

## G protein activation by uncaging of GTP- $\gamma$ -S in the leech giant glial cell

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### Summary

Glial cells can be activated by neurotransmitters via metabotropic, G protein-coupled receptors. We have studied the effects of 'global' G protein activation by GTP- $\gamma$ -S on the membrane potential, membrane conductance, intracellular  $\text{Ca}^{2+}$  and  $\text{Na}^+$  of the giant glial cell in isolated ganglia of the leech *Hirudo medicinalis*. Uncaging GTP- $\gamma$ -S (injected into a giant glial cell as caged compound) by moderate UV illumination hyperpolarized the membrane due to an increase in  $\text{K}^+$  conductance. Uncaging GTP- $\gamma$ -S also evoked rises in cytosolic  $\text{Ca}^{2+}$  and  $\text{Na}^+$ , both of which were suppressed after depleting the intracellular  $\text{Ca}^{2+}$  stores with cyclopiazonic acid ( $20 \mu\text{mol l}^{-1}$ ). Uncaging inositol-trisphosphate evoked a transient rise in cytosolic

$\text{Ca}^{2+}$  and  $\text{Na}^+$  but no change in membrane potential. Injection of the fast  $\text{Ca}^{2+}$  chelator BAPTA or depletion of intracellular  $\text{Ca}^{2+}$  stores did not suppress the membrane hyperpolarization induced by uncaging GTP- $\gamma$ -S. Our results suggest that global activation of G proteins in the leech giant glial cell results in a rise of  $\text{Ca}^{2+}$ -independent membrane  $\text{K}^+$  conductance, a rise of cytosolic  $\text{Ca}^{2+}$ , due to release from intracellular stores, and a rise of cytosolic  $\text{Na}^+$ , presumably due to increased  $\text{Na}^+/\text{Ca}^{2+}$  exchange.

Key words:  $\text{K}^+$  conductance, cytosolic  $\text{Ca}^{2+}$ , cytosolic  $\text{Na}^+$ ,  $\text{Na}^+/\text{Ca}^{2+}$  exchange, BAPTA.

### Introduction

A large variety of cellular responses in neurons and glial cells is mediated by metabotropic membrane receptors linked to G proteins (Gilman, 1987; Brauner-Osborne et al., 2007). One of the most prevalent pathways initiated by activation of G proteins is the activation of phospholipase C (PLC), which leads to the formation of inositol-trisphosphate ( $\text{IP}_3$ ) and the release of  $\text{Ca}^{2+}$  from intracellular stores (Berridge et al., 2000). As electrically inexcitable cells, glial cells use this pathway in response to many kinds of stimuli, including neurotransmitters, neuromodulators, growth factors and cytokines (Lohr and Deitmer, 2006; Fiocco and McCarthy, 2006). In the nervous system, glial cells often respond to active neurons releasing neurotransmitters, which activate metabotropic receptors in the glial cells. Recent evidence suggests that glial cells may themselves release transmitters such as glutamate and ATP, mediated by cytosolic  $\text{Ca}^{2+}$  transients (Araque et al., 2001; Pascual et al., 2005). Due to the presence of several G proteins, which initiate or suppress different signalling cascades (for a review, see Luttrell, 2006), we tried to elucidate the dominant pathways initiated by global activation of G protein.

We have previously shown in the leech giant glial cell *in situ* that stimulation of the Leydig neuron elicits a membrane hyperpolarization due to an increased  $\text{K}^+$  conductance in the giant glial cell (Britz et al., 2002). We were able to show that Leydig neurons release the peptide myomodulin, which activates metabotropic membrane receptors in the glial cell membrane and results in the activation of G protein-coupled adenylyl cyclase, leading to an increase in  $\text{K}^+$  conductance and

a membrane hyperpolarization (Britz et al., 2004). A similar signalling cascade to that of Leydig neuron stimulation and myomodulin application is initiated by 5-hydroxytryptamine (5-HT) (Britz et al., 2005), a major neurotransmitter and neuromodulator in the leech central nervous system (Lent et al., 1991). The action of both ligands, myomodulin and 5-HT, was suppressed after blocking G proteins by injecting GDP- $\beta$ -S into the glial cell. These studies raised the question of whether the negative membrane potential in this and other glial cells may in part be attributable to neuronal activity by activating G protein-coupled glial membrane receptors. Since this  $\text{K}^+$  conductance was not affected by inhibition of protein kinase A (PKA), we suggested that it is mediated by ion channels directly gated by cyclic nucleotides (Britz et al., 2004; Britz et al., 2005).

As 5-HT also evokes cytosolic  $\text{Ca}^{2+}$  transients in this glial cell (Lohr and Deitmer, 1999), another signalling cascade can be triggered by the same ligand, presumably due to the expression of different types of 5-HT receptors in the leech central nervous system. Although the  $\text{K}^+$  conductance induced by 5-HT was not dependent on external  $\text{Ca}^{2+}$  (Britz et al., 2005), the question remained of whether conductance changes evoked by agonists of metabotropic transmitters and modulators, which are mediated by G protein activation, are principally independent of changes in cytosolic  $\text{Ca}^{2+}$ , even if cytosolic  $\text{Ca}^{2+}$  rises at the same time.

In the present study, we activated G proteins in the leech giant glial directly by injecting caged GTP- $\gamma$ -S, which was released by brief UV illumination. We assume that this method would not discriminate between different G proteins in the giant glial

cell and would give us the effects of 'global' G protein activation. This should also give us an idea about the most dominant effects mediated by G protein activation in this glial cell. Our results show that glial cells readily hyperpolarize due to an increase of the membrane  $K^+$  conductance and evoke cytosolic  $Ca^{2+}$  and  $Na^+$  transients, suggesting that the glial membrane potential is under metabotropic control of neuronal activity.

### Materials and methods

The experiments were performed on exposed giant glial cells in the neuropil of isolated ganglia of the leech *Hirudo medicinalis* L. For recording with intracellular conventional and ion-selective microelectrodes, and for confocal imaging in the giant glial cell, the ganglionic capsule and some overlying neuronal cell bodies of the ganglia were mechanically removed on the ventral side after prior enzymatic treatment with collagenase/dispase ( $2 \text{ mg ml}^{-1}$  for up to 30 min) and fine micro-dissection. The steps of dissection and the preparation have been described before (Britz et al., 2002). Isolated ganglia were stored in leech saline at  $4^\circ\text{C}$ , before being transferred into the experimental chamber.

#### *Injection of caged GTP- $\gamma$ -S and $IP_3$ into the giant glial cell and uncaging of these molecules in the cell*

The non-hydrolyzable G protein activator GTP- $\gamma$ -S, dissolved in  $H_2O$ , was injected into both giant glial cells as inactive 'caged' compound, S-(DMNPE-caged) {guanosine 5'-O-(3-thiotriphosphate),  $P^{3(S)}$ -[1-(4,5-dimethoxy-2-nitrophenyl)-ethyl]ester, triammonium salt} (Molecular Probes, Eugene, OR, USA) by constant or pulsed (2 s at 0.25 Hz) iontophoretic currents of  $-5 \text{ nA}$  for 10 min *via* a microelectrode containing  $10 \text{ mmol l}^{-1}$  caged GTP- $\gamma$ -S. After the injection, 30 min was allowed for diffusion of GTP- $\gamma$ -S within the giant glial cell before the caged compound was uncaged. Photolysis of caged GTP- $\gamma$ -S was carried out by illumination using a mercury arc lamp (model USH-102D; Ushio Electric, Tsukuba, Japan) at 355 nm. The light was focussed onto the ganglion and carefully adjusted to allow full photolysis of the caged compound without affecting neuronal and glial properties (e.g. membrane potential changes) when applied alone. The UV photolysis of caged GTP- $\gamma$ -S results in the release of the active, non-hydrolyzable, GTP analogue in the glial cells (Dolphin et al., 1988).

Caged  $IP_3$  (NPE-caged Ins-1,4,5- $P_3$ ) (Molecular Probes), dissolved in  $H_2O$ , was injected into the glial cells, and photolysis was induced in a similar way to caged GTP- $\gamma$ -S.

#### *Injection of GDP analogues*

For injection of GDP- $\beta$ -S, which blocks G protein-mediated responses, or, as a control, GDP, a micropipette was filled with either  $10 \text{ mmol l}^{-1}$  GDP- $\beta$ -S or  $10 \text{ mmol l}^{-1}$  GDP (Sigma-Aldrich Chemie, Taufkirchen, Germany), dissolved in  $0.5 \text{ mol l}^{-1}$  K-acetate. In order to minimize leakage of the negatively charged drugs from the pipette, a positive backing current was delivered by clamping the cells at a holding potential  $1\text{--}3 \text{ mV}$  positive to the resting membrane potential. Injection was then obtained with sustained or repetitive hyperpolarizing voltage pulses of  $3 \text{ mV}$  for 8 min.

### Electrophysiology

Electrophysiological recordings were performed with intracellular microelectrodes filled with  $2 \text{ mol l}^{-1}$  K-acetate/ $20 \text{ mmol l}^{-1}$  KCl or  $3 \text{ mol l}^{-1}$  KCl for membrane potential measurements. In two-electrode voltage-clamp experiments, the microelectrodes were filled with  $3 \text{ mol l}^{-1}$  KCl for potential recording and  $2 \text{ mol l}^{-1}$  K-citrate, adjusted to pH 7.0 with HCl, for current injection (resistance  $1\text{--}3 \times 10^7 \Omega$ ). For current and voltage-clamp recordings, Axoclamp 2A and/or 2B amplifiers (Axon Instruments, Union City, CA, USA) were used. Membrane currents were recorded by the built-in current measurement circuit of the headstages. The experimental bath was grounded *via* a chloride-covered silver wire in agar dissolved in normal saline. For further details, see Deitmer and Schneider (Deitmer and Schneider, 1995). The membrane input resistance was measured by injecting hyperpolarizing current pulses ( $10\text{--}20 \text{ nA}$  for  $1\text{--}2 \text{ s}$ ).

#### *Stimulation*

A Leydig neuron was stimulated with single square current pulses ( $2\text{--}4 \text{ nA}$ ,  $10\text{--}40 \text{ ms}$ ) *via* an intracellular sharp microelectrode, controlled by digitimers (D4030 and DS2; Digitimer Limited, Welwyn Garden City, UK), to induce firing frequencies of 10 Hz (for details, see Britz et al., 2002; Britz et al., 2004).

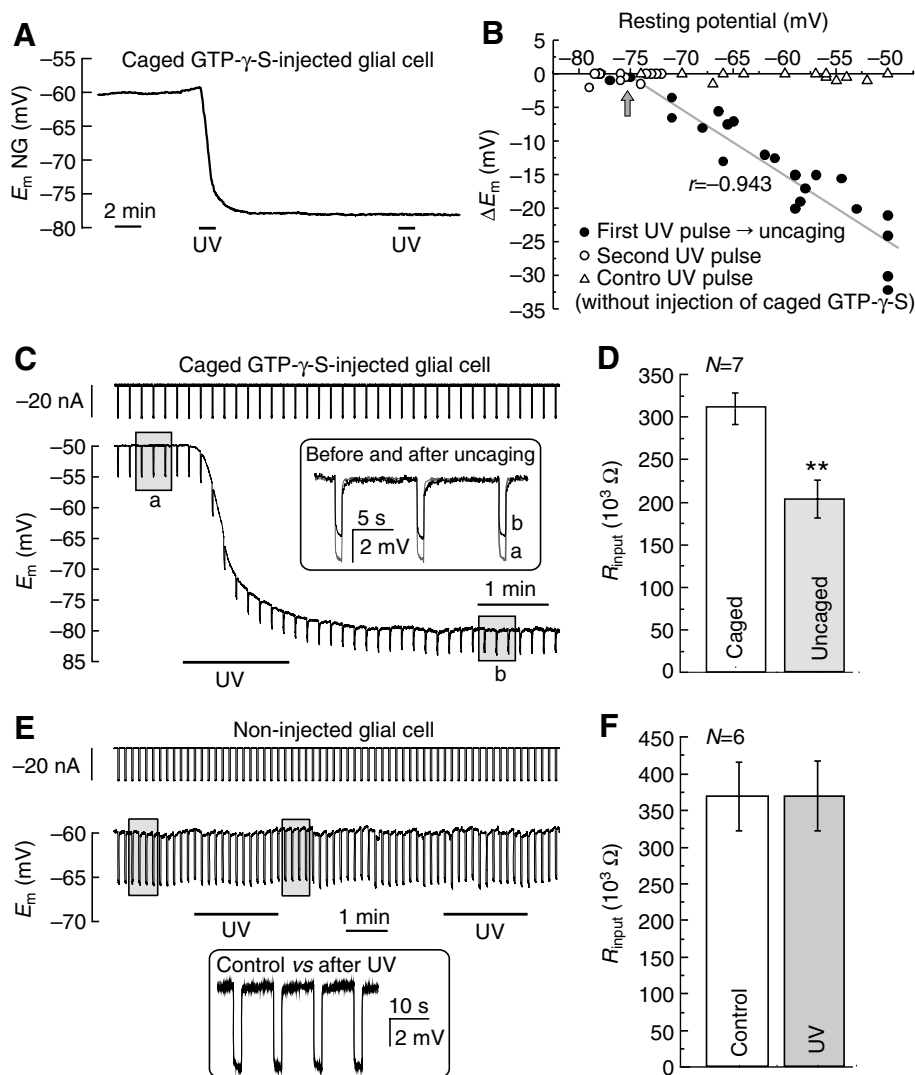
#### *$Na^+$ -selective microelectrodes*

For measurements of the intracellular  $Na^+$  activity and the membrane potential, single-barrelled  $Na^+$ -selective and voltage-sensing reference microelectrodes were used. The ion-selective electrode was silanized with a drop of 5% tri-*N*-butylchlorosilane in 99.9% pure carbon tetrachloride, backfilled into the tip. The micropipette was baked for 4.5 min at  $450^\circ\text{C}$  on a hotplate. For  $Na^+$ -selective microelectrodes, a drop of  $Na^+$ -cocktail [mixture of  $Na^+$ -ionophore (Fluka 71739), organic solvent (Fluka 73732) and a lipophilic salt (Fluka 72018) at a mass percent ratio of 10:89.5:0.5, respectively] was backfilled into the silanized barrel of the electrode, which was then filled up with  $0.1 \text{ mol l}^{-1}$  NaCl/ $10 \text{ mmol l}^{-1}$  3-(*N*-morpholino) propanesulphonic acid (MOPS), pH 7.0. The reference electrode was filled with  $3 \text{ mol l}^{-1}$  KCl. The ion-selective and the reference electrodes were connected by chloride-covered silver wires to the headstages of an electrometer amplifier. The electrodes were calibrated in leech salines with different  $Na^+$  concentrations ( $85 \text{ mmol l}^{-1}$ ,  $15 \text{ mmol l}^{-1}$ ,  $10 \text{ mmol l}^{-1}$ ,  $8.5 \text{ mmol l}^{-1}$ ,  $5 \text{ mmol l}^{-1}$ ) prior to and at the end of every experiment. In calibrating solutions with reduced  $Na^+$  concentration,  $Na^+$  was replaced by equimolar amounts of *N*-methyl-D-glucamine (NMDG, Fluka). Only electrodes with a response greater than  $50 \text{ mV}$  for a 10-fold change in the  $Na^+$  concentration were used for the experiments.

#### *Confocal $Ca^{2+}$ imaging*

The calcium-sensitive fluorescent dye Oregon Green 488 BAPTA-1 (OGB; Molecular Probes) was dissolved in water at a concentration of  $5 \text{ mmol l}^{-1}$  and used to fill a microelectrode. The resistance of the microelectrodes filled with the dye solution was  $120\text{--}150 \times 10^6 \Omega$ . The microelectrodes were positioned with a micromanipulator (UM-3FC, U Ltd, Tokyo, Japan), fixed at the stage of an upright microscope (Leica

Fig. 1. Changes in membrane potential ( $E_m$ ) and membrane resistance following uncaging of GTP- $\gamma$ -S. (A) Membrane potential recording of a giant glial cell injected with caged GTP- $\gamma$ -S, following moderate UV pulses (UV), the first of which hyperpolarized the cell membrane. (B) Changes in membrane potential following UV pulses; after a first (filled circles) and a second (open circles) UV pulse onto GTP- $\gamma$ -S-injected cells and after UV pulses of non-injected cells (open triangles). (C) Membrane input resistance ( $R_{input}$ ) measured by injection of square constant current pulses (upper trace), leading to brief deflection of the membrane potential (lower trace) before and after uncaging of GTP- $\gamma$ -S by a UV pulse. The inset shows superimposed potential traces at enhanced time scale before and after the UV pulse (indicated by shaded areas), showing a loss of input resistance after uncaging of GTP- $\gamma$ -S (D). (E,F) UV pulses onto non-injected cells had no effect on membrane potential or membrane resistance. \*\* $P < 0.01$ .



DMRB, Bensheim, Germany). An individual giant glial cell was impaled by microelectrodes with a piezo stepper (P-840.20, Physik Instrumente, Waldbronn, Germany). The dye was injected iontophoretically into the glial cell by a negative current of  $-3$  nA for about 10 min. 15–30 min after the termination of the dye injection, the fluorescence in the cell body and the cell processes reached constant values, indicating that the dye was equally distributed within the cell. OGB was excited by the 488 nm line of a Krypton–Argon laser, and images were taken with a confocal laser scanning microscope (Leica TCS 4D) at a sample rate of 0.25–4.7 Hz. The excitation light and the emission light were separated by a dichroic mirror at 510 nm. The emission light was truncated by a 515–545 nm band-pass filter. Regions of interest were defined in the first of a sequence of images, and the normalized fluorescence changes,  $\Delta F$  (in %), were measured throughout the image sequence.

### Solutions

The standard physiological leech saline for the experiments contained (in mmol  $l^{-1}$ ): NaCl, 85; KCl, 4;  $CaCl_2$ , 2;  $MgCl_2$ , 1; Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 10; pH adjusted to 7.4 with NaOH. In saline containing 10 mmol  $l^{-1}$  or 40 mmol  $l^{-1}$   $K^+$ , KCl was exchanged for an equivalent amount of NaCl. Cyclopiazonic acid (CPA) (Alexis Biochemicals, Lörrach, Germany) was dissolved in dimethyl sulfoxide (DMSO) as stock solutions and added to saline to make a final concentration of 20  $\mu$ mol  $l^{-1}$ . The DMSO concentration never exceeded 0.2% in the experimental saline. The leech myomodulin was synthesized according to Wang et al. (Wang et al., 1998) as described

before (Britz et al., 2002). All experiments were done at room temperature (22–24°C).

### Statistics

All data are given as means  $\pm$  standard error of the mean (s.e.m.),  $N$  indicates the number of experiments. The glial responses were measured either in the anterior or in the posterior giant glial cell, and the results were pooled, since no differences in the responses of the two glial cells can be observed (Schmidt and Deitmer, 1999; Britz et al., 2002). The statistical significance of the differences between mean values was tested using a standard, or a paired,  $t$ -test, if applicable. Differences were indicated in some figures as statistically significant for an error probability of  $P < 0.05$ ,  $P < 0.01$  or  $P < 0.001$ , as indicated by one, two or three asterisks, respectively.

### Results

#### Activation of G proteins by uncaging GTP- $\gamma$ -S

Persistent activation of G proteins was attempted by cell loading with the non-hydrolyzable GTP analogue GTP- $\gamma$ -S. We injected the GTP- $\gamma$ -S ionophoretically in caged form into both giant glial cells. When the ganglion was illuminated with

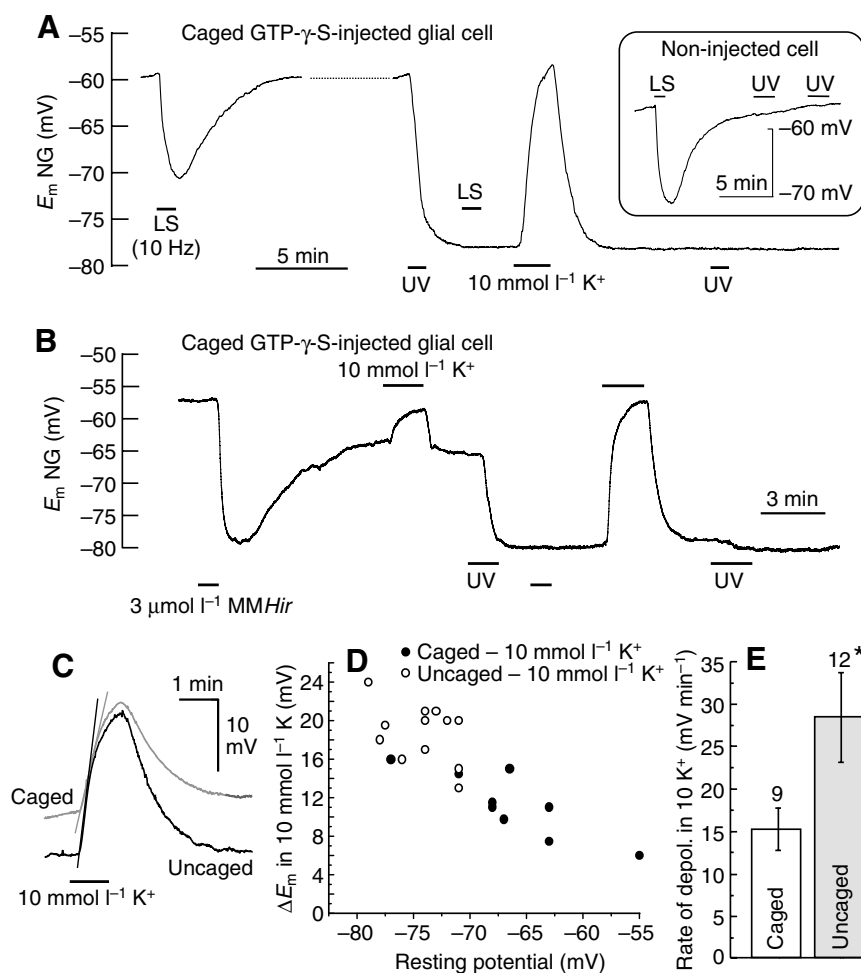


Fig. 2. Uncaging of GTP- $\gamma$ -S increases the relative K<sup>+</sup> conductance of the membrane. (A) Effects of Leydig neuron stimulation (LS) before and after a UV pulse (UV) to uncage GTP- $\gamma$ -S on the membrane potential. After the membrane hyperpolarization following uncaging of GTP- $\gamma$ -S, LS had no additional effect on the membrane potential, while raising the extracellular K<sup>+</sup> concentration from 4 to 10 mmol l<sup>-1</sup> evoked a large depolarization. The inset shows the effect of LS and UV pulses on a non-injected cell. (B) Effects of 3  $\mu$ mol l<sup>-1</sup> myomodulin (MMHir) and 10 mmol l<sup>-1</sup> K<sup>+</sup> before and after a UV pulse uncaging GTP- $\gamma$ -S on the membrane potential. (C–E) The membrane depolarization induced by 10 mmol l<sup>-1</sup> K<sup>+</sup> is larger (C,D), and faster rising (C,E), after uncaging of GTP- $\gamma$ -S, indicating an increased relative K<sup>+</sup> conductance. \* $P$ <0.05.  $N$  represents the number of experiments.

contrast, non-injected glial cells showed no change in their input resistance before ( $369 \pm 46$  k $\Omega$ ) and after ( $370 \pm 48$  k $\Omega$ ) UV illumination ( $N=6$ ) (Fig. 1E,F). These results indicate that uncaging GTP- $\gamma$ -S induces a persistent membrane hyperpolarization and an increase in membrane conductance of the glial membrane.

Iontophoretic injection of caged GTP- $\gamma$ -S alone (without uncaging) had no effect on the glial cell, and Leydig interneuron stimulation (10 Hz, 1 min) (Fig. 2A), myomodulin (MMHir) (3  $\mu$ mol l<sup>-1</sup>) (Fig. 2B) and 5-HT (20  $\mu$ mol l<sup>-1</sup>; not shown) still evoked a membrane hyperpolarization in the glial cell,

as described previously (Britz et al., 2002; Britz et al., 2004). After uncaging of GTP- $\gamma$ -S and the subsequent membrane hyperpolarization, neither MMHir nor Leydig neuron stimulation had any additional effect on the glial membrane potential (Fig. 2 A,B). Repeated UV illumination had only a small or no effect on the glial membrane potential once the glial cell membrane had hyperpolarized. In glial cells not injected, Leydig neuron stimulation elicited a membrane hyperpolarization, but UV pulses had no effect (Inset, Fig. 2A).

The K<sup>+</sup> permeability was evaluated by the membrane depolarisation induced by raising the extracellular K<sup>+</sup> concentration from 4 to 10 mmol l<sup>-1</sup> for 1–2 min (see Fig. 2A–C). The amplitude and the rate of rise of this depolarization give an indication of the relative K<sup>+</sup> permeability (dV/dt indicative of the induced membrane current). The glial membrane depolarization to 10 mmol l<sup>-1</sup> K<sup>+</sup> depended on the resting membrane potential but was considerably larger in glial cells, which had been hyperpolarized following the uncaging of GTP- $\gamma$ -S (Fig. 2D). The rate of rise of the glial membrane depolarisation in 10 mmol l<sup>-1</sup> K<sup>+</sup> (dV/dt), indicative of the current flow upon raising the K<sup>+</sup> concentration, was almost doubled, rising from  $15.3 \pm 2.5$  mV min<sup>-1</sup> to  $28.5 \pm 5.3$  mV min<sup>-1</sup> ( $N=9-12$ ;  $P<0.05$ ) (Fig. 2E) after uncaging GTP- $\gamma$ -S. These results show that the membrane hyperpolarization induced by uncaging GTP- $\gamma$ -S was accompanied by a rise in the relative K<sup>+</sup> conductance of the glial

moderate UV light ('UV pulse') for 60 s to uncage GTP- $\gamma$ -S, a rapid membrane hyperpolarization was measured, which did not reverse during the duration of the experiment of up to 1 h (Fig. 1A). UV illumination of ganglia without prior injection of caged GTP- $\gamma$ -S into the glial cell, had no effect on the glial membrane potential ( $N=13$ ) (Fig. 1E). The UV illumination as used here had no effect on the resting membrane potential and on the firing frequency and kinetics of spontaneous action potentials of Leydig neurons or N-cells ( $N=4$ ; not shown).

The size of the glial membrane hyperpolarization following the uncaging of GTP- $\gamma$ -S was dependent on the resting potential of the cell; the less negative the resting potential, the larger was the glial response to uncaging of GTP- $\gamma$ -S ( $N=22$ ) (Fig. 1B). The regression line (coefficient of correlation  $r=-0.943$ ) indicated a reversal potential of the glial response of  $-75$  mV, which is close to the K<sup>+</sup> equilibrium potential (Britz et al., 2005). A second UV pulse ( $N=11$ ), or a UV pulse on non-injected cells ( $N=13$ ), had no effect on the glial membrane potential (open symbols in Fig. 1B).

We also measured the input resistance of the glial membrane before and after UV illumination of caged GTP- $\gamma$ -S-injected and non-injected glial cells. The input resistance, recorded by repeated negative currents injected into the glial cell, decreased after uncaging GTP- $\gamma$ -S (Fig. 1C), on average by 36% from  $315 \pm 32$  k $\Omega$  to  $200 \pm 28$  k $\Omega$  ( $N=7$ ;  $P<0.01$ ) (Fig. 1D). By

cell membrane, indicating that G protein activation increases the  $K^+$  permeability of the glial cell membrane, as had been shown to be induced by Leydig neuron stimulation, myomodulin and 5-HT (Britz et al., 2004; Britz et al., 2005).

#### Injection of GDP- $\beta$ -S or GDP

The increase in  $K^+$  conductance induced by the uncaging of GTP- $\gamma$ -S was not due to the hyperpolarization *per se*, since the glial cell current–voltage relationship is nearly linear over the potential range of  $-50$  to  $-100$  mV (not shown here) (Munsch and Deitmer, 1994). The glial responses to myomodulin and Leydig neuron stimulation and to 5-HT could be blocked by the injection of the non-hydrolyzable G protein inhibitor GDP- $\beta$ -S (Britz et al., 2004; Britz et al., 2005). We measured the membrane current and membrane resistance following G protein blockade by injecting the G protein inhibitor GDP- $\beta$ -S into the glial cell. In voltage-clamped glial cells, GDP- $\beta$ -S, but not GDP, led to a net inward current (Fig. 3A). The membrane conductance of the glial membrane decreased after injection of GDP- $\beta$ -S by 20%, from  $4.9 \pm 0.4$   $\mu$ S to  $3.9 \pm 0.3$   $\mu$ S (Fig. 3B,C). Injection of the hydrolyzable analogue GDP had no effect on membrane potential and membrane resistance of the giant glial cell (Fig. 3D,E).

#### Cytosolic $Ca^{2+}$ rises following uncaging of GTP- $\gamma$ -S

The cytosolic  $Ca^{2+}$ , monitored by the use of the  $Ca^{2+}$  indicator dye Oregon Green BAPTA-1 (OGB) injected into the leech giant glial cell, and with confocal microscopy, increased following uncaging of GTP- $\gamma$ -S by a UV pulse (Fig. 4A,C). A second UV pulse, or UV illumination to a cell not injected with caged GTP- $\gamma$ -S, had no effect on the cytosolic  $Ca^{2+}$ . By contrast, raising the extracellular  $K^+$  concentration from 4 to 40 mmol  $l^{-1}$ , which depolarized the cell to near  $-30$  mV, evoked a  $Ca^{2+}$  transient in all cells injected and not injected with GTP- $\gamma$ -S (Fig. 4A–C). This  $Ca^{2+}$  rise had been shown to be due to voltage-dependent  $Ca^{2+}$  influx (Lohr and Deitmer, 2006; Munsch and Deitmer, 1992). The amplitude of the  $Ca^{2+}$  transients amounted to about 30% fluorescence change for both stimuli (Fig. 4C). After depletion of the intracellular  $Ca^{2+}$  stores with cyclopiazonic acid (CPA; 20  $\mu$ mol  $l^{-1}$ ), GTP- $\gamma$ -S still evoked a membrane hyperpolarization but did not elicit any cytosolic  $Ca^{2+}$  change (Fig. 4D,E). This indicates that the  $Ca^{2+}$  rise evoked by GTP- $\gamma$ -S can be attributed to release of  $Ca^{2+}$  from intracellular stores. Membrane depolarization by 40 mmol  $l^{-1}$   $K^+$  still induced a rise in cytosolic  $Ca^{2+}$ , which amounted to  $18 \pm 2\%$  fluorescence change ( $N=4$ ) (Fig. 4E). Our experiments suggest that the membrane hyperpolarization following release of GTP- $\gamma$ -S was independent of the increase in cytosolic  $Ca^{2+}$ , and presumably due to a  $Ca^{2+}$ -independent  $K^+$  conductance increase, as described for the 5-HT-evoked, cAMP-mediated,  $K^+$  conductance in this cell (Britz et al., 2005). We confirmed this conclusion further by the injection of BAPTA into the giant glial cell, which suppressed the  $Ca^{2+}$  rise (not shown) but did not abolish the membrane hyperpolarization elicited by uncaging GTP- $\gamma$ -S (Fig. 4F). The membrane potential was  $-53.5 \pm 1.5$  mV before and  $-51.4 \pm 1.6$  mV after injection of BAPTA (not significant;  $N=10$ ); subsequent uncaging of GTP- $\gamma$ -S hyperpolarized the membrane to  $-74.3 \pm 1.3$  mV ( $P<0.01$ ,  $N=10$ ).

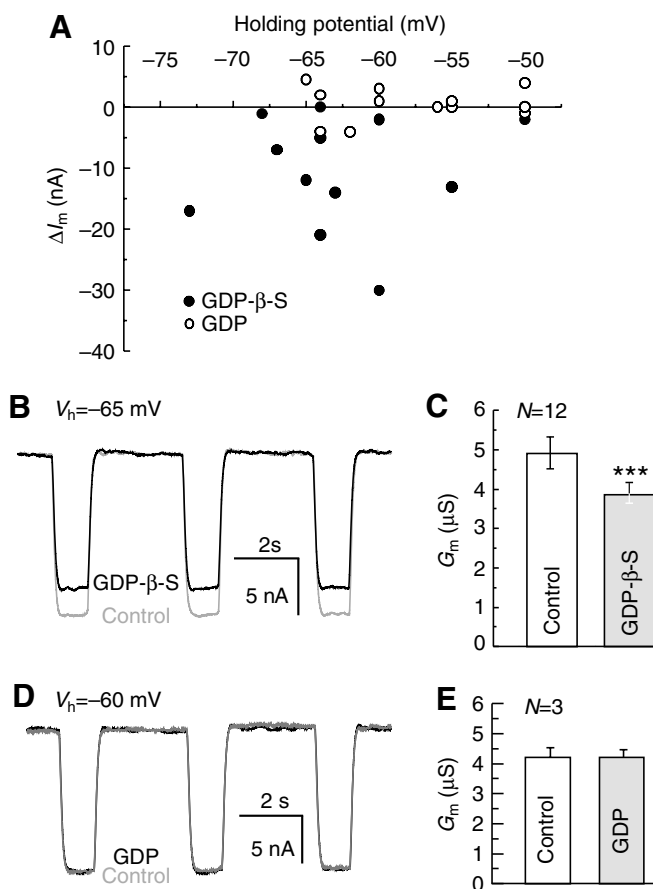


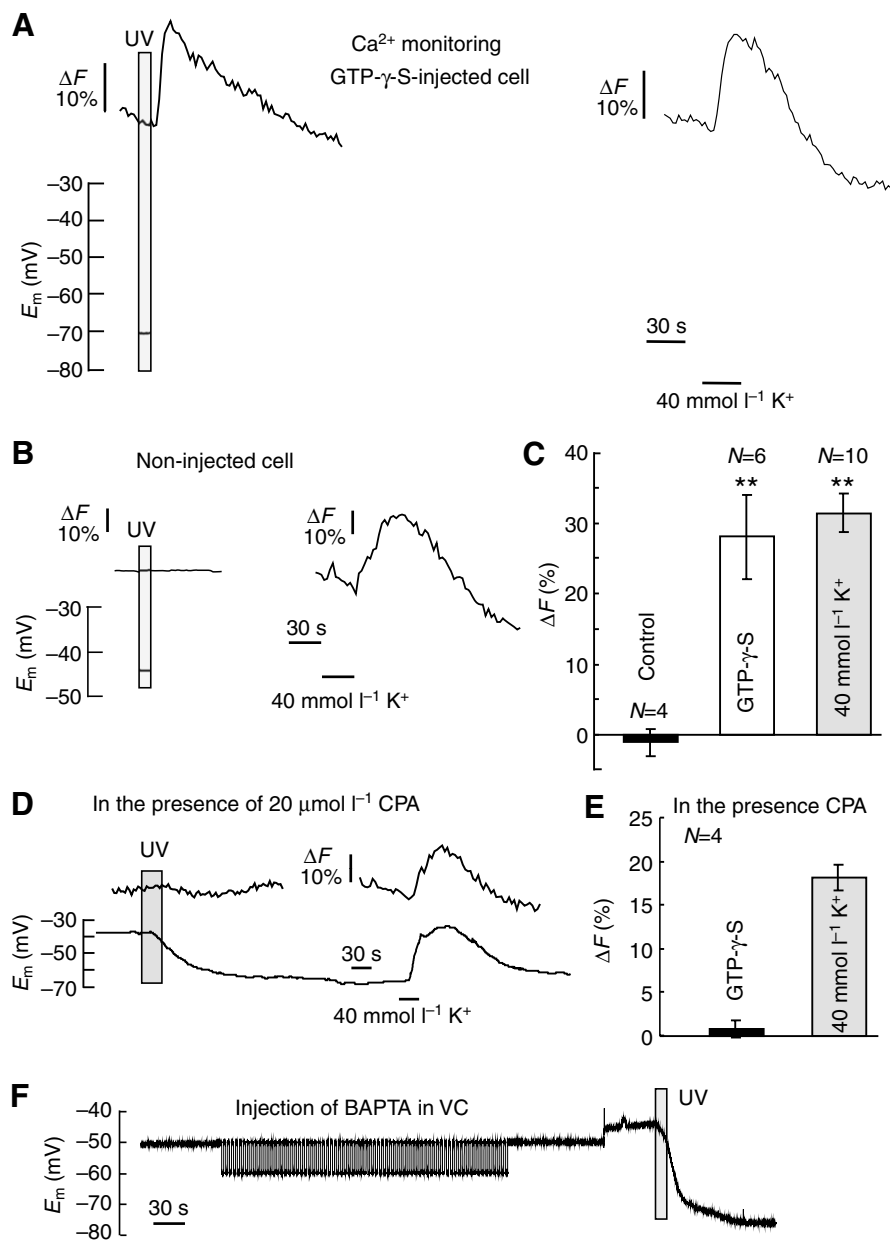
Fig. 3. Inhibition of G proteins after injection of GDP- $\beta$ -S. (A) Change in holding current ( $I_m$ ) after injection of the non-hydrolyzable inhibitor of G proteins GDP- $\beta$ -S (filled circles) and of GDP (open circles) of a glial cell voltage-clamped to different holding potentials (set near to the zero current potential of each cell). (B–E) Currents during hyperpolarizing voltage ( $V_h$ ) steps before and after injection of GDP- $\beta$ -S (B) or GDP (D), from which the membrane conductance ( $G_m$ ) was calculated (C,E). \*\*\* $P<0.001$ .

#### Cytosolic $Na^+$ rises following uncaging of GTP- $\gamma$ -S

The release of GTP- $\gamma$ -S by a UV pulse also affected the cytosolic  $Na^+$  as measured with ion-selective microelectrodes in the leech giant glial cell. During the membrane hyperpolarization following uncaging of GTP- $\gamma$ -S, the cytosolic  $Na^+$  concentration increased from  $7.6 \pm 1.8$  mmol  $l^{-1}$  to  $13.2 \pm 1.7$  mmol  $l^{-1}$  ( $N=8$ ,  $P<0.01$ ) (Fig. 5A,B). In cells not injected with GTP- $\gamma$ -S, UV illumination had no effect on the cytosolic  $Na^+$  (Fig. 5C). Application of 5-HT (20  $\mu$ mol  $l^{-1}$ ), which also hyperpolarizes the cell membrane of the giant glial cell, did not change the cytosolic  $Na^+$  level, suggesting that it was not the membrane hyperpolarization *per se* that induced the rise in cytosolic  $Na^+$  (not shown here).

In order to check if the rise in  $Na^+$  was linked to the release of  $Ca^{2+}$  from intracellular stores, we depleted these stores with CPA prior to releasing GTP- $\gamma$ -S. The cytosolic  $Na^+$  did not change after the addition of CPA, nor did it change upon uncaging GTP- $\gamma$ -S in the presence of CPA (Fig. 5D). As described above, the membrane hyperpolarization evoked by uncaging GTP- $\gamma$ -S was not affected by CPA. These experiments

Fig. 4. The effect of uncaging GTP- $\gamma$ -S on the cytosolic  $\text{Ca}^{2+}$ . (A) Glial cell injected with the fluorescent  $\text{Ca}^{2+}$  indicator Oregon Green BAPTA-1 and caged GTP- $\gamma$ -S showed a transient increase in cytosolic  $\text{Ca}^{2+}$  ( $\Delta F$ ) and a sustained membrane hyperpolarization following a first UV pulse (UV), while a second UV pulse remained without effect. Membrane depolarization induced by raising the extracellular  $\text{K}^+$  concentration from 4 to 40 mmol  $\text{l}^{-1}$  induced a  $\text{Ca}^{2+}$  transient of similar amplitude. (B) Effect of UV pulse and 40 mmol  $\text{l}^{-1}$   $\text{K}^+$  on a cell not injected with GTP- $\gamma$ -S. (C) Summary of cytosolic  $\text{Ca}^{2+}$  changes as shown in A and B. (D) Changes in cytosolic  $\text{Ca}^{2+}$  and membrane potential following uncaging of GTP- $\gamma$ -S and during membrane depolarization in 40 mmol  $\text{l}^{-1}$   $\text{K}^+$  after depletion of the intracellular  $\text{Ca}^{2+}$  stores with cyclopiazonic acid (CPA, 20  $\mu\text{mol l}^{-1}$ ). While high- $\text{K}^+$  still evoked a  $\text{Ca}^{2+}$  rise, the uncaging of GTP- $\gamma$ -S did not change the cytosolic  $\text{Ca}^{2+}$  after store depletion (E) but still elicited the sustained membrane hyperpolarization. (F) Injection of the  $\text{Ca}^{2+}$  chelator BAPTA into the glial cell did not suppress the membrane hyperpolarization following the uncaging of GTP- $\gamma$ -S by a UV pulse.  $**P < 0.01$ .



suggest that the rise of intracellular  $\text{Na}^+$  following uncaging of GTP- $\gamma$ -S was secondary to the release of  $\text{Ca}^{2+}$  from intracellular stores, presumably due to enhanced  $\text{Na}^+/\text{Ca}^{2+}$  exchange activity, and not due to a  $\text{Na}^+$  leak of the cell membrane, which would be expected to increase during the membrane hyperpolarization due to the larger  $\text{Na}^+$  gradient generated.

#### Uncaging of inositol-trisphosphate

In order to check the effects of a  $\text{Ca}^{2+}$  increase alone, we injected caged inositol-trisphosphate ( $\text{IP}_3$ ) into the giant glial cell and uncaged it with a moderate UV pulse. This should lead to the release of  $\text{Ca}^{2+}$  from intracellular stores *via* activation of  $\text{IP}_3$  receptor channels. Monitoring cytosolic  $\text{Ca}^{2+}$  indicated that uncaging of  $\text{IP}_3$  evoked a  $\text{Ca}^{2+}$  rise of similar amplitude as membrane depolarization with 40 mmol  $\text{l}^{-1}$   $\text{K}^+$  (Fig. 6A–C). A fast rise of cytosolic  $\text{Ca}^{2+}$  was followed by a rapid recovery, and a second UV pulse had no effect on the recovered level of intracellular  $\text{Ca}^{2+}$ . The membrane potential was not affected by uncaging  $\text{IP}_3$ .

Uncaging of  $\text{IP}_3$  also evoked a small but significant rise in cytosolic  $\text{Na}^+$  by 1.4 mmol  $\text{l}^{-1}$  from  $8.5 \pm 0.5$  mmol  $\text{l}^{-1}$  to  $9.9 \pm 0.6$  mmol  $\text{l}^{-1}$  ( $N=7$ ;  $P < 0.05$ ) (Fig. 6D,E). A second UV pulse had only a small or no effect on the cytosolic  $\text{Na}^+$  level. Thus,  $\text{Ca}^{2+}$  release from intracellular stores evokes an  $\text{Na}^+$  rise, which supports the notion that this  $\text{Na}^+$  is due to an increase of  $\text{Na}^+/\text{Ca}^{2+}$  exchange. These experiments also indicate that, although uncaging  $\text{IP}_3$  elicited a transient rise in cytosolic  $\text{Ca}^{2+}$

and  $\text{Na}^+$ , these ion changes were not sufficient to induce a membrane hyperpolarization.

#### Discussion

G protein activation by extracellular signalling molecules is one of the most frequently employed mechanisms to activate cells, including neurons and glial cells in the brain (Gilman, 1987; Brauner-Osborne et al., 2007). Our experiments show that activation of G proteins by uncaging of GTP- $\gamma$ -S mimics the effects of several neurotransmitters/neuromodulators activating metabotropic receptors in the leech central nervous system. Uncaging GTP- $\gamma$ -S resulted in membrane hyperpolarization, due to a  $\text{K}^+$  conductance increase, and a rise in cytosolic  $\text{Ca}^{2+}$ , due to release from intracellular stores, and cytosolic  $\text{Na}^+$ , presumably attributable to increased  $\text{Na}^+/\text{Ca}^{2+}$  exchange. Our study shows that 'global' activation of G proteins in the giant glial cell leads to the activation of a number of signalling

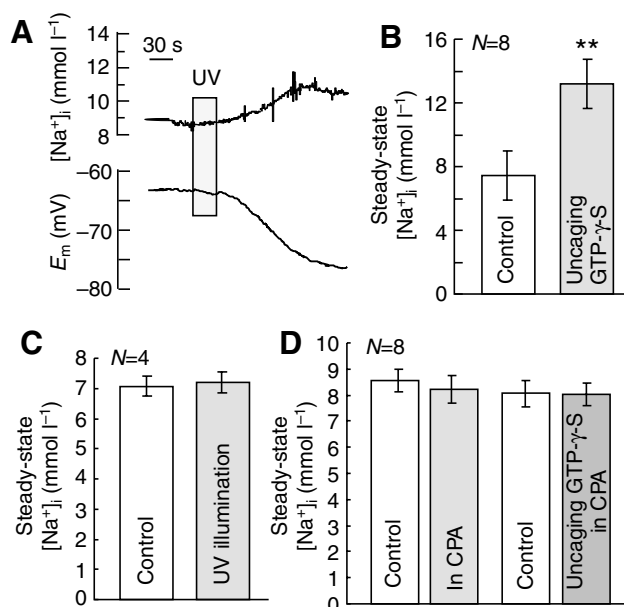
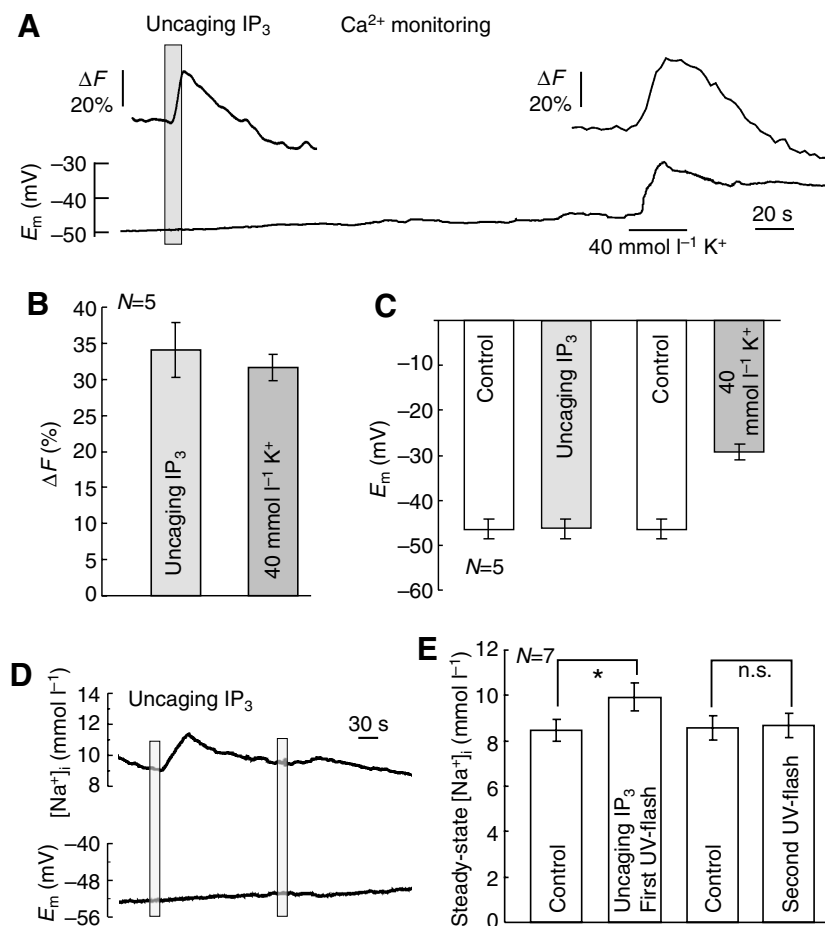


Fig. 5. The effect of uncaging GTP- $\gamma$ -S on cytosolic Na<sup>+</sup>. (A) Recording of intracellular Na<sup>+</sup> and membrane potential before and after uncaging GTP- $\gamma$ -S by a UV pulse. (B–D) Steady-state cytosolic Na<sup>+</sup> concentration before (control) and during uncaging GTP- $\gamma$ -S (B), before (control) and after a UV pulse on non-injected cells (C), and in the absence (control) and presence of cyclopiazonic acid (CPA, 20  $\mu$ mol l<sup>-1</sup>), before (control) and after uncaging GTP- $\gamma$ -S in the presence of CPA (D). \*\* $P$ <0.01.

cascades, which in turn affects various ion gradients and membrane conductance.

#### Activation of G proteins by uncaging of GTP- $\gamma$ -S

Ligands usually activate metabotropic receptors, which are linked to specific, excitatory or inhibitory G proteins (Luttrell, 2006). However, one or more G proteins can be activated by simultaneous activation of several types of metabotropic receptors (Werry et al., 2003). This may trigger multiple signalling cascades in a given cell, leading to the activation of diverse cell functions. The technique of uncaging caged molecules by UV illumination has become a powerful technique to increase the non-hydrolyzable G protein activator GTP- $\gamma$ -S in a relatively short time to initiate G protein-mediated cellular activity (Dolphin et al., 1988; Oberhauser et al., 1998). Since we studied effects that were relatively slow in this large cell, we used moderate UV illumination to avoid the side effects of strong UV light. For example, ion channels can be sensitive to UV, and strong illumination may therefore result in a change in neuronal activity (Hof and Fox, 1983; Middendorff et al., 2000). We could observe changes in membrane potential and firing frequency of several neurons in the leech central nervous system when we used strong UV pulses. Therefore, we used moderate UV pulses of longer duration (up to 2 min), which still lead to the uncaging of intracellularly injected caged compounds but do not alter neuronal activity. UV illumination without prior injection of GTP- $\gamma$ -S also had no effect on the membrane potential of the giant glial cell.



#### 'Global' activation of G proteins triggers different signalling cascades

The increase in the membrane K<sup>+</sup> conductance and the subsequent hyperpolarization following uncaging of GTP- $\gamma$ -S was likely to be attributable to cAMP produced by the activation of adenylyl cyclase. This had been studied in detail for the neurotransmitters 5-HT and myomodulin, which both evoked a GDP- $\beta$ -S-sensitive increase in the membrane K<sup>+</sup> conductance of the leech giant glial cell, and which were blocked by inhibition of adenylyl cyclase and mimicked by membrane-permeant cAMP analogues (Britz et al., 2004; Britz et al., 2005). It can be concluded that 'global' activation of G proteins by uncaging of GTP- $\gamma$ -

Fig. 6. Effect of uncaging inositol-trisphosphate (IP<sub>3</sub>) on cytosolic Ca<sup>2+</sup> and Na<sup>+</sup>. (A) Uncaging IP<sub>3</sub> with a UV pulse elicited a cytosolic Ca<sup>2+</sup> rise (ΔF) but no membrane potential change. A second UV pulse changed neither cytosolic Ca<sup>2+</sup> nor membrane potential, and membrane depolarization in 40 mmol l<sup>-1</sup> K<sup>+</sup> still evoked a normal transient Ca<sup>2+</sup> rise. (B,C) Summary of effects of uncaging IP<sub>3</sub> and of 40 mmol l<sup>-1</sup> K<sup>+</sup> on cytosolic Ca<sup>2+</sup> (B) and on the membrane potential (C). (D,E) Uncaging of IP<sub>3</sub> elicited a reversible intracellular Na<sup>+</sup> rise but no membrane potential change. A second UV pulse had no effect on either Na<sup>+</sup> or membrane potential. \* $P$ <0.05.

S activates the same signalling pathways leading to the conductance increase as initiated by 5-HT and myomodulin and following Leydig neuron stimulation. Since inhibition of PKA did not inhibit this effect, the  $K^+$  conductance was suggested to be due to activation of cyclic nucleotide-gated channels in the glial membrane (Britz et al., 2004; Hirth and Deitmer, 2006). The  $K^+$  conductance elicited by 5-HT was also shown to be independent of  $Ca^{2+}$  (Britz et al., 2005), which is supported by our present findings that the membrane hyperpolarization induced by uncaging of GTP- $\gamma$ -S was neither suppressed by injection of the fast  $Ca^{2+}$  chelator BAPTA nor by prior depletion of intracellular  $Ca^{2+}$  stores by CPA.

The other signalling pathway activated by uncaging GTP- $\gamma$ -S in the giant glial cell resulted in the release of  $Ca^{2+}$  from intracellular stores mediated by phospholipase C (PLC) and the production of  $IP_3$  (Berridge et al., 2003). We could show that uncaging of  $IP_3$  elicited a similar transient  $Ca^{2+}$  rise as uncaging of GTP- $\gamma$ -S. The rise of cytosolic  $Na^+$ , however, was apparently secondary to the cytosolic  $Ca^{2+}$  increase, since it was abolished after the intracellular  $Ca^{2+}$  stores were depleted by CPA, suggesting increased  $Na^+/Ca^{2+}$  exchange as the source for the elevated cytosolic  $Na^+$ .  $Na^+/Ca^{2+}$  exchange is one of the main processes to regulate cytosolic  $Ca^{2+}$  and is activated by increased intracellular  $Ca^{2+}$  concentrations (Annunziato et al., 2004; DiPolo and Beaugé, 2006). In the leech giant glial cell, we could show that both plasmalemmal  $Ca^{2+}$ -ATPase and  $Na^+/Ca^{2+}$  exchange contribute to the recovery of  $Ca^{2+}$  from a cytosolic rise (Nett and Deitmer, 1998a). In rat cerebellar astrocytes,  $Na^+/Ca^{2+}$  exchange was shown to be responsible for maintaining a low resting cytosolic  $Ca^{2+}$  (DiPolo and Beaugé, 2006). We hypothesize that a transient intracellular  $Na^+$  rise may generally follow  $Ca^{2+}$  release from stores and global cytosolic  $Ca^{2+}$  signalling in glial cells and other cell types, as initiated by activation of metabotropic receptors. A rise of cytosolic  $Na^+$  would be expected to reduce membrane transport processes driven by the  $Na^+$  gradient across the glial cell membrane, such as the excitatory amino acid transporter EAAT, which is instrumental for the clearance of synaptically released glutamate. Hence, cytosolic  $Ca^{2+}$  rises in glial cells may modulate glutamatergic transmission by decreasing  $Na^+$ -dependent glutamate uptake (Marcaggi and Attwell, 2004).

Uncaging  $IP_3$  elicited cytosolic  $Ca^{2+}$  and  $Na^+$  transients, as uncaging of GTP- $\gamma$ -S did, but no membrane hyperpolarization, confirming the above conclusion that the G protein-mediated increase in  $K^+$  conductance and membrane hyperpolarization is not  $Ca^{2+}$ - or  $Na^+$ -dependent. In the leech giant glial cell, an increased  $K^+$  conductance can be elicited by removal of external  $Na^+$ , which is  $Ca^{2+}$  independent and presumably attributable to a decreased cytosolic  $Na^+$  concentration (Nett and Deitmer, 1998b). The experiments also support the notion that it was the release of  $Ca^{2+}$  from intracellular stores that caused the rise in intracellular  $Na^+$ , and not G protein activation by GTP- $\gamma$ -S *per se*. It can be concluded that the cAMP-mediated  $K^+$  conductance in the leech giant glial cell, elicited either by neurotransmitters or, as in the present study, by G protein activation, is neither  $Ca^{2+}$ - nor  $Na^+$ -dependent. The rise in cytosolic  $Na^+$  may itself affect  $Na^+$ -dependent transport processes, in particular glial glutamate uptake, which is greatly dependent on the  $Na^+$  gradient across the membrane. This would leave more glutamate

for a longer while in synaptic areas and thus may be a glial feedback response to the synaptic activity between neurons.

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