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Augmentation of Calcium Channel Currents in Response to G Protein Activation by GTPγS in Chick Sensory Neurons

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G protein–mediated downregulation of current through neuronal voltage-gated Ca^{2+} channels is well known. We now report that G protein activation by GTPγS increases the Ba^{2+} conductance of high-voltage-activated Ca^{2+} channels of chick dorsal root ganglion (DRG) cells. This occurs with a delay of minutes during which the channels are inhibited by the activated G proteins. The Ba^{2+} current (I_{Ba}) showed an absolute enhancement by a factor near 2, 15 min after GTPγS application. However, by utilizing prior observations of the voltage dependence of the inhibitory action we could demonstrate that the G protein–inhibited component of I_{Ba} was still present. Moreover, the achieved amount of I_{Ba} disinhibition showed little variation throughout the experiments. This indicates that the increase in I_{Ba} is not due to a relief of the inhibitory action of activated G proteins but to the slow appearance of a distinct upregulating action, probably through a different pathway. Augmentation of I_{Ba} was eliminated by pertussis toxin (PTX) infusion or pretreatment, but was also prevented by intracellularly infusing protein kinase C (PKC) inhibitors together with GTPγS. The upregulation of neuronal Ca^{2+} channels thus appears to be exerted through a messenger pathway upstream of PKC activation that involves G proteins. Augmentation of Ca^{2+} currents (I_{Ca}) was observed only with strong intracellular [Ca^{2+}] buffering, which suggests a control of the upregulating action by even moderate increase in intracellular [Ca^{2+}].

[Key words: calcium channels, upregulation, guanosine-5′-O(3-thio)triphosphate, G proteins, protein kinase C, dorsal root ganglion neurons]

Receptor-triggered activation of guanine nucleotide–binding (G) proteins depresses Ca^{2+} channel activity in peripheral and central neurons. The inhibition of calcium channel currents was obtained directly by intracellular application of substituted and differently activated G proteins (G_o, G_i) and their α subunits (Hescheler et al., 1987; Ewald et al., 1989; Kleuss et al., 1991), and disappeared after ADP ribosylation of the endogenous G proteins by pertussis toxin (PTX). A general criterion for the G protein–mediated modulation of cellular function and channel activity is that the same effect can be obtained by intracellular application of nonhydrolyzable analogs of GTP such as GTPγS.

In neurons, the overall response to the application of GTP analogs is the activation of PTX-sensitive G (G_o and G_i) proteins and inhibition of calcium channel currents. Similar results have been observed after the activation of various receptor-linked G proteins (for review, see Anwyl, 1991). The current depression depended on voltage (Bean, 1989; Grassi and Lux, 1989; Kasai and Aosaki, 1989; Scott and Dolphin, 1990) and was effectively and quickly removed with strong depolarizing prepulses in dorsal root ganglion (DRG) cells as used here. These findings are in agreement with a membrane-delimited modulation of calcium channels (Brown and Birnbaumer, 1990). In addition, voltage-independent PTX-sensitive as well as PTX-insensitive depression of Ca^{2+} channels has been observed in sympathetic neurons (Beech et al., 1992; Hille, 1992) and has been attributed to the secondary activation of unidentified messenger systems. The neuronal mechanism appears to differ from that in cardiac muscle in which G protein activation by GTPγS upregulates Ca^{2+} channel activity (Yatani et al., 1987; Shuba et al., 1990). This occurs through G_s activation that usually abolishes a probable inhibitory effect of the activation of G_i (for review, see Trautwein and Hescheler, 1990).

The question has not been investigated directly whether or not G proteins could stimulate or stabilize Ca^{2+} channel activity through some pathway in neurons. Since the inhibitory effect of G protein activation and Ca^{2+} channel current rundown counteracts the potential stimulation of the channel activity, A relative increase of GTPγS-suppressed Ca^{2+} channel activity over long recording times as observed by Elmslie (1992) may not be uncommon but could alternatively be attributed to a loss of the inhibitory agent by dialysis. Here we report an absolute augmentative effect of internal GTPγS after a period of inhibition of Ba^{2+} currents through Ca^{2+} channels in DRG cells. The effect is highly Ca^{2+} sensitive, eliminated by PTX treatment and protein kinase C (PKC) inhibitors, and is caused by the appearance of Ca^{2+} channels that are free of G protein–mediated inhibition.

Materials and Methods

Neurons were mechanically dissociated from DRGs of chick day 10–12 embryos, plated on poly-L-ornithine–coated dishes containing Eagle's basal medium (BME) with 5% fetal calf serum/5% horse serum, and used 6–12 hr after plating.

The standard bath solution contained (in mM) 20 $BaCl_2$, 105 choline chloride, and 10 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), at pH 7.4 adjusted with CsOH. The neurons were continuously superfused with bath solution using a two-barreled pipette placed 200 μm away from the cell. One barrel contained equimolar $CaCl_2$ for eventually recording Ca^{2+} currents. The pipette solution contained (in mM) 145 N-methyl-D-glucamine (NMDG), 4 $MgCl_2$, 10 ethylene-bis(oxonitrilo)tetraacetate (EGTA), 20 HEPES, 3 adenosine triphosphate, magnesium salt (Mg-ATP), and 0.5 guanosine triphosphate, sodium salt (Na-GTP); adjusted to pH 7.4 with HCl. In some experiments internal solution contained 20 mM BAPTA or 100 mM EGTA for high

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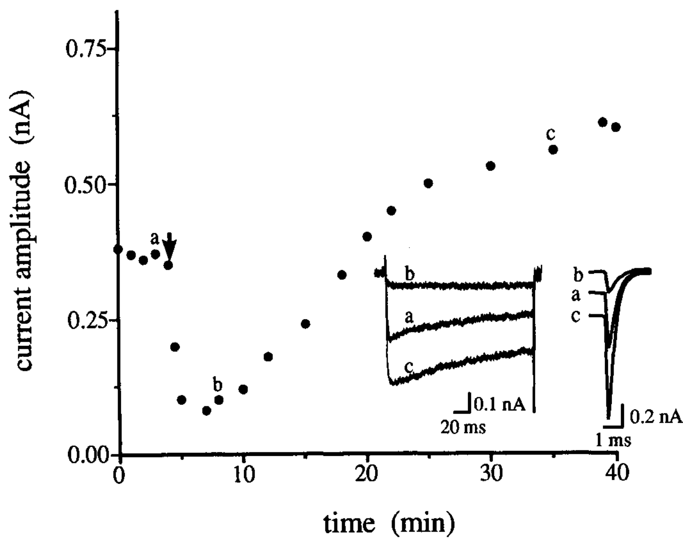

Figure 1. Early inhibition and late enhancement of I_{Ba} in response to infusion of GTPγS. Ba^{2+} currents were elicited by test pulses (V_t) to 0 mV from −70 mV holding potential (V_h). GTPγS at 250 μM was applied by the intrapipette perfusion method. The *arrow* marks the beginning exchange of the pipette medium by a solution containing 250 μM GTPγS. The *insets* show I_{Ba} recordings (*left inset*) at times indicated by *a, b,* and *c.* The tail currents (*right inset*) are displayed at a different time base. The traces were taken 0, 4, and 32 min after infusion of GTPγS.

buffering of intracellular Ca^{2+}. In this case NMDG was equimolarly subtracted. Pipette solutions containing GTPγS and PTX were prepared fresh from stock solutions of 10 mM and 100 μg/ml, respectively. For intracellular application, PTX was activated by adding dithiothreitol (5 mM) at 35°C for 15 min and then diluted to 0.5 μg/ml in the internal solution containing 1 mM nicotinic acid adenine dinucleotide (Surprenant et al., 1990).

With the exception of PKC inhibitor peptide (PKC 19-36) purchased from Bachem (Heidelberg, Germany), all chemicals and drugs were obtained from Sigma (Deisenhofen, Germany), and culture reagents from GIBCO (Eggenstein, Germany). The protein kinase A (PKA) inhibitor PKIm 16-26 was a gift of Dr. F. Hofmann (Institut für Pharmakologie und Toxikologie, Technische Universität München, München). Phorbol ester, PKC 19-36, and H7 were dissolved in ethanol, 0.5 M acetic acid, and chloroform, respectively. The stock solutions of phorbol ester (10 mM), PKC 19-36 (1 mM), and H7 (100 mM) were stored at −20°C. These stock solutions were diluted to their final concentrations just before use. The vehicular solutions had a final concentration of 0.1%, and had no effect on the calcium channel currents. PKIm 16-26 was dissolved in patch pipette solution at 1 mM.

Pipettes of 5–10 MΩ resistance were used and cells with seals below 20 GΩ after breakthrough were disregarded. Currents were obtained under whole-cell voltage clamp using a Dagan 3900A, 3911A expander, patch-clamp amplifier (Dagan, Minneapolis, MN) at room temperature (22–24°C). For voltage command pulses and data acquisition we used a microcomputer running PCLAMP software (Axon Instruments). Voltage pulse with 180 msec duration was then applied every 30 sec. The potential of 0 mV was chosen because it is close to potential of maximal activation of high-threshold-activated I_{Ba}. Currents were digitized at 10 kHz, filtered at 3 kHz, and analyzed with AUTESP program (Garching Instruments, Munich). Serial resistance was compensated as much as possible (>60%). Current recordings were corrected by subtracting linear components of leak and capacitive currents using sample averages of small hyperpolarizing pulses. When settling times of uncorrected capacitive transients eventually increased beyond 1 msec, suction was applied to reestablish the original conductive and diffusional access to the cell. If this was unsuccessful the cell was not used. Except for initial recordings with 30 sec intervals, voltage pulses and blocks of pulses for *I–V* determination were separated by at least 1 and 2 min, respectively. No correction was done for rundown of currents. All results are expressed in means and standard deviations.

Several experiments utilized the intrapipette perfusion technique of Soejima and Noma (1984). Two quartz glass capillaries with 30-μm-diameter tips at 100–200 μm distance to the patch pipette tip served to perfuse the pipette cone by applying positive pressure (20–40 cm H_2O) to the back end of the delivery barrel while similarly sized suction was applied to the other. Solution changes were reached within 1–2 min.

Results

Time course and voltage dependence

Figure 1 illustrates the time course of Ba^{2+} currents (I_{Ba}) during test pulses to 0 mV from −70 mV holding potential before and during intracellular application of 250 μM GTPγS. Calcium channel currents had a biphasic response to application of GTPγS. I_{Ba} started to decrease within 0.5 min after starting the exchange of the electrode solution. The GTPγS-depressed current (Fig. 1, left inset, trace b) displayed a slightly slowed onset compared with control current at the same potential (Fig. 1, left inset, trace a). This feature persisted throughout the recording time in the presence of GTPγS and was apparent as a rounding of the peak of I_{Ba} even during the augmentation at later times (Figs. 1, trace c in inset; 2*B*). The deactivation (tail) currents confirmed the conductance changes during depression and augmentation of the Ca^{2+} channels (Fig. 1, right inset). Kinetic changes of tail currents could not be found. The rise of I_{Ba} developed 6–10 times slower than its depression by GTPγS. With the same perfusion in two other cells I_{Ba} increased over control by 45% and 70%, 12 and 15 min, respectively, after GTPγS application. Initially, I_{Ba} was depressed by about 40% in these cases.

Breaking into the cells with GTPγS-containing pipette solution produced a similarly fast block and slow rise of I_{Ba} (Fig. 2). An actual control is unavailable in these cases, since the intracellular diffusion of the active substrate could be fast enough to prevent a recording of the undisturbed I_{Ba}, as suggested by the results of the intrapipette perfusion method. Taking an early current recording as reference could easily result in an overestimation of the observed rise of I_{Ba}. Therefore, the peak I_{Ba} amplitudes obtained at 0.5 and 1.0 min were extrapolated back to zero time (t_0). Compared with the extrapolated value at t_0, I_{Ba} showed an enhancement by a factor of 1.92 ± 0.42, 15 min after GTPγS infusion. It should be noted that currents were isochronally measured by averaging between 6 to 8 msec after the voltage steps. These times enclose the peak time of the control current at 0 mV. The measurements at times of peak I_{Ba} of the controls were preferred to determine peak amplitudes of the GTPγS-modulated currents. Our intention was to account for the noninhibited current component as well as that modified by GTPγS. As a result of this measurement the dependence on voltage of the inhibited and the augmented I_{Ba} appeared to be similar (Fig. 2*C*). Peak current measurements under GTPγS would additionally exhibit a considerable voltage shift in consequence to the voltage- and time-dependent relief of inhibition that could alternatively be viewed as delayed gating under GTPγS (Bean, 1989). Under the same conditions, variations of control currents were studied using a GTPγS-devoid but otherwise identical pipette solution. A directional preference of the changes of currents with time was not apparent in the averages within the first 20 min but rundown became significant thereafter (Fig. 2*A*). We conclude that after its initial depression I_{Ba} absolutely increases in a late response to perfusion of GTPγS, which is evidently not explained by a loss of inhibitory substrate.

The depression of Ca^{2+} channel currents by GTPγS and by G protein–activating agonists such as baclofen and dopamine

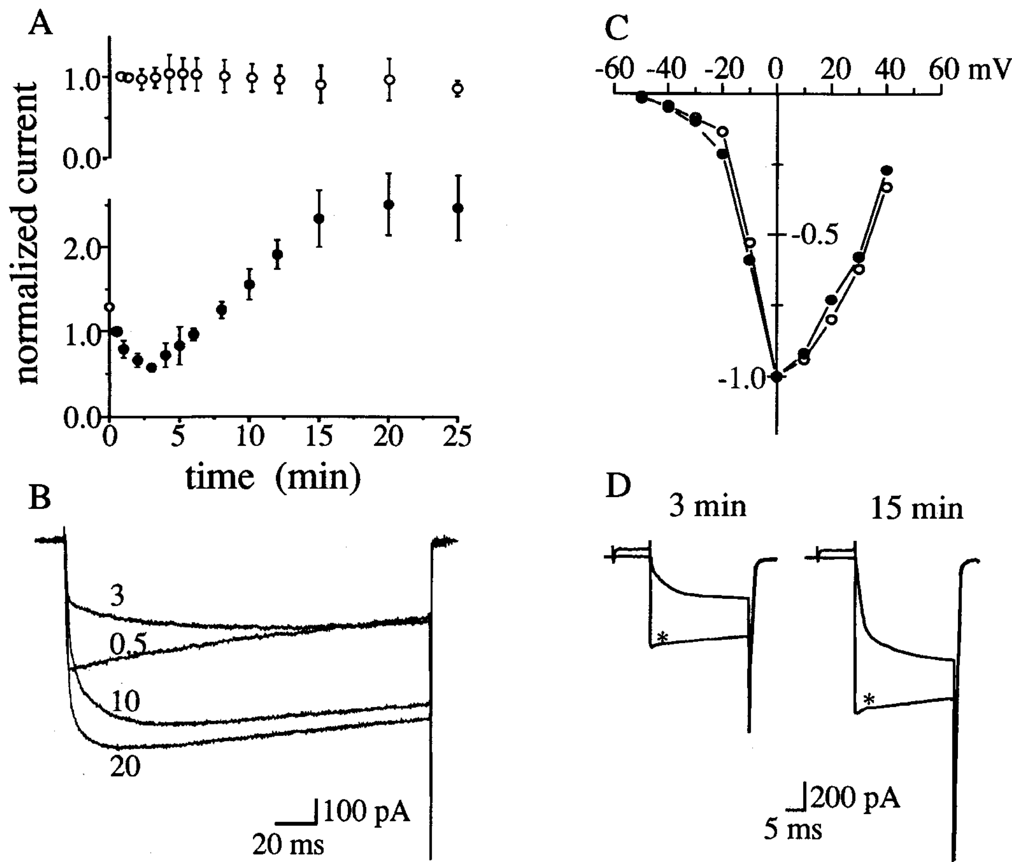

Figure 2. Kinetic changes of I_{Ba} and current–voltage relationships during G protein–mediated inhibition and augmentation. *A*, Time course of I_{Ba} in the absence (○, n = 10) and presence (●, n = 7) of 250 μM GTPγS. The current amplitudes were normalized to the initial values of I_{Ba} and extrapolated back to zero time (*open cycle in bottom plot*). *B*, I_{Ba} recordings with a patch pipette containing 250 μM GTPγS. *Numbers* give times (min) after breaking into the cell. V_h = −70 mV; V_t = 0 mV in *A* and *B*. *C*, Isochronal current–voltage relationships of I_{Ba} (current averaged 6–8 msec after voltage step) during perfusion with 250 μM GTPγS, 2.5 min (○) and 12 min (●) after seal breaking. The I_{Ba} amplitudes were normalized to the maximum values at 0 mV. I_{Ba} at 12 min was greater by a factor of 2.8 than that at 2.5 min. *D*, I_{Ba} traces elicited by test pulses to 0 mV from −70 mV without and with (*) a preceding pulse of 10 msec to +100 mV membrane potential. The prepulse depolarization removed similar fractions of I_{Ba} at 3 min and 15 min despite marked inhibition and late augmentation during perfusion with 250 μM GTPγS.

is known to be largely removed by short pulses to positive membrane potentials (Grassi and Lux, 1989). Thus, prepulses of 10 msec duration to +100 mV were applied to investigate the amount of inhibition of I_{Ba}. The time required to restore I_{Ca} and I_{Ba} is short, with a time constant of 4.5 msec at +40 mV and 3 msec at +100 mV (not shown). Thus, 10 msec prepulses to +100 mV were expected to be sufficient to remove the voltage-dependent depression of I_{Ba} by GTPγS. The prepulse-restored current amplitudes at times of maximum I_{Ba} depression appeared useful as additional controls, and were found to be within ±12% (n = 6) of the extrapolated values for the control currents. It was interesting to observe with this method that the GTPγS-inhibited part of the current failed to increase in proportion to the late rise in I_{Ba}. Figure 2*D* displays the largest inhibition found during the augmented I_{Ba} and exemplifies that the augmentation of I_{Ba} is expressed by a current component that is largely insensitive to the depressing action of GTPγS. The amount of the G protein–mediated depression determined by the prepulse method was on the average 10 ± 12% (n = 6) smaller during augmentation of I_{Ba} than that at early times with the maximum inhibition of I_{Ba}. These results clearly show that the late rise of I_{Ba} is not due to a relief of the inhibitory action of activated G proteins but to a distinct, Ca^{2+} channel–stimulating mechanism that suggests a different way of action.

Two receptor agonists, baclofen and dopamine (50 μM each), in DRG cells were observed by us to produce similar (20–50%) increases in I_{Ba} (but not I_{Ca}) after prolonged (10 min) or repeated (5 sec every min) application about 7–15 min after the early inhibitory action. The underlying mechanism is probably similar to that found with internal application of GTPγS but was not investigated in further detail.

PTX eliminates modulation of I_{Ba}*, and PKC inhibitors prevent its augmentation*

Not only inhibition but also the late rise of I_{Ba} under GTPγS was almost absent in cells that were preincubated for 20–24 hr at 37°C with 0.5 μg/ml PTX in the culture medium. I_{Ba} amplitudes at 3 min and 15 min after breakthrough were 101 ± 11% and 103 ± 14%, respectively, of the initial values (n = 5). The prevention by PTX of the inhibitory effect of photoreleased GTPγS on Ca^{2+} channel currents is known (Dolphin et al., 1988). Since the cell samples could have undergone changes in metabolic conditions during prolonged exposure to the toxin, it could be argued that the loss of an augmenting response to GTPγS was of a nonspecific nature. For this reason untreated neurons were intracellularly perfused with both 0.5 μg/ml PTX and 250 μM GTPγS. With this application I_{Ba} became depressed as usual in the first 5 min. There was a tendency of the inhibition to recover but not even control amplitudes were reached within the recording time (Fig. 3, bottom trace). This observation was found to differ from that with agonist-induced depression of I_{Ba} (by 100 μM baclofen, not shown), in which case the inhibited current recovered nearly to control values 5–10 min after infusing PTX, similar to the results of Surprenant et al. (1990) with somatostatin-produced inhibition of Ca^{2+} channels in submucosal neurons. When, by intrapipette perfusion, the neuron was first dialyzed with PTX for at least 5 min, the subsequent application of GTPγS was found to be ineffective (Fig. 3, upper

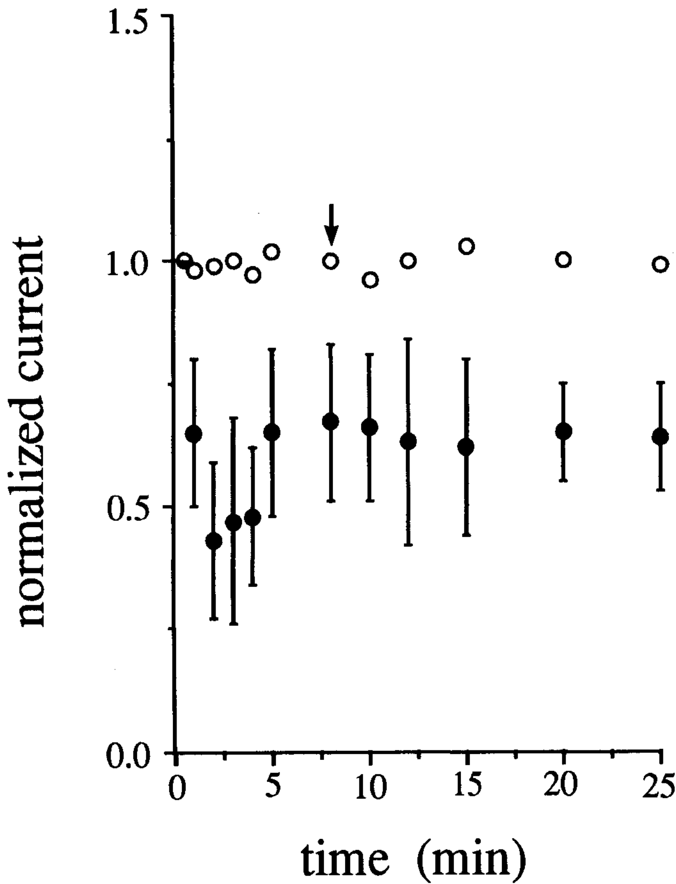

Figure 3. Involvement of PTX-sensitive G proteins in I_{Ba} augmentation. Internal application of PTX eliminated I_{Ba} modulation by GTPγS. *Upper plot,* Normalized I_{Ba} of a neuron first dialyzed with 0.5 μg/ml PTX for 8 min. The subsequent addition of 250 μM GTPγS by intrapipette perfusion (after *arrow*) was ineffective. *Lower plot,* With simultaneous application of 0.5 μg/ml PTX and 250 μM GTPγS, I_{Ba} amplitudes became initially depressed but thereafter showed a tendency to recover from inhibition without reaching control values (n = 5). V_h = −70 mV; V_t = 0 mV.

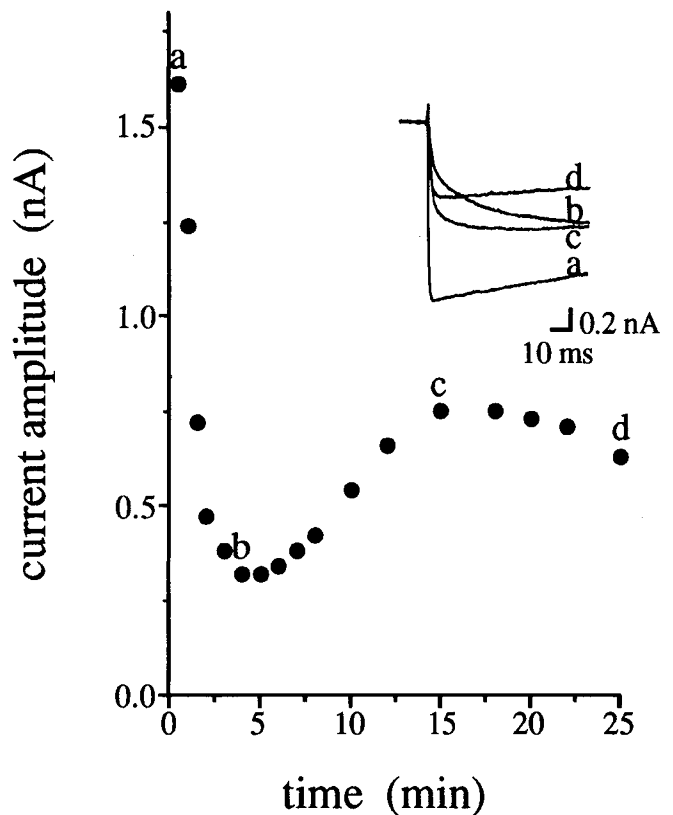

Figure 4. Impairment of phosphorylating actions affect G protein-mediated inhibition. AMP-PNP at 5 mM was infused together with 250 μM GTPγS. The *inset* shows I_{Ba} recordings of the neuron taken at times *a–d* of the amplitude–time plot. The voltage-dependent inhibition in early recordings is displayed by a slowed onset of I_{Ba} (compare *traces a* and *b*). This kinetic characteristic of the G protein–mediated inhibition disappeared over tens of minutes while the current amplitudes only partially recovered. V_h = −70 mV; V_t = 0 mV.

trace). These results indicate that the sequence of PTX and GTPγS application may not be ambiguous for the effects to be investigated. The faster loading of the cells and occupation of G protein by GTPγS could possibly hinder PTX ribosylation of the same G protein (Mattera et al., 1987). This would explain the persistence of the inhibition. The complete absence of augmentation of I_{Ba} suggests that the underlying action of G proteins is more strongly affected by PTX.

The argument that the late rise of I_{Ba} was due to the permanent occupation rather than activation of G protein is not consistent with the inefficacy of the GDP analog GDPβS, in spite of the strong binding and replacement of GDP from G proteins. The augmentation was unaltered with additional infusion of 100 μM cAMP (data not shown). However, in five experiments aimed at reducing the phosphorylation potency by infusing 5 mM 5′-adenylylimidodiphosphate (AMP-PNP), an absolute augmentation was absent but the initial I_{Ba} inhibition by GTPγS was offset, at least partially (Fig. 4), with considerable variations in size and onset (4–10 min) after perfusion.

The late rise but not the early inhibition by GTPγS appeared to be reduced by applying protein kinase inhibitors. A complete removal of the augmenting response was achieved with additional dialysis of the isoquinodine H7 (100 μM) (Fig. 5*A*). Regarding that H7 and other compounds acting at ATP-binding sites of kinases poorly discriminate between protein kinases, the PKC pseudosubstrate (PKC 19-36) was dialyzed at a 3 μM concentration. The result was entirely similar to that with H7, with the preservation of the early inhibition and loss of the late rise in I_{Ba} (Fig. 5*B*). An inhibitory effect of its own on Ca^{2+} channels was not observed with the PKC pseudosubstrate (Fig. 5*B*, upper trace).

To investigate for specificity a specific protein kinase A inhibitor, PKIm 16-26, was applied (with GTPγS) at a higher concentration (1 mM) than that of PKC 19-36. This treatment could not suppress the augmentation of I_{Ba}, although its amplitudes were less than in the untreated samples with factors between 1.1 and 1.6 (n = 8). To test whether phorbol esters as preferential activators of PKC (Castagna et al., 1982; Niedel et al., 1983) could reproduce a similar augmentation of I_{Ba}, the cells were superfused for 20 min during recording with bath solution containing 10 μM phorbol 12-myristate 13-acetate 4-*o*-methyl ether. A small (10% and 15%) increase in I_{Ba} was seen in two of five cells. However, intracellular application of the phorbol ester was followed with delays of 5–10 min by an increase of I_{Ba} by 32 ± 25%, comprising four ineffective trials out of 10. These results were thus considered to be of less significance than those with GTPγS. When cells were bathed in external

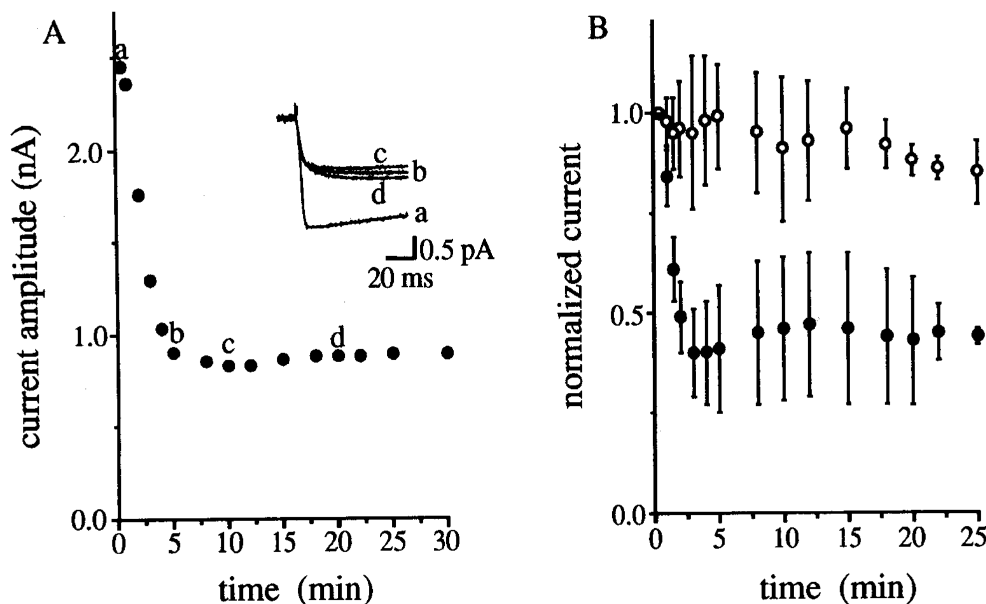

Figure 5. Disappearance of G protein–mediated augmentation during the additional perfusion with protein kinase inhibitors. *A*, Lasting inhibition of I_{Ba} during intracellular perfusion with 100 μM H7 and 250 μM GTPγS. The I_{Ba} recordings (*inset*) at times *a–d* of the amplitude data show a persistently slowed onset of I_{Ba}. *B*, Effects of internal PKC 19-36 (3 μM) in the absence (○, *n* = 5) and presence of 250 μM GTPγS (●, *n* = 6). The GTPγS-induced depression of I_{Ba} continued in the cells treated with PKC 19-36, which hardly affected I_{Ba} when given alone. V_h = −70 mV; V_t = 0 mV.

solution containing Ca^{2+} and internally supplied with the phorbol ester, no increase was observed in I_{Ca} ($n = 5$).

The fact that the late rise of I_{Ba} is abolished by PKC inhibitors and to a limited extent reproduced by a PKC activator (see also Swartz et al., 1993; Yang and Tsien, 1993) suggests that the GTPγS-induced augmentation of I_{Ba} is at least in part mediated by protein kinase C. Its effect probably involves the activation of a second messenger pathway downstream of the G_o, G_i, and G_q proteins (Kikuchi et al., 1986; Berstein et al., 1992). PKC activator–induced upregulation of I_{Ba} could possibly be attributed to direct phosphorylation of Ca^{2+} channels (Ahlijanian et al., 1991; Chang et al., 1991).

Counteraction by Ca^{2+} entry

When Ca^{2+} was used as current carrier, inhibition of I_{Ca} was always observed; but an example of a rising in Ca^{2+} conductance in response to GTPγS dialysis failed to be encountered (Fig. 6*A*). When Ba^{2+} in the bath solution was replaced for minutes by Ca^{2+}, the increase of I_{Ba} after reexchange of the bath solution occurred only slowly and partially during the next 20 min (data not shown). To intercept transient rises in $[Ca^{2+}]_i$, the Ca^{2+} buffer capacity was increased. EGTA was used at a concentration of 100 mM ($n = 3$) and a fast-reacting buffer, BAPTA (20 mM, $n = 3$), was employed. Under these conditions, a significant rise in I_{Ca} (overall by a factor of 1.56 ± 0.34) was in fact observed (Fig. 6*B*).

Discussion

G protein–mediated upregulation of neuronal Ca^{2+} channels has not received as much attention as downregulation. In the present work, we found that I_{Ba} through Ca^{2+} channels in chick DRG neurons was nearly doubled by G protein activation by GTPγS. Several neurotransmitters such as noradrenaline, isoproterenol, and NMDA are reported to increase Ca^{2+} channel current in

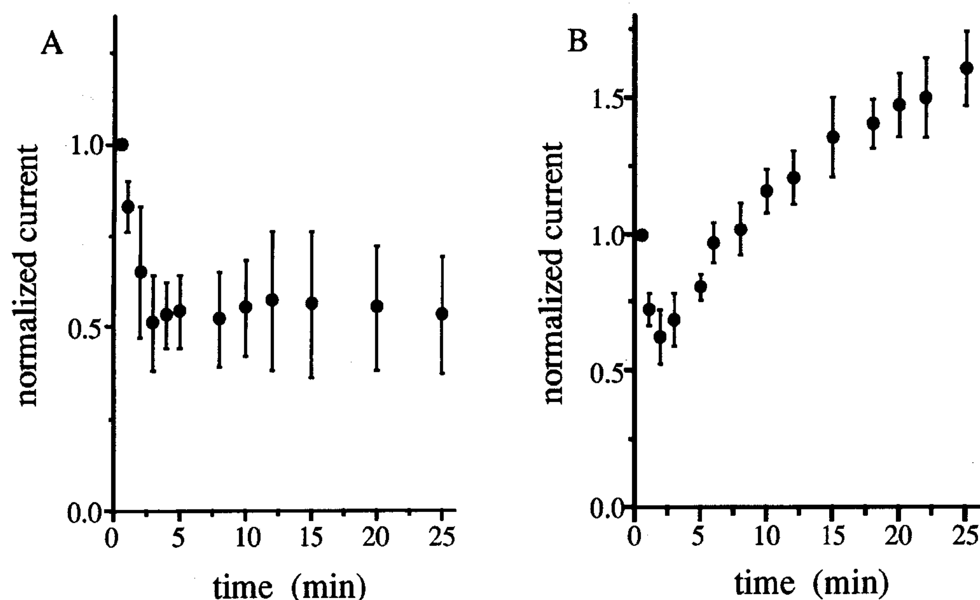

Figure 6. Dependence of GTPγS-induced augmentation of Ca^{2+} currents on strong intracellular Ca^{2+} buffering. *A*, GTPγS at 250 μM permanently inhibited Ca^{2+} channel currents when Ca^{2+} ions served as current carrier (*n* = 7). The pipette solution contained 10 mM EGTA for $[Ca^{2+}]_i$ buffering. *B*, Perfusion with 250 μM GTPγS produced a late rise of I_{Ca} with pipettes containing 20 mM BAPTA. The normalized I_{Ca} amplitudes (*n* = 3) increased by factors between 1.3 and 1.7, 25 min after perfusion. V_h = −70 mV; V_t = 0 mV.

hippocampal neurons (Gray and Johnston, 1987; Fisher and Johnston, 1990; Chetkovich et al., 1991). The enhancement without preceding Ca^{2+} channel suppression, predominantly studied with Ba^{2+} ions, was moderate and suggested to result from a cAMP-dependent mechanism (Gray and Johnston, 1987; Fisher and Johnston, 1990; Chetkovich et al., 1991). This was less apparent in our preparation because the specific PKA inhibitor fails to prevent the enhancement of calcium channel currents. This suggests that a cAMP-dependent system may be less active in the embryonic DRG neurons that were freshly dissociated and short-time cultured.

G proteins are suggested to be involved in the downregulation of Ca^{2+} channels in several ways, including a direct or membrane-delimited G protein–channel interaction (Brown and Birnbaumer, 1990) that is consistent with our observations, and indirect ones that employ second messengers (Beech et al., 1991, 1992; Hille, 1992; Diversé-Pierluissi and Dunlap, 1993). Our results provide evidence for the involvement of G proteins in an upregulating action on Ca^{2+} channels. We cannot rule out the possibility of allosteric interactions between the sites responsible for the inhibition and augmentation of Ca^{2+} channel currents. However, the target sites appear to differ since the augmenting effect involved Ca^{2+} channels that were obviously insensitive to inhibition. The observations on the time requirement of the upregulation of high-voltage-activated Ca^{2+} channels by GTPγS and its prevention by specific kinase antagonists point to an indirect way of action.

Hypotheses on possible mechanisms should incorporate the finding that G proteins are able to upregulate the activity of phospholipase C (Kikuchi et al., 1986; Berstein et al., 1992), with the generation of diacylglycerol, which subsequently activates PKC. The kinase phosphorylates Ca^{2+} channels (Ahlijanian et al., 1991; Chang et al., 1991). Independence of the two roles of G proteins is suggested by the fact that inhibitory action of activated G proteins persists while the Ca^{2+} channel current increases. It was found as well that the amplitudes of inhibitory effects were far too small to account, by their relief, for the augmentation. The upregulated Ca^{2+} channels can thus be defined as an entity insensitive to direct action of G proteins. They might possibly lack a close association with G proteins.

PKC activators such as phorbol esters are reported to either depress (Rane et al., 1986, 1989; Hockberger et al., 1989; Diversé-Pierluissi and Dunlap, 1993) or increase neuronal Ca^{2+} channel currents (DeRiemer, 1985; Leonard et al., 1987; Sigel and Baur, 1988; Swartz et al., 1993; Yang and Tsien, 1993). It is interesting to note that upregulation was described in these studies when Ba^{2+} currents were studied while downregulation was observed on currents carried by Ca^{2+} ions. Our results thus agree with these previous results. These data indicate that Ca^{2+} currents and thus Ca^{2+} entry may interfere with the upregulation of Ca^{2+} channels by a presumed PKC activation.

The results with intracellular Ca^{2+} buffering led to the conclusion that increases in $[Ca^{2+}]_i$ could severely counteract the augmentative actions on Ca^{2+} channels. Dephosphorylating actions are assumed to be involved in a slow Ca^{2+}-dependent inactivation process of Ca^{2+} channel currents and in an eventually irreversible rundown (Eckert and Chad, 1984; Ono and Fozzard, 1992). A Ca^{2+}-dependent dephosphorylating action characterizes several phosphatases (Gutierrez et al., 1988) and Ca^{2+} channels may well be one of their targets. The protective effect regarding rundown of increasing the Ca^{2+} buffer concentrations is probably due less to the increased buffering capacity than to an earlier capture of entering Ca^{2+} ions, such as during activated Ca^{2+} current. The largest variations in $[Ca^{2+}]_i$ would occur near the cytoplasmic side of the membrane, and the speed of complexation of Ca^{2+} ions is expected to be the primary factor in the spatial and temporal extension of the local increase in $[Ca^{2+}]_i$ (Gutnick et al., 1989). This in turn suggests that increases in free $[Ca^{2+}]$ in regions next to the membrane evoke a process that counteracts the augmentating action. *In vivo,* at moderate neuronal activity, a transient increase in intracellular $[Ca^{2+}]$ by activated Ca^{2+} channels may not be strong enough to counteract G protein–mediated upregulation of Ca^{2+} channels.

Although upregulation of presynaptic Ca^{2+} channels is not yet demonstrated in synaptic potentiation phenomena, it is known that the activation of PKC by phorbol esters increases transmitter release in sympathetic neurons (Wakade et al., 1985) and induces synaptic potentiation in the hippocampus (Malenka et al., 1986). If an upregulation of Ca^{2+} channels is responsible for these findings the control of the intracellular Ca^{2+} activity could well be a significant factor for the underlying regulatory mechanism.

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