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# Different effects of baclofen and GTPγS on voltage-activated $Ca^{2+}$ currents in rat hippocampal neurons in vitro

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**Abstract**

Reduction of voltage-activated $Ca^{2+}$ currents by intracellular application of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) through ultraviolet (UV) photolysis of the caged compound, is followed by a re-augmentation to control levels within 10 min, independently of the divalent cation used. The $Ca^{2+}$ current inhibition by the γ-aminobutyric acid type B ($GABA_B$) receptor agonist baclofen, which is also thought to be mediated by a GTP-binding protein (G-protein), is potentiated when GTPγS is uncaged during agonist superfusion. The authors suggest that GTPγS activates G-protein-dependent pathways that are not activated by the baclofen receptor.

*Keywords:* $Ca^{2+}$ current; GTPγS; Baclofen; Photolysis, ultraviolet; G-protein; Hippocampus, rat

## 1. Introduction

The inhibition of voltage-activated $Ca^{2+}$ channels by γ-aminobutyric acid (GABA) is thought to be mediating the presynaptic inhibitory action of this transmitter in the hippocampus (Scholz and Miller, 1991; Thompson et al., 1993).

The extracellular application of $GABA_B$ receptor agonists, such as baclofen, reduces the amplitude of voltage-activated $Ca^{2+}$ currents in vertebrate neurons presumably via a G-protein-dependent pathway (Anwyl, 1991; Deisz and Lux, 1985; Doroshenko and Neher, 1991; Dunlap and Fischbach, 1981; Huston et al., 1990; Sah, 1990). A similar action is induced when G-proteins are permanently activated by the hydrolysis-resistant GTP analogue GTPγS (Brown and Birnbaumer, 1990; Dolphin and Scott, 1987; Gandia et al., 1993; Lux et al., 1992; Plummer et al., 1991; Toselli et al., 1989).

We used the patch clamp method in whole-cell configuration to investigate whether these effects are due to the same underlying G-protein coupled mechanism, or whether different pathways are involved.

## 2. Materials and methods

Hippocampal cells from 17-day-old rat embryos were mechanically dissociated, and plated in a medium with a density of approximately 30000/$cm^2$. The cells were used 11–13 days after plating. The bath solution contained (in mM): NaCl 125, KCl 1, $CaCl_2$ 5 or $BaCl_2$ 5, $MgCl_2$ 1, Hepes 20, glucose 8. pH was 7.35. Tetrodotoxin (1 μM) was added to eliminate Na currents. Patch pipettes were filled with (mM): CsCl 110, tetraethylammonium chloride 30, EGTA 10, Hepes 10. 2 mM ATP was added to increase the stability of the $Ca^{2+}$ currents. Baclofen was applied by the focal perfusion method (Veselovsky et al., 1995) directly onto the cell soma, while the caged form of GPTγS was intracellularly applied via the patch pipette. Holding potential was −70 mV. Currents were measured with a List EPC7 amplifier using the whole-cell patch clamp configuration and filtered at 3 kHz. Serial resistance was compensated as much as possible (> 60%). Current recordings were corrected by subtracting linear

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components of leak and capacitive currents using sample averages of small hyperpolarizing pulses. A Labmaster data acquisition and analysis board and a 386-based microcomputer with pCLAMP software (Axon Instruments, Forster City, CA, USA) were used to acquire the data. Data analysis was done with Autesp (H. Zucker, Garching Instrumente, Germany).

The investigation of the action of GTPγS by applying it directly via the patch pipette does not allow a full understanding of its effect, because it starts to activate G-proteins immediately after disrupting the membrane, which obviously makes it impossible to record control currents. Therefore we made use of the caged form of GTPγS which can be applied to the intracellular medium in its inactive form and, after control recordings, transformed into its active state by an intense flash of ultraviolet (UV) light focused through the microscope objective. UV illumination was performed with a high-power continuous Xenon lamp and a 340 nm bandpass filter.

## 3. Results

We first examined the effects of intracellularly applied GTPγS on $Ca^{2+}$ currents activated by voltage steps to 0 mV of 20 ms duration. These steps will be referred to as 'test pulses' in the following text. Immediately after establishment of the whole cell configuration, pulses were applied with a frequency of 1/min. Over the first 5–10 min a run up of the $Ca^{2+}$ current was observed. This was probably due to the exchange of the intracellular $K^+$ by the cesium in the patch pipette over that period. As long as there is a significant amount of $K^+$ in the cytoplasm the inward $Ca^{2+}$ current and the voltage-activated outward $K^+$ current will compensate each other.

Approximately 15 min after the first test pulse a run down of the $Ca^{2+}$ current was observed with a $t_{1/2}$ of $40 \pm 5$ min (S.E.M., 20 cells, data not shown). The recording time in these control experiments was roughly 1 h.

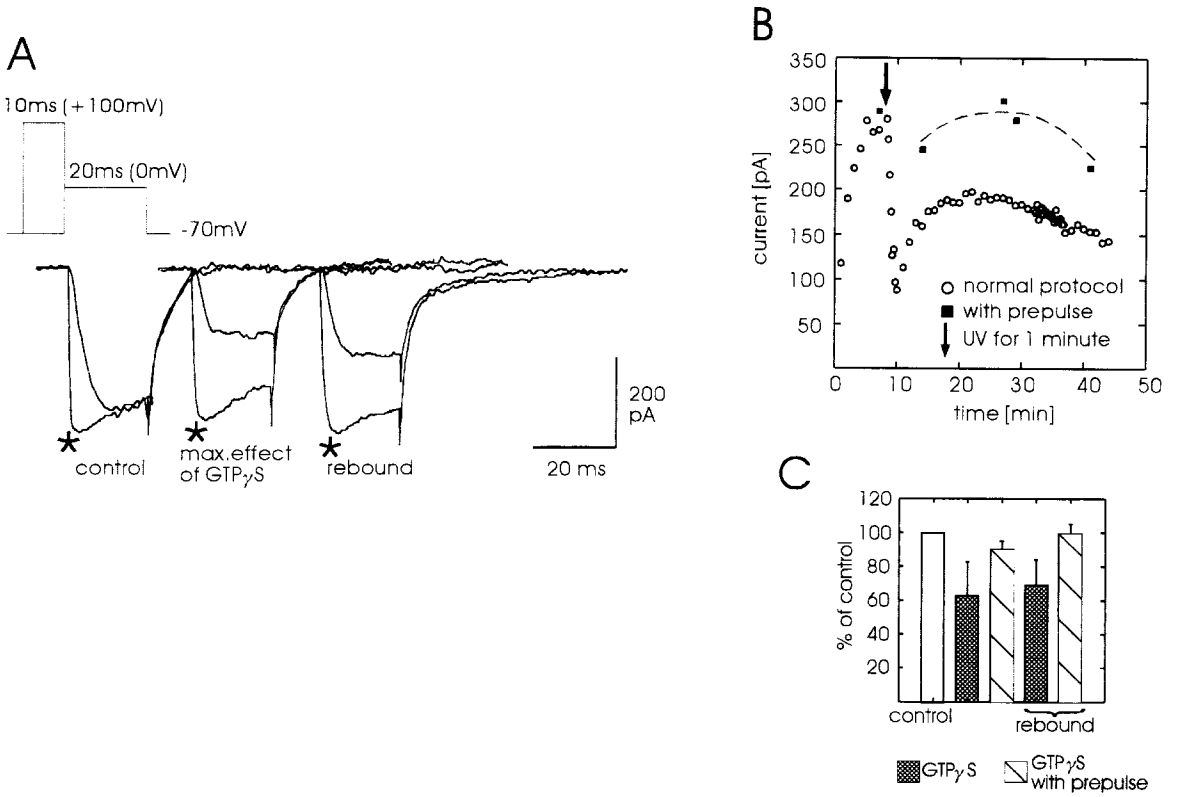


Fig. 1. (A) Superimposed currents resulting from voltage protocols with (indicated by asterisks) and without a prepulse. Intracellular application of GTPγS (UV-photolysation of the caged compound) leads to a pronounced decrease in $Ca^{2+}$ current. A preceding prepulse largely attenuates the depressive effect, indicating that the GTPγS-effect is voltage dependent and thus membrane delimited. In more than 50% of the cells a distinct re-augmentation appeared that was visible with and without prepulse and therefore probably due to some augmentative effect through a different and voltage-independent pathway. (B) The action of intracellularly applied GTPγS on $Ca^{2+}$ currents (circles): after activation of the caged compound with UV-light (arrow), the current decreases quickly to values between 30% and 55%, and within 10 min rebounds up to 60–70% of the initial value. The depressive effect is strongly counteracted by a preceding prepulse (squares). The rebound, however, is clearly visible with both voltage protocols (circles and squares – dashed line is drawn by hand). (C) Bar chart showing averaged values over nine cells (means ± S.E.M.).

After stabilisation of the current amplitude (usually 10 min) GTPγS was activated. This provided an intracellular concentration of free GTPγS between 20 and 200 μM and reduced control currents with a $t_{1/2}$ of 2 min to 70–30%, in accordance with previous observations (Dolphin et al., 1988). In more than 50% of the cells the initial reduction was followed, within 10 min, by a significant re-augmentation of the current (rebound) (Fig. 1). This rebound appeared independently of the divalent cation used ($Ba^{2+}$ or $Ca^{2+}$).

When a caged substance is liberated with a brief flash of UV-light and only the cell and the very tip of the patch pipette are illuminated, the activated substance in the intracellular medium will quickly be replaced by the still caged compound in the rest of the patch pipette. To counteract this effect we exposed the cell continuously (for the rest of the experiment) to UV light; we did not find a difference between the effects of short and long illumination. This can be readily explained by the fact that GTPγS irreversibly binds to

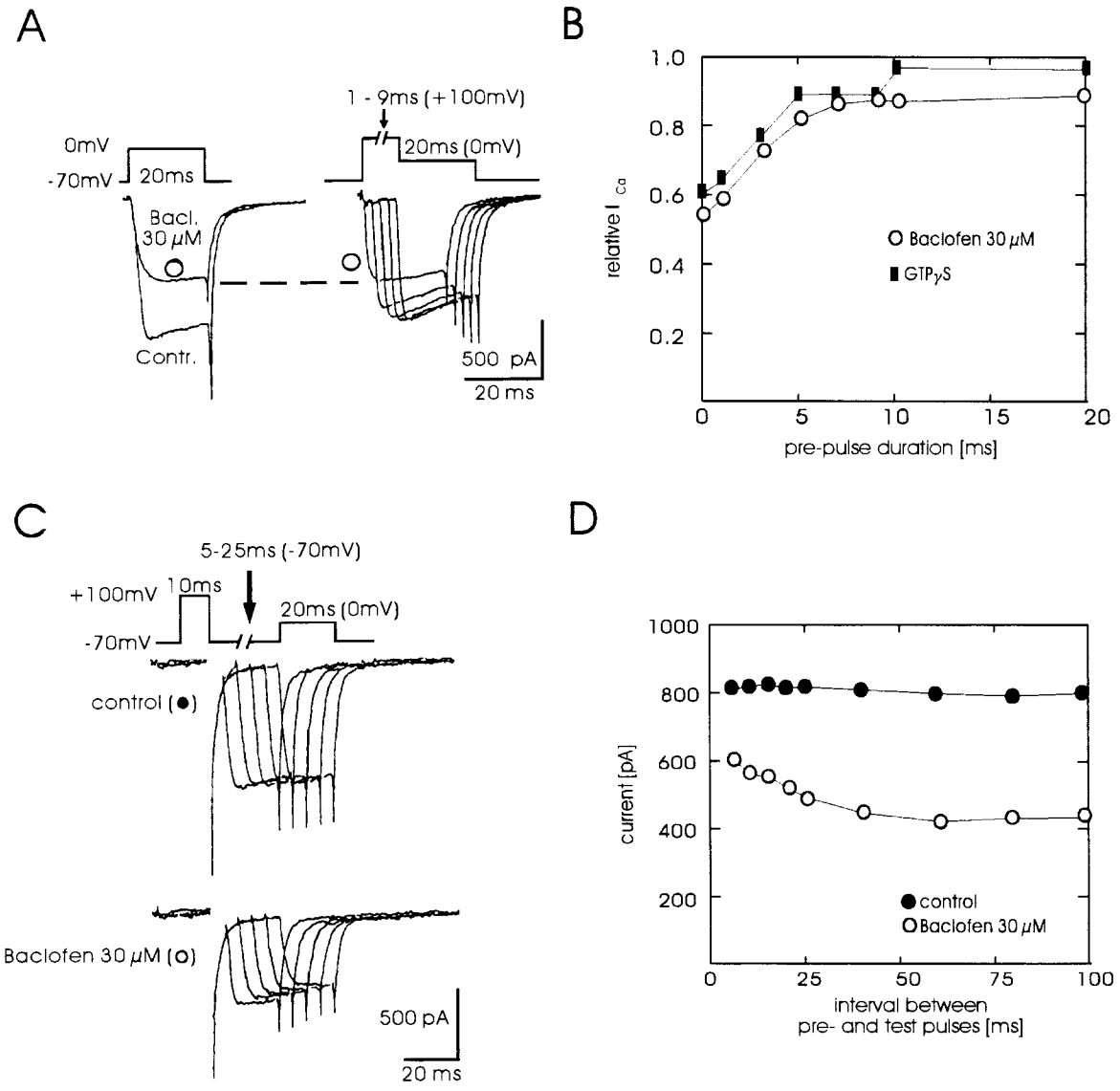


Fig. 2. (A) Left – superfusion of the cell with 30 μM baclofen reduces the voltage-activated $Ca^{2+}$ current to roughly 50%. Right – when the test pulse up to 0 mV is directly preceded by brief voltage step to +100 mV the effect of baclofen is relieved, which indicates that a major part of the G-protein's action is membrane delimited. The magnitude of the relief depends on the length of the prepulse. (B) For both baclofen and GTPγS, a prepulse of 5 ms duration (immediately before the test pulse) was sufficient for a half maximal reduction of the drugs' effect and at 10 ms the inhibition was at its maximum. On the ordinate the value 1.0 corresponds to the amplitude of the control current. (C) When the prepulse precedes the test pulse with a certain time-lag (5–25 ms) its modulative strength on the baclofen induced depression gradually decreases. No effect is visible on control currents with 0 μM baclofen superfusion. (D) A time lag of 20 ms between prepulse and test pulse reduces the inhibitory effect to half of its maximal value and with a 60 ms interval no effect is visible anymore.

G-proteins and thus a transient rise of the GTPγS concentration should have a very similar effect to a constant elevation.

We can rule out that UV-light on its own has any influence on $Ca^{2+}$ channel behaviour because continuously illuminating five control cells for more than 30 min did not produce any significant effect.

G-protein activation by focal perfusion with the $GABA_B$ receptor agonist baclofen (30 μM, up to 5 min) quickly and reversibly decreased the voltage-activated $Ca^{2+}$ current to about 50% (compare Dolphin and Scott, 1987; Fig. 2A). The fact that the baclofen-triggered G-proteins still function without any GTP in the intracellular solution can be explained by the remaining endogenous GTP in the cell interior. Complete washout of GTP can be prevented or slowed down by e.g. compartmentalisation of the cell interior, binding of GTP to intracellular structures or resynthetization of GTP by the cell itself.

No significant baclofen or GTPγS induced shift in the I-V curves of voltage-activated $Ca^{2+}$ currents was found in previous experiments on sensory neurons and bovine chromaffin cells (Dolphin and Scott, 1987; Dolphin et al., 1988; Doroshenko and Neher, 1991). We can confirm these findings in rat hippocampal neurons (data not shown) and assert thus that the observed reduction of the $Ca^{2+}$ current is not merely a result of a shift in the voltage dependency.

The depression of $Ca^{2+}$ channel currents by GTPγS and by G-protein-activating agonists such as baclofen was observed to be largely removed by short steps to positive membrane potentials prior to the activation of the $Ca^{2+}$ current (Grassi and Lux, 1989). The reason for this is presumably that a large part of the G-protein-coupled mechanism is voltage-dependent (i.e. membrane delimited) and can thus be modified by changing the membrane potential. In other words a strong prepulse can be used as a tool to examine in how far the action of the G-protein on the $Ca^{2+}$ channels is voltage-dependent and thus limited to the cell membrane.

In accordance with earlier experiments on the subject (Grassi and Lux, 1989), we found that a short depolarising prepulse (10 ms, +100 mV) almost completely neutralised the inhibitory effect of GTPγS and had a comparable, yet somewhat lesser effect on the

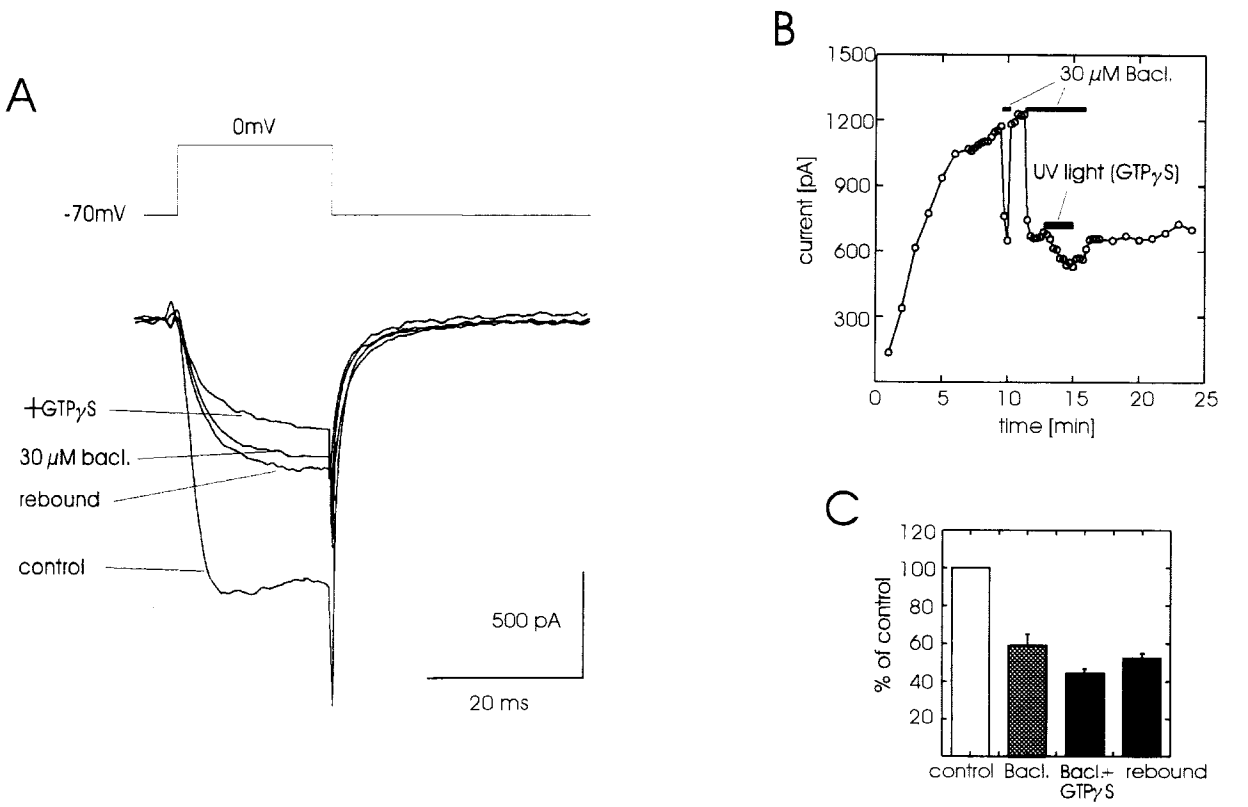


Fig. 3. (A) Superimposed traces: 30 μM baclofen quickly decreases the $Ca^{2+}$ current to 50% of its initial value. When caged GTPγS is photolysed during baclofen perfusion, a further decrease in the current appears and the reduction due to baclofen superfusion becomes irreversible. Within 10 min the same kind of re-augmentation as in Fig. 2 becomes visible. (B) When 30 μM baclofen is applied for 1 min, the $Ca^{2+}$ current decreases reversibly to about 50%. When GTPγS is applied during the superfusion, the current shows a further decrease and within few minutes rebounds up to the level dictated by the baclofen-induced depression which becomes irreversible in the presence of the non-hydrolysable GTPγS. (C) Bar chart showing averaged values over four cells (means ± S.E.M.).

baclofen-induced depression (Fig. 2B). The dependence of this current recovery on the length of the prepulse and on the time between prepulse and test pulse is shown in Fig. 2: a prepulse of 5 ms duration (immediately before the test pulse) was sufficient for a half maximal relief of the drugs' effect and at 10 ms the current showed a recovery almost up to control levels. Fig. 2C,D describes in how far the effect of the prepulse is weakened when the potential is stepped back to −70 mV for a certain period of time before the start of the test pulse. A time lag of 20 ms subdues the prepulse's effect by 50% and with a 60 ms interval the prepulse was entirely without consequence. Very similar dependencies were observed by Grassi and Lux (Grassi and Lux, 1989) on chick dorsal root ganglion (DRG) neurons.

It is particularly interesting that the rebound of the $Ca^{2+}$ current amplitude, that was observed with the test pulse on its own (Fig. 1B, circles), was also visible when the voltage-dependent inhibitory action of the G-protein was relieved with the help of the depolarising prepulse (Fig. 1B squares, dashed line). In other words, the difference between the two current amplitudes remained the same. This indicates that the rebound effect is not simply due to a rundown of G-protein efficiency, since in that case it would have been expected that the two current amplitudes converged.

The combined effects of GTPγS and baclofen are described in Fig. 3: photolyzing caged GTPγS during perfusion with baclofen induced a further decrease in the $Ca^{2+}$ current, similar to the one observed with GTPγS alone. Here too it was possible to observe a re-augmentation of the current, but it was roughly limited to the values that were reached during the preceding baclofen-induced depression. The inhibitory effect of baclofen became irreversible in the presence of activated GTPγS (see also Mintz and Bean, 1993; Scholz and Miller, 1991) which was a good control for the successful activation of the caged substance and its subsequent tight and irreversible binding to the G-protein(s). Since baclofen on its own has a quite reliable and steady depressive effect on the $Ca^{2+}$ current (data not shown), we think it unlikely, that the reaugmentation is merely due to a decrease in the depressive power of the $GABA_B$ receptor agonist.

## 4. Discussion

We interpret the rebound as an additional, augmentative effect of GTPγS with slower activation kinetics and through a different, voltage-independent and thus non-membrane-delimited pathway. Similar observations were made by Zong and Lux (Zong and Lux, 1994) who found a significant rebound (up to 200%) of $Ba^{2+}$ current amplitude upon intracellular application of GTPγS in DRG neurons and by Scott et al. (Scott et al., 1990) who observed an increase in $Ca^{2+}$ current at low concentrations of baclofen (2 μM) and GTP (6 μM) when applied to rat DRGs, and only at higher concentrations (100 μM, 20 μM) did they see the usually detected depression.

Our explanation for the further reduction of the current amplitude evoked by GTPγS during perfusion with baclofen is that GTPγS activates additional, G-protein-dependent, second messenger pathways that do not rely on the activation of the baclofen receptor. This is corroborated by the work of Elmslie (Elmslie, 1992) who came to the conclusion that multiple G-proteins can produce identical changes in $Ca^{2+}$ channel gating.

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