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Photoactivation of intracellular guanosine triphosphate analogues reduces the amplitude and slows the kinetics of voltage-activated calcium channel currents in sensory neurones

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Abstract. The influence of guanine nucleotide analogues on calcium channel currents in cultured rat dorsal root ganglion neurones has been studied using a technique in which the rate of diffusion of the analogues to their site of action is by-passed by photochemical release of the analogues within the neurones. The 1(2-nitrophenyl)ethyl P^3-ester derivatives of guanosine 5′-0(3-thio)triphosphate (caged GTP-γ-S) and 5′-guanylylimidodiphosphate (caged GMP-PNP) were synthesised and found to be completely photolysable by light, yielding free GTP-γ-S and GMP-PNP. Calcium channel currents were recorded using the whole cell patch technique and either caged GTP-γ-S or caged GMP-PNP (2 mM) were included in the patch pipette. Stable currents were recorded for 5–10 min, and a single pulse of 300–350 nm irradiation was directed using a liquid light guide onto the recording dish. Calcium channel currents were then recorded every 30–120 s following photochemical release of approximately 20 μM GTP-γ-S. The peak calcium channel current was reduced by about 70% with a slow time course [$t_{1/2}$ 1.5 ± 0.2 min (mean ± SEM); n = 5]. The transient component of the peak current was usually completely abolished, whereas the sustained current measured at the end of the 100 ms depolarising pulse was less affected. Qualitatively similar effects were observed on photolysis of caged GMP-PNP. These results suggest that the channels underlying the transient and the sustained components of the whole cell current may be differentially molulated by GTP analogues.

Key words: Guanine nucleotide analogue – Calcium current – Flash photolysis – Guanine nucleotide binding protein – dorsal root ganglion neurone

Introduction

Guanine nucleotide binding proteins (G proteins, Rodbell 1985; Spiegel et al. 1985; Gilman 1987) were first discovered in relation to receptor-mediated adenylate cyclase activation (G_s) (Pfeuffer and Helmreich 1975) and subsequently its inhibition (G_i) (Jakobs et al. 1983). More recently it has been found that G proteins are also involved in receptor-mediated changes in other second messenger systems (Gomperts 1983) and in activation and inhibition of ion channels (Breitwieser and Szabo 1985; Pfaffinger et al 1985; Scott and Dolphin 1986; Holz et al. 1986).

Voltage-activated calcium channel currents may be modulated by neurotransmitters in several cell types. In atrial and ventricular myocardial cells, calcium currents are enhanced by β-adrenergic receptor agonists and inhibited by muscarinic agonists; and these effects are thought to be associated with elevation and reduction respectively of the intracellular level of cyclic AMP (Bean et al. 1985; Breitwieser and Szabo 1985; Hescheler et al. 1986).

Calcium channel currents in dorsal root ganglion (DRG) neurones cultured from several species, including rat and chick, can be inhibited by neurotransmitters acting at several classes of receptor, including noradrenaline (α_2), opiates (κ), GABA (B) and adenosine (A_1) (Dunlap and Fischbach 1981; Dolphin and Scott 1986; MacDonald and Werz 1986). There is evidence in most cases that these effects are not due to inhibition of adenylate cyclase (Forscher and Oxford 1985; Forscher et al. 1986; Dolphin and Scott 1987a), since they can be elicited in the presence of intracellular cyclic AMP or forskolin. However, there is good evidence that pertussis toxin-sensitive G proteins are involved in coupling these responses to inhibition of calcium channel activity, since they can be enhanced by intracellular GTP analogues (Scott and Dolphin 1986; Dolphin and Scott 1987a), and reduced by a GDP analogue (Holz et al. 1986; Scott and Dolphin 1986; Dolphin and Scott 1987a) and by pertussis toxin (Holz et al. 1986; Dolphin and Scott 1987a). We have also observed that GTP analogues have the ability to modify whole cell calcium channel currents in the absence of agonist, slowing the current activation and abolishing the initial transient component of the current (Scott and Dolphin 1986; Dolphin and Scott 1987a).

Studies of single calcium channels have recently revealed three types of channel in DRGs (Nowycky et al. 1985). T and N type channels underlie the transient whole cell currents and are both inactivated by holding at potentials more positive to −60 mV, and require strongly negative potentials to remove inactivation. T type channels are activated at low threshold (Carbone and Lux 1984) and N at high threshold potentials. The third type of unitary channel observed, L, underlies sustained whole cell currents activated at high threshold potentials, and is not inactivated by depolarization of cells to −60 mV. It is possible that the effect of GTP analogues on whole cell currents occurs as a result of inhibition of flux through N and T type channels,

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leaving only the non-inactivating whole cell current passing through L type channels, which may be less sensitive to inhibition by GTP-γ-S (Scott and Dolphin 1986; Dolphin and Scott 1987a, b). However, it is likely from our results that the rate of activation of the L current is also reduced by GTP-γ-S (Dolphin and Scott 1987a, Fig. 2).

The present experiments were undertaken in order to examine in a single cell the effect of guanine nucleotide analogues on the amplitude and kinetics of calcium channel currents. Novel caged analogues of GTP, guanosine 5′-0-3-thiotriphosphate (GTP-γ-S) and 5′-guanylylimidodiphosphate (GMP-PNP) have been synthesised, which are photolysed by light at 300–350 nm. These have been included in the patch pipette, so that once inside the cell they can be activated rapidly by photolysis, allowing the kinetics of their effect on the calcium channel currents to be examined.

Methods

Synthesis of 1(2-nitrophenyl)ethyl P^3 ester of guanosine 5′-0(3-thio)triphosphate (caged GTP-γ-S). The method of Walker, Read and Trenthan (personal communication) was employed to synthesize the caged derivatives of GTP-γ-S. The tetralithium salt of GTP-γ-S (Boehringer-Mannheim, Mannheim, FRG, containing approximately 26% of GDP as estimated by HPLC) was used without further purification. Fifteen μmol as GTP-γ-S in 1.0 ml of H_2O was adjusted to pH 4.4 with dilute HCl and stirred vigorously for 22 h with 2 ml of freshly prepared 85 mM 1(2-nitrophenyl)diazoethane in $CHCl_3$, at room temperature and protected from light. The nucleotide in the aqueous phase of the reaction mixture (pH 5.7) was analysed by HPLC (see below) and contained 30% caged GTP-γ-S, with GDP (caged and free) comprising the bulk of the remaining nucleotides, reflecting further hydrolysis of free GTP-γ-S. The aqueous phase was washed three times with 1–2 volumes of $CHCl_3$ and applied directly to a reverse phase HPLC C18 Bondapak (Waters, Northwich, UK) preparative column pre-equilibrated and eluted with 20% methanol (v/v) in 10 mM aqueous KH_2PO_4, at pH 5.5. Uncaged nucleotides emerged without retardation followed by second and third peaks which were well resolved. The second peak contained the bulk of the caged GTP-γ-S and the third the caged GDP as demonstrated by generation of the corresponding nucleotides on photolysis and by analytical HPLC.

The caged GTP-γ-S peak was applied at 4° C directly to a 1.5 × 50 cm anion exchange column of DEAE cellulose (Whatman DE52) pre-equilibrated with 10 mM triethylamine bicarbonate (TEAB) buffer at pH 7.5, and eluted with a 1.5 l linear gradient of TEAB from 10 mM to 700 mM at a rate of 60 ml · h^{-1}. The product, monitored at 254 nm, eluted as a double peak with maxima at 480 and 525 mM TEAB (Fig. 1a). The peaks were divided into three pools of equal volume. In each, solvent and TEAB were removed by rotary evaporation under vacuum and co-evaporation with four changes of methanol. The samples were stored in water at −20° C protected from light. The estimated overall yield was 28% based on absorption at 255 nm. The UV spectrum of caged GTP-γ-S is shown in Fig. 1b. ε = 16,900 $mM^{-1}cm^{-1}$ was assumed at the λ_{max} of 255 nm in 25 mM in organic phosphate at pH 7. This is based on the sum of ε of GTP and 1(2-nitrophenyl)ethyl phosphate at 255 nm.

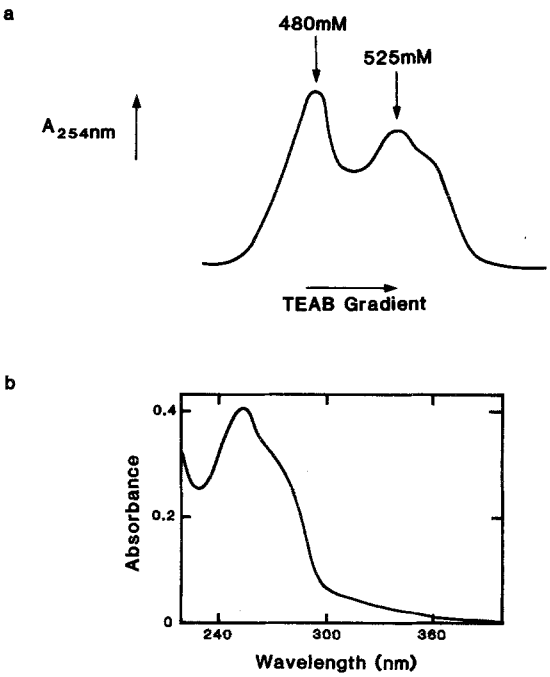

Fig. 1. a Elution profile of caged GTP-γ-S from a DEAE cellulose column. The complex elution profile is ascribed to the multiple isomers of caged GTP-γ-S (see text). **b** Absorption spectrum of 24 μM caged GTP-γ-S measured in a 1 cm path cuvette at 22° C and pH 7.0 and in aqueous 25 mM P_i

Analysis by HPLC on a Whatman SAX column in 0.7 M $(NH_4)_2HPO_4$ adjusted to pH 4.0 with HCl showed that the nucleotide from the first pool eluted predominantly as a single peak with a retention time of 5.9 min, and from the third pool with a retention time of 5.5 min. The second pool had both components in approximately equal amounts. In each case the nucleotide, when photolysed in the presence of 1 mM dithiothreitol (DTT), formed GTP-γ-S which had a retention time of 7.1 min. Better resolution of the three fractions from GTP-γ-S (retention time 7.0 min) was obtained if 10% methanol (v/v) was added to the elution solvent, but then all three fractions had the same retention time of 3.0 min.

These properties closely resemble those of caged ATP-γ-S which has been more fully characterized by ^{31}P and ^{1}H NMR spectroscopy (Walker et al. personal communication). Based on this we assign the first peak shown in Fig. 1a to be S-caged GTP-γ-S and the second to be O-caged GTP-γ-S, where the S- and O-prefixes denote the atom through which the 1(2-nitrophenyl)ethyl group is bonded to the GTP-γ-S.[1]

S-caged GTP-γ-S is a mixture of two diastereoisomers because the 1(2-nitrophenyl)ethyl group contains a chiral centre. The second peak in Fig. 1b is the O-caged GTP-γ-S which is a mixture of four diastereoisomers because a second chiral centre is introduced at the γ-phosphorus atom in addition to the chiral centre at the benzylic carbon. In the electrophysiological studies described here, the S-caged

Note added in press: this conclusion has now been confirmed by ^{31}P NMR of S- and O-caged GTP-γ-S)

GTP-γ-S and O-caged GTP-γ-S were pooled in order to prepare a substantial amount of the caged molecule of common composition.

Synthesis of the 1(2-nitrophenyl)ethyl P^3 ester of 5'-guanylyl imidodiphosphate (caged GMP-PNP). Following the general protocol of Walker et al. (personal communication) for the synthesis of caged AMP-PNP, 13.4 mg of the tetralithium salt of GMP-PNP (Boehringer-Mannheim) in 1.0 ml of water was adjusted to pH 4.0 with dilute HCl and stirred for 19 h with 1.1 ml of 200 mM 1(2-nitrophenyl)diazoethane in $CHCl_3$.

Caged GMP-PNP was obtained in 30% yield, purified and stored as for caged GTP-γ-S. Caged GMP-PNP was analysed by HPLC on the SAX column using 0.7 M $(NH_4)_2HPO_4$ adjusted to pH 4.0 with HCl as eluting solvent. Caged GMP-PNP had a retention time of 6.3 min compared to a GMP-PNP retention time of 2.6 min.

Both GMP-PNP and GTP-γ-S were obtained as essentially pure nucleotides on photolysis of caged GMP-PNP and caged GTP-γ-S, whereas both commercial materials had 25–35% nucleotide contaminants, largely GDP (with GDP-NH_2 also present as a contaminant of GMP-PNP).

The other caged compounds used in this study (caged ATP and ADP) were preparaed according to Walker et al. (personal communication). All caged compounds were stored at 25 mM as their triethylamine salts in water at −20° C.

Photolysis equipment and analysis. Photolysis was effected by a Chadwick Helmuth Strobex Model 278 xenon arc flash lamp apparatus (200 W/s maximum output) mounted in an ALH215 PRA lamp housing. The light pulses (0.5 ms duration) were transmitted through a water heat filter and a Corning 9863 glass band pass filter (300 – 350 nm) and focussed onto a 5 mm diameter UV transmitting (> 270 nm) liquid light guide (Oriel). The tip of the light guide was positioned 5 mm above the surface of the bath used for electrophysiological recording. In all experiments the light pulses used were at maximum output of the lamp.

Quantum yields (Q), and $t_{1/2}$ for release of GTP-γ-S and GMP-PNP from their caged precursors were measured by comparison with caged P_i (Q = 0.54) (Kaplin et al. 1978; McCray et al. 1980). Q for caged GMP-PNP was 0.6, and was about 0.35 for both O-cagedGTP-γ-S and S-caged GTP-γ-S. The reason why Q for both forms of caged GTP-γ-S was only about half that observed for caged ATP-γ-S is unknown. The $t_{1/2}$ for release of GMP-PNP from caged GMP-PNP was 3 ms, and about 10 ms for the release of GTP-γ-S from the mixture of caged GTP-γ-S used in the physiological experiments under the same solvent conditions.

The efficiency of light-induced hydrolysis in this experimental system was estimated by placing a 20 µl droplet of 2 mM caged ATP or ADP, either separately or together with 2 mM caged GTP-γ-S on a coverslip in place of the cells. Each flash photolysed approximately 1% of the caged GTP-γ-S (producing about 20 µM GTP-γ-S), and 2% of the caged ATP and ADP as estimated by reverse-phase HPLC.

Electrophysiology. Rat dorsal root ganglia were dissociated as previously described (Dolphin et al. 1986), and cells were grown on collagen-coated coverslips and maintained for between 2 and 6 weeks in culture, before use in electrophysiological experiments. Experiments were performed in dim illumination when caged guanine nucleotide analogues were used, and voltage clamp recordings were made using an Axoclamp-2 switching voltage clamp amplifier, operated at 10–20 kHz; using the whole cell recording technique (Hamill et al. 1981). Patch pipettes of between 1 and 4 MΩ resistance were used. Calcium channel currents were recorded using a bathing solution containing in mM: NaCl 130, KCl 3, $MgCl_2$ 0.6, $BaCl_2$ 2.5, Hepes 10, glucose 4, tetraethylammonium bromide 25 and tetrodotoxin 0.0025. The pH was adjusted to 7.4 with NaOH and the osmolarity to 320 mosM with sucrose. The patch pipette solution contained in mM: Cs acetate 140, $CaCl_2$ 0.1, EGTA 1.1, ATP 2, $MgCl_2$ 2 and Hepes 10. The pH was adjusted to 7.2 with Tris and the osmolarity to 310 mosM. The pCa of this solution was 8.0. Results were stored on FM tape and analysed using a PDP 11-23 computer. All current traces are shown following subtraction of linear leakage and capacitative currents. As an estimate of the contribution of the transient component to the whole cell calcium channel current, the percentage inactivation during the 100 ms voltage step was calculated of the peak maximum current, following leakage subtraction.

The caged compounds were diluted in patch pipette solution on the day of use and kept at 0° C. After commencing whole cell recording, cells were allowed to equilibrate for at least 5 min with the patch pipette solution containing caged compounds before the flash. Previous studies have shown that the by-product 2-nitrosoacetophenone relased upon photolysis of the caged compounds can react with biological preparations to varying extents (Kaplan et al. 1978; Goldman et al. 1984). Deleterious effects can be eliminated by the addition of thiol reagents. For this reason, preliminary experiments were performed in the presence of 10 mM DTT in the patch solution, to react with the nitrosoacetophenone released by photolysis of the caged compounds (Goldman et al. 1984). Similar results utilising caged GTP-γ-S and GMP-PNP were obtained in the presence and absence of DTT suggesting that insufficient nitrosoacetophenone was generated by photolysis in the present experiments to produce any injurious effects. All the results presented here were obtained in the absence of DTT. In further preliminary studies, the effect was examined of photolysis of 2 mM caged ATP included in the patch pipette. Either no effect was observed following photolysis of this compound or a small increase in the maximum calcium channel current was seen ($n = 3$), ruling out the possibility that release of nitrosoacetophenone was responsible for the effects observed following photolysis of caged GTP-γ-S and GMP-PNP. Further control experiments are described in the Results section and shown in Fig. 5.

Results

Effect of GMP-PNP on calcium channel currents

Inclusion of GMP-PNP (0.5 mM) in the patch pipette produced a time-dependent decrease in the whole cell calcium channel current activated from a holding potential (V_H) −80 mV. The maximum calcium channel current evoked at a clamp potential (V_{clamp}) of between 0 and +10 mV immediately after breaking the membrane seal between the patch pipette contents and the cell cytoplasm, was rapidly activating and showed a marked component of inactivation during the 100 ms voltage step command. After 5–10 min

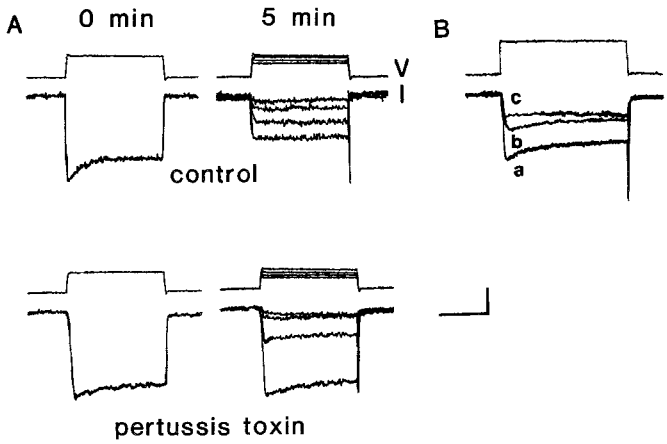

Fig. 2A, B. The effect of GMP-PNP on calcium channel currents in rat DRGs. In **A**, maximum calcium channel current (V_H = −80 mV, V_{clamp} = +5 mV for *upper panel*, and *0 mV* for *lower panel*) was recorded immediately after breaking the seal between the patch pipette containing 500 μM GMP-PNP and the cell contents (*left panel*), and 5 min later a current-voltage relationship was performed (*right panel*). In control cells steps to −25, −15, −2 and +5 mV are illustrated (*upper traces*); and in cells which had been incubated for 3 h with pertussis toxin (500 ng · ml^{-1}) steps to −40, −25, −10 and 0 mV are illustrated (*lower traces*). In **B**, the maximum calcium channel current was recorded 5 min after commencing recording with caged GMP-PNP (2 mM) in the patch pipette (*trace a*). The current recorded 70 ms after flash photolysis is superimposed on (*a*). The trace labelled (*b*) was recorded 5 min later, and that labelled (*c*) 15 min later. V_H = −80 mV. The *calibration bars* are 100 mV or 1 nA and 50 ms (**A**) and 60 mV or 1.2 nA and 40 ms (**B**)

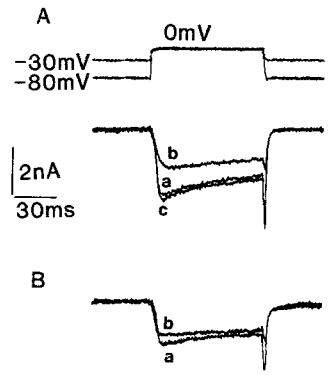

Fig. 3A, B. The response of calcium channel currents evoked from different holding potentials to photochemically released GMP-PNP. **A** Maximum calcium channel currents were recorded from V_H = −80 mV (*a, c*) and V_H = −30 mV (*b*) before photolysis of caged 2 mM GMP-PNP present in the patch pipette solution. **B.** Maximum calcium channel currents were recorded from V_H = −80 mV (*a*) and −30 mV (*b*) 10 min after 3 flashes, 30 s apart

the maximum calcium channel current was markedly reduced in amplitude, from 4.45 ± 0.75 nA to 2.10 ± 0.21 nA (mean ± SEM), and the percentage inactivation was reduced from 42 ± 6% to 13 ± 7%, with 3 out of 8 cells now showing no inactivation during the 100 ms step (mean ± SEM, n = 8). An example of this time-dependent differential inhibition by GMP-PNP of the transient whole cell calcium channel current is shown in Fig. 2A (upper current traces). This inhibition was prevented by prior treatment of the cells with pertussis toxin (Fig. 2A, lower current traces). In pertussis toxin treated cells, the percentage inactivation of the calcium channel current was initially 29 ± 4% and after 5 min equilibration with GMP-PNP was 35 ± 6% (mean ± SEM, n = 8).

Photolysis of caged GMP-PNP

When caged GMP-PNP (2 mM) was included in the patch pipette, the maximum calcium channel current remained stable in amplitude and degree of inactivation which were 2.3 ± 0.2 nA and 31 ± 7% initially, and 2.3 ± 0.2 nA and 34 ± 6% after 5 min (mean ± SEM, n = 8). In the example illustrated in Fig. 2B, the maximum calcium channel current activated 70 ms after a single light flash was unchanged compared to that before the flash (superimposed traces labelled a). Calcium channel currents were then activated every minute, and showed a gradual reduction over about 15 min in amplitude and degree of inactivation. The currents illustrated were recorded 5 (b) and 15 (c) min after photolysis.

Effect of different holding potentials on calcium channel currents recorded before and after photolysis of caged GMP-PNP

In order to monitor the amount of inactivating current present before and after photolysis of caged GMP-PNP, a protocol was used of activating the maximum calcium channel current from either V_H = −80 mV or −30 mV, since at the depolarised holding potential the transient calcium channel current is rapidly inactivated. An example of results obtained using this protocol is shown in Fig. 3. With caged GMP-PNP (2 mM) in the patch pipette, the cell was held at V_H = −80 mV and the maximum calcium channel current was recorded by stepping to 0 mV (Fig. 3A, current trace labelled a). The cell was then held at V_H = −30 mV, and the maximum current recorded at the same V_{clamp}, 0 mV (Fig. 3A, current trace labelled b). To monitor recovery from inactivation the cell was returned to V_H = −80 mV and the maximum current again recorded (Fig. 2A, current trace labelled c).

The cell was then given three pulses of illumination, 30 s apart, and the effect on the maximum calcium channel currents recorded from V_H = −80 mV and −30 mV was examined. The currents shown in Fig. 3B were recorded 10 min after the last light flash. The trace labelled (a) was recorded from V_H = −80 mV, and that labelled (b), from V_H = −30 mV.

Liberation of GMP-PNP by photolysis has clearly reduced the amount of inactivating calcium channel current, represented by that which is lost between V_H = −80 mV and −30 mV. This was 1.2 nA measured at the peak current before photolysis and 0.4 nA after photolysis. Similar results were obtained in 3 other cells. A reduction in amplitude of the sustained calcium channel current measured at V_H = −30 mV was also observed following photolysis. The reduction was by 19% in the cell illustrated in Fig. 2B and the mean reduction was 38 ± 11% (n = 4). This suggests a component of the current measured at the end of the voltage step command can be inactivated by depolarizing the holding potential to −30 mV and inhibited by GTP-γ-S or GMP-PNP, as is also clear from Fig. 2b.

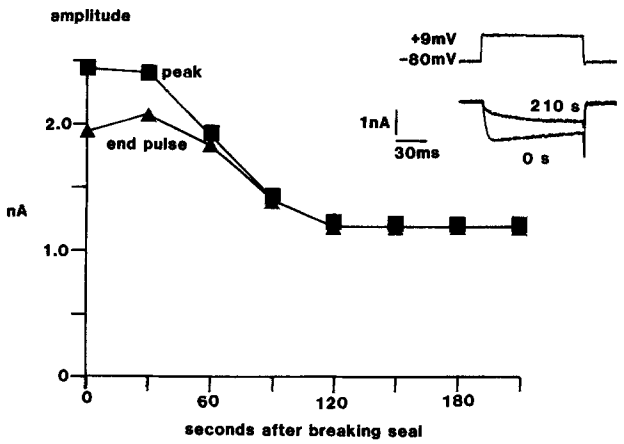

Fig. 4. The time-dependence of the inhibition of calcium channel currents by 100 μM GTP-γ-S. The *graph* shows the reduction with time after commencing whole cell recording of the maximum calcium channel current ($V_H = -80$ mV, $V_{clamp} = +9$ mV) measured at its peak (■) and at the end of the voltage step command (▲). In some cases in the presence of GTP-γ-S (e.g. see *inset*) the current showed no maximum during the 100 ms step, and its amplitude was only measured at the end of the step. The *inset currents* were recorded immediately and 210 s after the start of the experiment

Effect of GTP-γ-S on calcium channel currents

GTP-γ-S has previously been shown to produce a response similar to that of GMP-PNP on the characteristics of calcium channel currents, although at 500 μM the effect was usually more rapid and greater in extent (Scott and Dolphin 1986; Dolphin and Scott 1987a). In the present study, the effect of a lower concentration of GTP-γ-S (100 μM) on calcium channel currents was examined in more detail. The time course of action of 100 μM GTP-γ-S on peak and "steady-state" (end of pulse) calcium channel currents in one cell following rupture of the seal between the pipette contents and cytoplasm is shown in Fig. 4. Similar results were obtained from 6 other cells; $t_{1/2}$ for the reduction in peak amplitude of the calcium channel current was 2.2 ± 0.3 min (mean ± SEM, $n = 7$). The residual current was also clearly slowed in activation in only 2 out of 7 cells (e.g. Fig. 4, inset current at 210 s), although slowed activation was universally observed when a higher concentration of GTP-γ-S (500 μM) was present in the patch pipette (Dolphin and Scott 1987a). However, the $t_{1/2}$ for reduction of peak calcium channel current by the higher concentration of GTP-γ-S (500 μM) was similar, being 2.0 ± 0.3 min (mean ± SEM, $n = 6$).

Photolysis of caged GTP-γ-S

The inclusion of caged GTP-γ-S (2 mM) in the patch pipette had no effect on calcium channel currents, which remained stable during a 5–10 min period of equilibration. The current amplitude was initially 4.7 ± 0.6 nA and was 4.7 ± 0.6 nA after 5 min (mean ± SEM). Following a single flash, the maximum calcium channel current was recorded in some cells 70 ms later, and then in all cells every successive 30–120 s. In the cell illustrated in Fig. 5A the two superimposed calcium channel currents labelled (a) were obtained 2 min before and 70 ms after the flash. The currents became successively smaller after the flash; those illustrated were recorded 0.5, 1.5, 2, 2.5, 3 and 5 min later. After recording the calcium channel current at 5 min (b), two more flashes were given, at an interval of 0.5 min, and the current recorded again 1 min later. No further change in calcium channel current was observed (Fig. 5A, upper trace labelled b).

As well as showing a reduction in amplitude, the maximum calcium channel currents also showed changes in their kinetics of activation and inactivation. The activation of the calcium channel currents illustrated in Fig. 5A could be approximately described by a single exponential; before flash photolysis of caged GTP-γ-S, τ_{act} was 2.6 ms, 0.5 min after the flash it had increased to 4.6 ms and after 5 min it was stable at 7.6 ms. The degree of inactivation of the maximum calcium channel current, during the 100 ms step, decreased from 28% before the flash to a completely non-inactivating current 5 min after the flash. Subtraction of the maximum calcium channel currents before and 5 min after flash photolysis in this cell reveals the current lost following photochemical release of GTP-γ-S (Fig. 5B). This current has a large component of inactivation (52%) which can be fitted by a single exponential with τ_{inact} of 29.1 ms.

The average rate of reduction of maximum calcium channel currents following flash photolysis of caged GTP-γ-S (releasing about 20 μM GTP-γ-S), is shown in Fig. 6. The $t_{1/2}$ for reduction of the peak calcium channel current amplitude was 1.5 ± 0.16 min (mean ± SEM; $n = 5$) whereas the effect of liberated GTP-γ-S on the sustained calcium channel current measured at the end of the pulse was smaller and slower ($t_{1/2}$ 3.0 ± 0.2 min), again suggesting a differential inhibition of the transient calcium channel current by GTP-γ-S.

Three control experiments were performed, in addition to those described in the Methods section. No effect was observed of the light flash on calcium channel currents in the absence of caged molecules in the patch pipette (e.g. Fig. 5C). The peak current and percentage inactivation were initially 4.0 ± 1.2 nA and 44.3%, and were 4.6 ± 1.4 nA and 42.8% after 5 min ($n = 5$). The normalised mean amplitude of peak calcium channel currents in control cells after a single flash is shown in Fig. 6.

Calcium channel currents were observed to remain stable for between 10 and 20 min when recorded in the presence of caged GTP-γ-S. An example is shown in Fig. 5D, where the initial current recorded is labelled (a). Over a period of 5 min, no change in peak amplitude was observed, and following a flash with the shutter closed so that no illumination reached the cell, there was a small increase in the degree of inactivation during the 100 ms voltage step. The current labelled (b) was recorded 5 min after the flash.

Pretreatment of cells with pertussis toxin prevented the effect of photochemically released GTP-γ-S on the transient component of the calcium channel currents. The percentage inactivation before and 5 min after a flash was 62 ± 4% and 55 ± 7% (mean ± SEM, $n = 3$) respectively. As previously observed (Dolphin and Scott 1987a), following exposure of cells to pertussis toxin, the calcium channel currents were often observed to have a marked component of inactivation. An example is given in Fig. 5E, where the maximum calcium channel current in the presence of caged GTP-γ-S (2 mM) was stable for 10 min (a). The calcium channel current 4 min

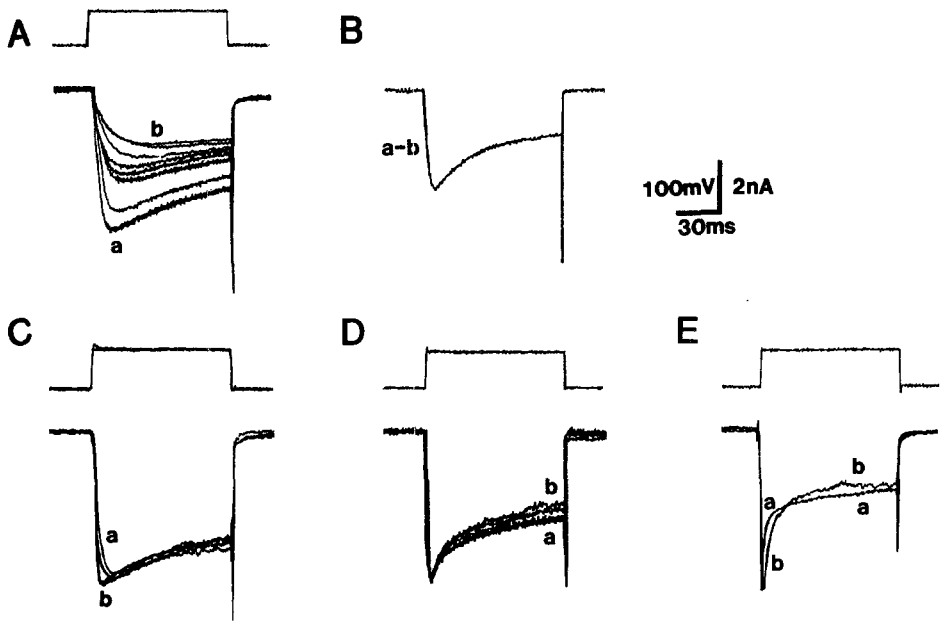

Fig. 5A–E. The effect of photochemically released GTP-γ-S on calcium channel currents. In **A**, the maximum calcium channel currents (V_H = 80 mV, V_{clamp} = 0 mV) were recorded from a cell in the presence of 2 mM caged GTP-γ-S, and 70 ms after flash photolysis [superimposed traces labelled (*a*)]. The currents shown in decreasing order of amplitude were recorded 0.5, 1.5, 2, 2.5, 3 and 5 min (*b*) after a single flash. Two further flashes were then given and the maximum current was recorded at 7 min (*b, upper trace*). In **B**, the maximum calcium channel current recorded 5 min after the flash (*b*) was subtracted from that recorded before the flash (*a*). In **C**, maximum calcium channel currents (V_H = −80 mV, V_{clamp} = +15 mV) were recorded from a control cell 2 and 0.5 min before a single flash (*a*) and 3 and 5 min after the flash (*b*). In **D**, maximum calcium channel currents (V_H = −80 mV, V_{clamp} = +5 mV) were recorded initially and 5 min later, in the presence of caged GTP-γ-S (2 mM) in the patch pipette (*a*), and 1 and 5 min after a single flash, with the shutter closed (*b*). In **E**, cells were pretreated with pertussis toxin (500 ng · ml^{-1}) for 3 h and the maximum calcium channel current was recorded in the presence of caged GTP-γ-S, before (*a*) and 4 min after a single flash (*b*). V_H = −80 mV, V_{clamp} = +5 mV. In this cell there was a small amount of rundown of the maximum calcium channel current following the flash, and the current (*b*) has therefore been scaled up by 15%, so that the currents (*a*) and (*b*) were matched at the end of the pulse. This resulted in the peak of (*b*) being slightly larger than (*a*), indicating no loss of the transient component

after the flash showed a small reduction in its amplitude, and has been scaled up by 15% (b) to illustrate the lack of effect on the degree of inactivation of the current during the 100 ms voltage step.

Effect of photolysis of caged GTP-γ-S on the calcium channel current/voltage relationship

This experiment was performed to examine whether photochemical release of GTP-γ-S produced any shift in the voltage dependence of activation of calcium channel current. All currents recorded from V_H = −80 mV show a degree of inactivation both in control cells and in the presence of caged GTP-γ-S (e.g. Fig. 7A, upper current traces). The current-voltage relationship (Fig. 7B) illustrates the voltage-dependence of the peak and "steady state" (end of pulse) calcium channel currents before and 6 min after flash photolysis of caged GTP-γ-S. Following liberation of GTP-γ-S, the maximum current is activated at the same V_{clamp}, and shows slower activation and a much reduced degree of inactivation at all potentials (Fig. 7A, lower current traces, Fig. 7B). Similar results were obtained in all other cells studied. The maximum current was activated at +4 ± 1 mV (mean ± SEM, n = 5) both before and after liberation of GTP-γ-S by photolysis.

Discussion

In our previous studies using guanine nucleotide analogues, we observed that, whereas control calcium channel currents were rapidly activating, and showed marked inactivation during the 100 ms voltage step command; in the presence of GTP-γ-S, the calcium channel currents showed slower activation, and were sustained and on average smaller than controls (Scott and Dolphin 1986; Dolphin and Scott 1987a). Opposite results were obtained with GDP-β-S (Dolphin and Scott 1987a). It was not possible to determine the rate of onset of the effect of guanine nucleotide analogues from these experiments since they were included in the patch pipette.

Caged derivatives of both GTP analogues were investigated in the present study, with caged GTP-γ-S being examined more extensively because it was found to have more rapid kinetics of action than GMP-PNP. The G proteins associated with adenylate cyclase are known to have a higher affinity for GTP-γ-S than for GMP-PNP (Pfeuffer and Helmreich 1975; Eckstein 1985), and it appears likely that this is also the case for the G protein proposed to be involved in modulation of calcium channel activity.

The half time for inhibition of calcium channel currents by 20 μM GTP-γ-S following its liberation by a single flash was 1.5 min whereas the $t_{1/2}$ for the effect of both 100 and 500 μM GTP-γ-S measured after breaking the seal between

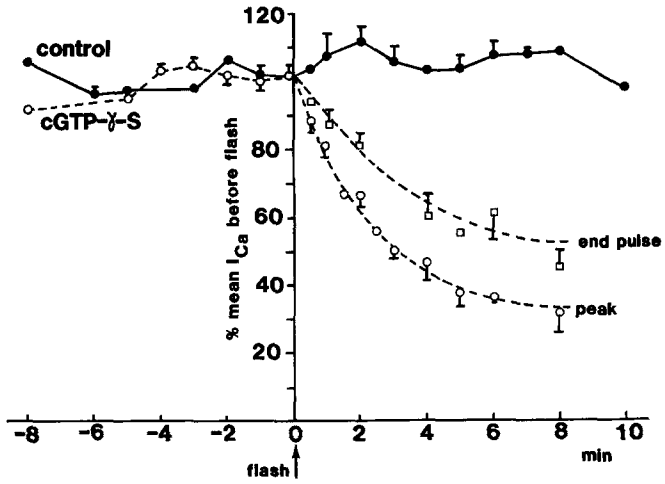

Fig. 6. The time course of the effect of caged GTP-γ-S, following flash photolysis. The maximum calcium channel current was recorded every min in control cells (●) and cells in the presence of caged GTP-γ-S (○, □). After 5–8 min of recording, in cells in which the maximum current was stable, a single flash was given at time zero. The maximum calcium channel current was then recorded at intervals of 30–120 s for between 5 and 10 min. The peak calcium channel current was measured for control cells, and both the peak current (○) and that at the end of the pulse (□) for cells in which 20 μM GTP-γ-S was released. The results are the mean ± SEM for 5 cells in each group, although for each point, *n* may equal less than 5, as recordings were not made every 30 s in all cells

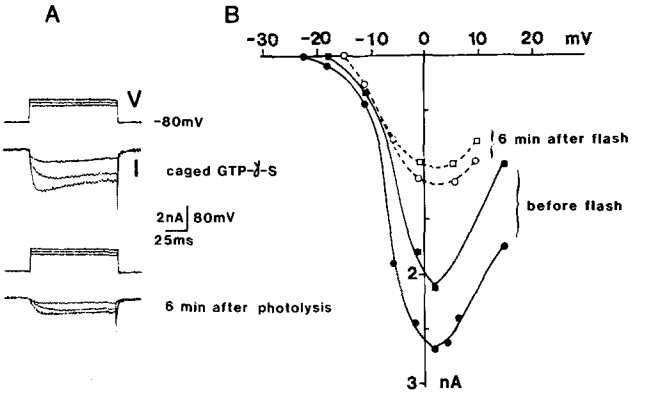

Fig. 7 A, B. The effect of photochemically released GTP-γ-S on the current-voltage relationship of the calcium channel currents. In **A**, a set of calcium channel currents activated by increasing voltage jumps from $V_H = -80$ mV is shown in the presence of caged GTP-γ-S (*upper current traces*) and 6 min after a single flash (*lower current traces*). In **B**, the corresponding current-voltage relationship is shown before (●, peak current; ■, end pulse) and 6 min after (○, peak current; □, end pulse) photochemical release of GTP-γ-S, to illustrate the reduction in the current as well as its lack of inactivation

patch pipette and cytoplasm was about 2 min. Several explanations may be put forward to account for the delayed response to GTP-γ-S. Firstly, commercially available GTP-γ-S contains about 30% contaminating GDP, which together with endogenous GDP would compete with GTP-γ-S, for binding to the G protein. Secondly, in the case of photochemical release of GTP-γ-S, a period of 5–10 min equilibration was allowed before photolysis, thus ensuring that the intracellular concentration of caged GTP-γ-S closely approximated to that in the patch pipette. The time course of the effect of GTP-γ-S liberated intracellularly by photolysis was thus not limited by diffusion down a concentration gradient from the patch pipette to its site of action. In contrast, the delayed response to free GTP-γ-S present in the patch pipette involves both diffusion into the cell as well as binding to G proteins. However, the finding that there was no difference between the time course of the responses to 100 and 500 μM GTP-γ-S, and that the response to 20 μM GTP-γ-S released photochemically was not significantly different from a 25-fold higher concentration in the patch pipette, suggests that diffusion of free GTP-γ-S from the patch pipette plays only a marginal role in the delay in response of calcium channel currents to GTP-γ-S.

This result is in agreement with those of Kurachi et al. (1986), who exposed inside-out membrane patches from heart cells to GTP-γ-S, and examined its effect on K^+ channel opening, which was slow in onset. It is possible that the rate of exchange of GDP by GTP-γ-S on the G protein(s) in question is the rate-limiting step in the direct activation of G protein by GTP-γ-S in the absence of agonist. Indeed, Ferguson et al. (1986) have observed that the rate of dissociation of GDP, bound tightly and stoichiometrically to G proteins, can entirely account for the slow rate of their activation by GTP-γ-S which does not show a linear relationship with GTP-γ-S concentration. A further biochemical correlate of these results is provided by the observations of Okada et al. (1986) that there is a lag period before activation of adenylate cyclase by GMP-PNP in rat cortical membranes which is abolished in the presence of agonist. Our result would be consistent with the hypothesis that despite high intracellular concentrations of GTP (in the micromolar range), calcium channels remain largely although not entirely (Dolphin and Scott 1987a) unaffected except in the presence of agonist which increases the rate of exchange of GDP by GTP on the G protein (Cassel and Selinger 1978). Some tonic inhibition of the transient component of the calcium channel current in control cells by endogenous GTP has been inferred because of the ability of GDP-β-S, and pertussis toxin to increase the transient component of the calcium channel current (Dolphin and Scott 1987a; see also Fig. 5E).

Evidence suggests that the response to GTP-γ-S observed in the present experiments does not involve modulation of adenylate cyclase activity and changes in cyclic AMP-dependent protein phosphorylation (Dolphin and Scott 1987a). However, it is also possible that other second messenger systems such as protein kinase C activation may be involved (Holz et al. 1986). These systems, if they are involved in the rapid neurotransmitter mediated inhibtion of Ca^{2+} channel currents, can be swiftly activated by agonist and thus are unlikely to account for much of the observed delay in the response to GTP analogues alone.

From the present experiments it is clear that photochemical liberation of GTP-γ-S results in a more rapid and greater inhibition of the peak transient calcium channel current than the sustained current. Under the present conditions, using Ba^{2+} as charge carrier, the transient component of the whole cell calcium channel current probably represents current passing largely through N type channels although T channels would also give some contribution, whereas the sustained component is due mostly to the opening of L type channels (Nowycky et al. 1985). While the

transient calcium channel current was completely abolished by photochemical liberaton of GTP-γ-S a plateau of about 50% inhibition of the sustained calcium channel current was reached at between 6 and 8 min after photolysis. It is unlikely that this reduction represents non-specific run-down of the sustained current, since it did not occur over the same time period in control cells. In several cells, additional flashes given at this time did not produce an additional decrease in the sustained calcium channel current, even though further GTP-γ-S would have been formed by the subsequent flashes. One possible explanation might be that not all L calcium channels can be inhibited by activated G protein, or that there is a finite rate of reactivation.

A question which is posed by this and our previous work (Scott and Dolphin 1986; Dolphin and Scott 1987a) is why GTP analogues differentially inhibit the transient calcium channel currents, whereas in these cells, agonists such as baclofen acting at $GABA_B$ receptors and 2-chloroadenosine acting at adenosine A_1 receptors, although inhibiting the transient current to a slightly greater extent, do not differentiate as markedly as GTP-γ-S (Scott and Dolphin 1986, 1987; Dolphin and Scott 1987a). It was thought possible that this might be explained by a spatial separation of the channels underlying the transient and sustained whole cell currents, between proximal neurites and cell soma. However, similar results have been found in freshly dissociated DRGs which have no neurites (A.C.D. and R.H.S., unpublished observations). Another possible explanation is that there exist sub-populations of G proteins associated with the different classes of calcium channel, which in the absence of agonists show different affinities for the GTP analogues or different off-rates for GDP from the GTP binding site. In the presence of agonist the activated receptor would increase the off-rate of GDP or the affinities of these G proteins for GTP and its analogues to a similar level. Of interest in this respect is the finding by Hescheler et al. (1987) that the calcium channel currents in a neuroblastoma × glioma hybrid cell line are probably coupled to opiate receptors by the α subunit of G_o, a subtype of pertussis-toxin sensitive G protein. The calcium channel current observed in these cells is almost completely inactivating, and probably represents current passing through N/T type calcium channels. It is possible that if this is also the case in DRG neurones, another class of G protein (e.g. G_i) may be associated with the L type channels underlying the sustained calcium channel current. As G_i has a greater affinity for GDP than G_o this may explain the lower sensitivity of the sustained calcium channel current to GTP-γ-S (Ferguson et al. 1986).

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