUDO, Y. AND AMANO, T. (1986). Cytosolic calcium elevation and cGMP production induced by serotonin in a clonal cell of glial origin. *J. Neurosci.* **6**, 2489–2494.

OWEN, C. S. (1991). Spectra of intracellular Fura-2. *Cell Calcium* **12**, 385–393.

PEARCE, B. AND MURPHY, S. (1988). Neurotransmitter receptors coupled to inositol phospholipid turnover and Ca^{2+} flux: consequences for astrocyte function. In *Glial Cell Receptors* (ed. H. K. Kimelberg), pp. 197–221. New York: Raven Press.

QUANDT, F. N. AND MACVICAR, B. A. (1986). Calcium activated potassium channels in cultured astrocytes. *Neuroscience* **19**, 29–41.

REISER, G., DONIÉ, F. AND BINMÖLLER, F.-J. (1989). Serotonin regulates cytosolic Ca^{2+} activity and membrane potential in a neuronal and in a glial cell line *via* 5-HT_3 and 5-HT_2 receptors by different mechanisms. *J. Cell Sci.* **93**, 545–555.

SALM, A. K. AND MCCARTHY, K. D. (1990). Norepinephrine-evoked calcium transients in cultured cerebral type 1 astroglia. *Glia* **3**, 529–538.

SCHLUE, W. R., SCHLIEP, A. AND WALZ, W. (1980). Fluorescence marking of neuropile glial cells in the central nervous system of the leech *Hirudo medicinalis*. *Cell Tissue Res.* **209**, 257–269.

SUGINO, H., OGURA, A., KUDO, Y. AND AMANO, T. (1984). Intracellular Ca^{2+} elevation induced by a neurotransmitter in a glial cell clone. *Brain Res.* **322**, 127–130.

TSIEN, R. Y. AND RINK, T. J. (1980). Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochim. biophys. Acta* **599**, 623–638.

WALLACE, B. G. (1981). Neurotransmitter chemistry. In *Neurobiology of the Leech* (ed. K. J. Muller, J. G. Nicholls and G. S. Stent), pp. 147–172. Cold Spring Habor Laboratory.

WALZ, W. (1988). Physiological consequences of activating serotonin receptors. In *Glial Cell Receptors* (ed. H. K. Kimelberg), pp. 121–130. New York: Raven Press.

WALZ, W. (1989). Role of glial cells in the regulation of the brain ion microenvironment. *Progr. Neurobiol.* **33**, 309–333.

WALZ, W. AND SCHLUE, W. R. (1982). Ionic mechanism of a hyperpolarizing 5-hydroxytryptamine effect on leech neuropile glial cells. *Brain Res.* **250**, 111–121.

WILLIAMS, D. A., HEAD, S. I., BAKKER, A. J. AND STEPHENSON, D. G. (1990). Resting calcium concentrations in isolated skeletal muscle fibres of dystrophic mice. *J. Physiol., Lond.* **428**, 243–256.