Guanosine-5'-*O*-(3-thiotriphosphate) modifies kinetics of voltage-dependent calcium current in chick sensory neurons

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ABSTRACT Internal perfusion with the G-protein activator guanosine-5'-*O*-(3-thiotriphosphate) (GTP-γS) mimics the effect of noradrenaline and dopamine on the voltage-dependent calcium current in chick dorsal root ganglion (DRG) cells. With 100 μM GTP-γS in the pipette, the current at +10 mV was depressed by ~50%, with a 10-fold increase of its time to peak. The activation time course of the control calcium current could be approximated with a single exponential curve, whereas with GTP-γS the activation time course was double exponential, with time constants τ_1 and τ_2. 2 mM Mg-ATP in the pipette prevented the GTP-γS-induced current decrease in 70% of the cells, but the time course of the current was always double exponential. From −50 mV, the current at +10 mV was best fitted with $\tau_1 = 1.7 \pm 0.5$ and $\tau_2 = 25.6 \pm 5.5$ in seven cells. Both time constants decreased with increasing depolarizations. In the first 2 min of recording, the current changed with time. However, both τ_1 and τ_2 were constant, whereas the relative contribution of the slow component increased from 10 to 70%. In addition, the effect was independent of the holding potential in the range from −100 to −30 mV. These results suggest that the activation of a G-protein causes a fraction of the high-threshold calcium channels to switch to a new closed state, with slower opening kinetics.

INTRODUCTION

Voltage-activated calcium current in neurons and endocrine cells are depressed by several neurotransmitters, including noradrenaline (1–3), dopamine (2), GABA (4, 5), enkephalin (6, 7), and somatostatin (7, 8). In many cases, the depression is accompanied by a slowing down of the time course of activation (2, 4–6). As a consequence, both the calcium influx and the action potential duration decrease, an effect which might be important in presynaptic inhibition (9). Previous studies indicate that this effect is mediated by a GTP-binding protein (5, 7, 10, 11), but the mechanism of the slowing down of the time course remains unclear. It has been proposed that it is due to a voltage-dependent release of the agonist from its binding site (2, 6). An alternative explanation comes from the finding that dorsal root ganglion (DRG) neurons express several types of calcium channels, different in kinetics and pharmacological sensitivity (12). The time course of the calcium current activation could be slower as a consequence of a selective block of a transient component (the N current, reference 12), which leaves almost unmodified another sustained component, the L current (5).

Internal perfusion with guanosine-5'-*O*-(3-thiotriphosphate) (GTP-γS), a nonhydrolyzable analogue of GTP, capable of activating G-proteins in a permanent way, induces similar changes in the calcium current (5, 10, 11, 13). Because internal GTP-γS can mimic the effect of receptor agonists, the slower time course of the current does not reflect a release of the agonist from its binding site, but is possibly related to the G-protein activation step. In this study, we examine the effect of internal GTP-γS on the calcium current in chick DRG neurons and propose an interpretive description to explain the slowing down of its time course. A preliminary communication of this work has appeared (14).

MATERIALS AND METHODS

Dorsal root ganglion cells were obtained from 10-d-old chick embryos, using a procedure similar to that described previously (2), but the dissociation was carried out in collagenase instead of trypsin. Ganglions were incubated in 0.1% collagenase (type CLS II, 89 U/mg, Worthington Biochemical Co., Freehold, NJ) in phosphate buffered saline for 15 min at 37°C. The digested tissue was mechanically triturated and isolated cells were plated on glass coverslips, as in reference 2. Cells were used 20–48 h after plating.

Current was measured with the patch-clamp technique in whole-cell configuration (15). Electrodes, pulled from glass capillaries (model 7052, Garner Glass, Claremont, CA), had resistance of 2–3 MΩ.

Cells were bathed in a solution containing (in millimolar): 150 TEA-Cl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 Hepes, and KOH to pH 7.4. The pipette solution contained (in millimolar): 124 CsCl, 11 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, and Trizma base to pH 7.4. When indicated, just before the experiment, this solution was supplemented with 2 mM Mg-ATP and/or with 100 μM GTP-γS (Sigma Chemical Co., St. Louis, MO). The concentration of GTP-γS in the pipette has to be taken as upper limit, because of the presence of GDP contamination in commercial GTP-γS (13).

Experiments were performed at room temperature.

0006-3495/89/12/1267/06 $2.00

Test pulses were generated by a personal computer (model AT, IBM Instruments, Inc., Danbury, CT) equipped with a 12-bit DACA D/A A/D converter, and the membrane currents were amplified and filtered at 3 kHz by an EPC 7 patch-clamp amplifier (List Medical Systems, Darmstadt, FRG). Capacitance artifacts and the series resistance error were compensated as much as possible by using the analogue circuitry on the amplifier. Current traces were stored on a VCR recorder and later digitized and analyzed by commercial laboratory software (Asystant+, Asyst Software Technologies, Rochester, NY). The response to either subthreshold or hyperpolarizing voltage pulses, were appropriately scaled, averaged, and subtracted from the records to correct for the leak current and the residual capacitive transient. However, when the corrected traces were fitted to models (as will be shown later), the results were not significantly different from those obtained with the uncorrected traces. Therefore, and because leak subtraction may introduce systematic errors due to the leak nonlinearity, most traces were fitted without correction and artifacts (<1ms) were blanked. Curves were fitted to functions using the Gauss-Newton approximation provided by the program.

RESULTS

In the first experiments (Fig. 1), the effect of internal perfusion with 100 μM GTP-γS on the calcium current was studied in the absence of ATP in the pipette. Currents were elicited by depolarizing pulses of 100 ms duration from a holding potential of either -90 or -50 mV. The first trace recorded immediately after breaking into the cell was taken as 'control' trace. This is justified by the observation that, in most cases, this first trace was undistinguishable from the current traces recorded at the same potential from cells in the absence of GTP-γS. From a holding potential of -90 mV, the 'control' current elicited by a voltage step to $+10$ mV reached a peak in 12 ms and then inactivated by 18% in 100 ms (Fig. 1 *a*). From -50 mV, the time course of activation was similar, but the current inactivated only by 5% in 100 ms (Fig. 1 *b*). The analysis was focused on the activation time course, and the inactivation was neglected. The 'control' time course to peak could be approximated with a single exponential curve and it was fitted with the equation:

$$I(t) = A[1 - \exp(-t/\tau)]. \quad (1)$$

The current changed with time and reached a steady state in ~3 min. After this time, the current from -90 mV was depressed by 64% at 10 ms, by 61% at 20 ms, and by 45% at 100 ms, and showed hardly any inactivation. From -50 mV, the current was depressed by 56% at 10 ms, by 48% at 20 ms, and by 30% at 100 ms. In both cases, the time course of activation was no longer adequately described by a single exponential function, but by the sum of two exponential components:

$$I(t) = A[1 - \exp(-t/\tau_1)] + B[1 - \exp(-t/\tau_2)]. \quad (2)$$

One component had a time constant close to that of the control curve, whereas the second component was 10–15 times slower. The slow component was observed in all the cells tested (n = 30), independently of the holding potential and of the presence of ATP (as will be discussed later). With GTP-γS and in the absence of ATP in the pipette, the current amplitude always decreased in amplitude with a similar time course (n = 5).

These observations are in agreement with the results of Dolphin et al. (5, 13) in rat DRG cells. These authors suggested that GTP-γS inhibits a transient calcium current, which is active only from hyperpolarized holding potential (N current, reference 12), but it also modulates a more sustained current (L current). To study the latter effect, we held the cell at -50 mV, to minimize the contribution of the transient components (12). In addition, the pipette solution was supplemented with 2 mM ATP, to prevent the irreversible loss of the L current, known as 'run down' (16, 17). In these conditions, in control cells (i.e., in the absence of GTP-γS in the pipette), the current increased slightly in the first minutes ('run up') and remained constant for 30 min (maximum length of recording). So, ATP addition appeared to be sufficient to prevent run down.

The time course of activation as a function of voltage was studied. Fig. 2 *a* shows single exponential fit with Eq. 1 for one control cell and the voltage dependence of the time constant, obtained from different cells. The time

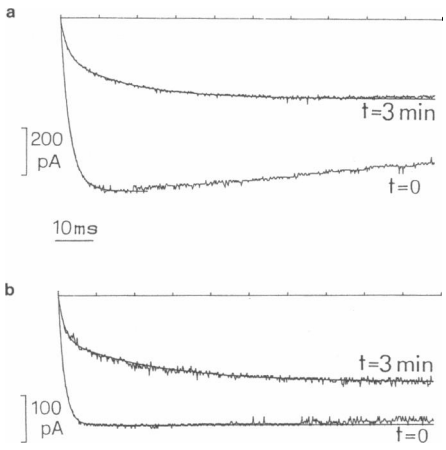


FIGURE 1 Effect of internal perfusion with 100 μM GTP-γS on the calcium current elicited by a step potential to +10 mV. The pipette solution contained standard CsCl, 100 μM GTP-γS, and no ATP. Current traces were sampled at 200 μs per point. The current traces in *a* and *b* are from two different cells held at (*a*) -90 and (*b*) -50 mV holding potential. In both examples, the traces recorded at $t = 0$ were fitted with Eq. 1 in the text. The best fit gave τ = 2.4 ms in *a* and τ = 1.6 ms in *b*. The traces recorded after 3 min were fitted with Eq. 2, with τ_1 = 1.82 ms and τ_2 = 16 ms in *a* and τ_1 = 1.6 ms and τ_2 = 25 ms in *b*.

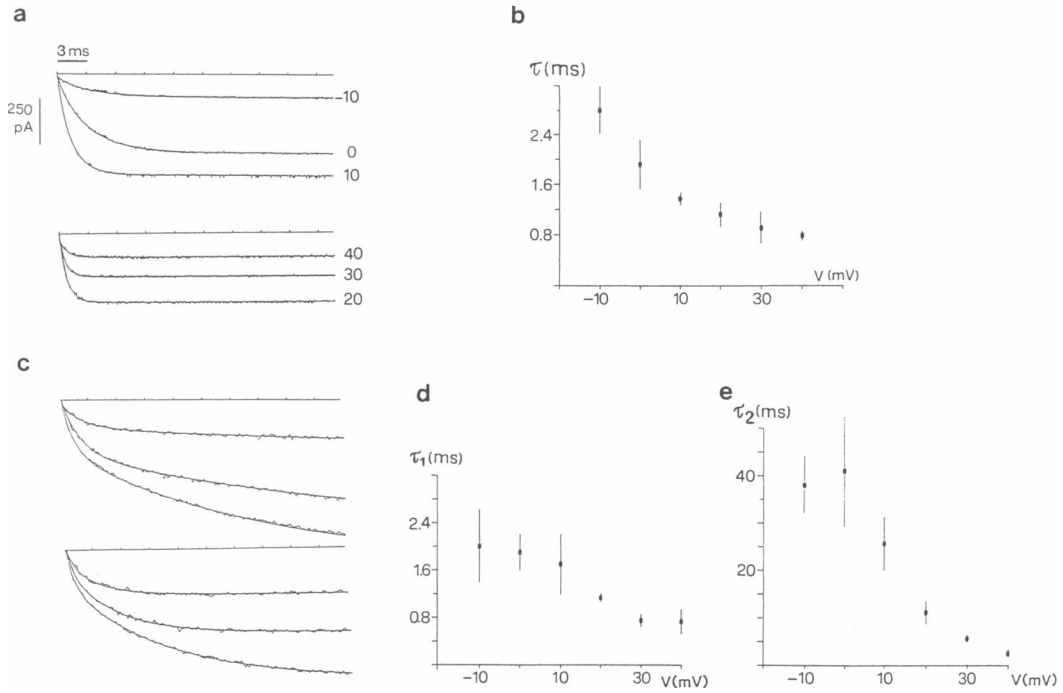


FIGURE 2 Analysis of the activation time course in control and GTP-γS treated cells. (*a*) Control calcium currents elicited by depolarizing steps to the indicated voltage from a holding potential of −50 mV. The pipette contained the standard CsCl solution with 2 mM ATP. Experimental traces were sampled at a frequency of 50 μs per point. Currents were fitted with the Eq. 1. The results of the fitting procedure are superimposed to the experimental records. (*b*) Time constant of current activation as a function of test potential. Points are the averages of values obtained in seven cells. Bars represent standard deviations. (*c*) Calcium currents recorded 5 min after breaking into the cell in the same conditions as in *a*, but with 100 μM GTP-γS in the pipette. Current traces were sampled at a frequency of 200 μs per point. The current and time scale, and the step voltages are the same as in *a*. Experimental traces were fitted with Eq. 2. The results of fitting procedure are superimposed to the experimental traces. (*d*) Values of the first time constant (τ_1), as a function of the test potential. Means and standard deviations for seven cells. (*e*) Values of the second time constant (τ_2) as a function of potential.

constant decreased with increasing depolarizations. When the pipette contained GTP-γS, the current traces at different voltages were fitted with Eq. 2 (Fig. 2 *b*). Both time constants decreased with increasing depolarizations. Note that also the difference between the two time constants decreased with depolarization. At 0 mV, the ratio τ_2/τ_1 was 20, while at 40 mV it was only 3. The relative weight of the slow component, $B/(A + B)$, was 0.63 ± 0.07 in the voltage range from 0 to +40 mV and did not show a clear voltage dependence.

To study the time development of the effect, the current at +10 mV was sampled every 5 s (Fig. 3). In the experiment shown, the pipette contained 100 μM GTP-γS and 2 mM Mg-ATP. The first trace recorded already had a double exponential time course, and it was impossible to compare a control curve with a modified one. However, the current changed with time in the first 3 min of recording. The relative contribution of the slower component increased from 10 to 70%, whereas both time constants did not vary with time, within experimental errors. This behavior was observed in three other cells with 100 μm GTP-γS, and in two cells with 500 μM GTP-γS, and the effect was independent of GTP-γS concentration in this range, suggesting that a dose of 100 μM already saturates the system. Note that, in the example shown, the total current was not depressed, but increased in amplitude. With ATP in the pipette, this 'run up' was observed in 40% of the GTP-γS perfused cells, whereas in 30% of the cells the current decreased with time.

The activation time course of the GTP-γS modified current was independent of the holding potentials shown in Fig. 4. The holding potential was varied from −100 to −30 mV, and in all cases the time course to peak was double exponential. A current trace from a control cell, held at −80 mV, is also shown for comparison. The amplitude of the current increased as the holding potential was made more negative and the fractional availability of calcium channels was half maximum at around −65 mV, independent of the pulse duration (Fig. 3 *b*).

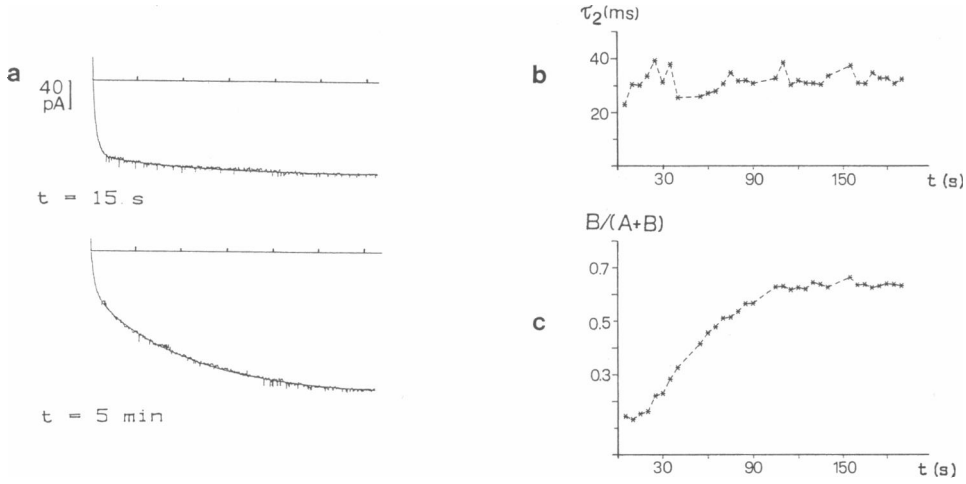


FIGURE 3 Effect of GTP-γS as a function of time. The holding potential was −50 mV and the test potential +10 mV. The pipette solution contained standard CsCl, 2 mM Mg-ATP, and 100 μM GTP-γS. (*a*) Current traces at t = 15 s and t = 5 min were fitted with Eq. 2. At t = 15 s, the best fit gave τ_1 = 1.2 ms, τ_2 = 28 ms, A = 83% and B = 17%. At t = 5 min, the current had reached a steady state. The best fit gave τ_1 = 1.2 ms, τ_2 = 31 ms, A = 33%, and B = 57%. Note that the total current amplitude increased with time in this cell. (*b*) Time dependence of the τ_2 time constant for the cell in *a*. The current was sampled every 5 s and fitted with Eq. 2 in the text. (*c*) Time dependence of the relative amplitude of the slow component.

DISCUSSION

Internal perfusion with the G-protein activator GTP-γS slowed down the time course of the voltage-dependent calcium current in all cells tested. This effect has been reported previously with internal GTP-γS (5, 13) and with external application of neurotransmitters and other modifiers (2, 4–6, 11), which affect the calcium channel through the activation of a G-protein (5, 10, 11).

Computer simulations of the time course of the current with exponential functions provide an interpretive description of the effect. In control, neglecting the very early phase of activation (16), currents elicited by depolarizations in the range from −10 to +40 mV had a single exponential time course. When the pipette contained 100 μM GTP-γS, the current time course was the sum of two exponential components: a fast component, whose time constant τ_1 was the same as the control time constant, within experimental error, and a slow component whose

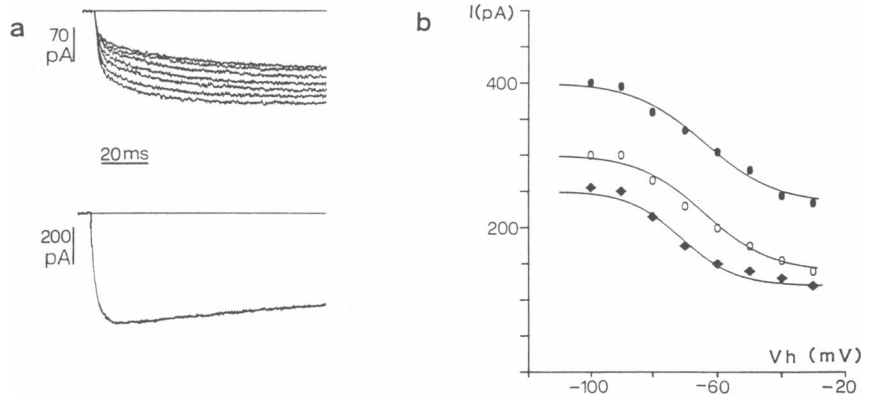
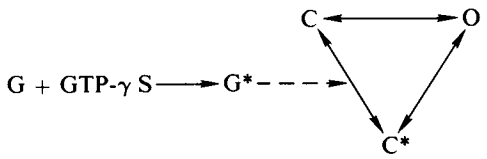


FIGURE 4 Effect of the holding potential on the GTP-γS modified calcium current. (*a*) Traces on top show the current from a GTP-γS perfused cell after 8 min from breaking into the cell. The holding potential was varied from −30 to −90 mV in 10-mV steps and the test potential was +10 mV. The current increased as the holding potential was made more negative. Bottom trace shows the current at +10 mV from a control cell held at −80 mV. Note the prolungation of the time to peak in the GTP-γS–treated cell. (*b*) Plot of the current amplitude at 5 (*diamonds*), 10 (*open circles*), and 100 ms (*solid circles*) against the holding potential value. Experimental points were fitted with the equation: $I(Vh) = (I_{max} - I_{min})/\{1 + \exp[(Vh - V^*)/K]\} + I_{min}$, with I_{max} = maximum current from −90 mV and I_{min} = minimum current (current from −30 mV). The best fit gave V^* = −68 mV, K = 8.5 mV at 5 ms; V^* = −64 mV, K = 10.5 mV at 10 ms; V^* = −64 mV, K = 10.8 mV at 100 ms.

time constant, τ_2, was an order of magnitude larger than τ_1. This simulation showed a good overlap with experimental data both when the cell was held at −90 mV and when it was held at −50 mV. From this last holding potential, the transient components, T and N currents (12, 13), although not totally inactivated, represent a small fraction of the total calcium current. Therefore the effect cannot be explained by a selective block of a transient component, and it was interpreted as a G-protein–mediated modulation of the L current.

The effect of GTP-γS developed with a relatively long time course and reached a steady state in ~2 min. This delay was longer that that seen with externally applied receptor agonists (1–5) and might be partly due to the diffusion of GTP-γS into the cell. However, Dolphin et al. (13) reported a delayed response also with photochemically released GTP-γS and suggested that the time limiting step is the binding of the activator to the G-protein. Our result indicates that, as more GTP-γS replaced GDP in binding the protein and the concentration of the activated protein G* increased, the relative weight of the slow component, $B/(A + B)$, also increased. Instead, the time constants, τ_1 and τ_2, did not change with time and therefore appeared to be independent of G* concentration. These observations suggest to describe the process with the following scheme:



where C and C* are two different nonconducting states of the channel and O is the conducting state. In control (no GTP-γS), depolarization favors the transition from C to O, according to a two-state kinetic scheme. The time constant of this transition decreases as depolarization increases from −10 to +40 mV. In the presence of GTP-γS, an effector molecule (possibly G* itself) interacts with the calcium channel and cause its transition to C* state. Both transition C ⟷ O and C* ⟷ O are voltage-dependent, as both τ_1 and τ_2 depend on the membrane voltage in the range from −10 to +40 mV. The rate constant of the reaction C ⟷ C* depends on G* concentration. As the time constants of activation were independent of the active G-protein concentration, the transition C ⟷ C* seems to occur in a time scale much longer than channel gating times, in agreement with the relatively slow development of the effect (Fig. 3) and with the result of Dolphin et al. (13). The resulting current is the sum of two mechanisms acting independently: the first one due to a fraction of channels unaffected by GTP-γS (C ⟷ O) and the second ascribed to a slower opening process from a different closed state (C* ⟷ O). The transition C ⟷ C* did not appear to depend on voltage in the range from −100 to −30 mV and our data give no evidence that depolarization favors this transition, because simulation with Eq. 2 did not show a significant change in the weight $B/(A + B)$ in the range from 0 to +40 mV. However, a voltage dependence of the transition rate of this reaction cannot be completely ruled out. A detailed evaluation of kinetic parameters would require additional data and the proposed scheme is to be interpreted as a phenomenological description.

We have also observed that, with 2 mM ATP in the pipette solution, the calcium current frequently increased with time, even in the presence of GTP-γS. An increase of current during the first minutes of whole-cell recording ('run up') has been previously reported when ATP was present in the pipette (3, 11, 18), and can be due to channel phosphorylation (16). GTP-γS did not seem to interfere with the mechanism which determine this 'run up', suggesting that the channel is modulated by phosphorylation and by a G-protein at two different sites. In addition, it is also possible that GTP-γS not only fails to prevent but even enhances the ATP-induced 'run up'. This second effect of GTP-γS could result from the activation of a different G-protein. In this respect, the action of GTP-γS is different from that of the receptor agonists (1–5), the latter being more specific.

The question of whether the G-protein acts directly on the calcium channel or affects it through one or more second messengers remains answered. Protein kinase C (PKC) activators, such as phorbol esthers and 1,2-oleoylacetyl-glycerol (OAG), depress calcium currents in chick DRG and pituitary cells (10, 18, 19), but this depression is not accompanied by the appearance of a slow component (18). Thus, although the mechanism of phorbol esthers and OAG-induced calcium current reduction is controversial (10, 18–20), it appears to be different from the G-protein–induced modulation described in this study. Although it is premature to rule out the involvement of other second messenger systems, most evidences suggest that a G-protein affects the channel directly.

We wish to thank Dr. Luciano Spadavecchia and Dr. Luigi Maga for their invaluable help with computer software.

Received for publication 20 April 1989 and in final form 17 July 1989.

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