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G-Protein Activation Mediates Prepulse Facilitation of Ca^{2+} Channel Currents in Bovine Chromaffin Cells

C.A. Doupnik*, R.Y.K. Pun

Department of Physiology and Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0576

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Abstract. The effects of G-protein activation were investigated on tonic, large depolarization-induced Ca^{2+} channel facilitation in cultured bovine adrenal chromaffin cells. Under whole-cell voltage clamp, activation of G proteins by intracellular dialysis with 200 μM GTP-γS did not significantly affect prepulse facilitation or whole-cell Ba^{2+} current (I_{Ba}) density. In contrast, inactivation of G proteins by intracellular GDP-βS or pertussis toxin (PTX) pretreatment completely abolished or markedly attenuated facilitation of I_{Ba}, respectively. GDP-βS dialysis resulted in nearly a threefold increase in peak I_{Ba} density, whereas PTX pretreatment resulted in a 50% increase. Our results indicate that under control recording conditions (200 μM intracellular GTP), G proteins are tonically activated and suppress high-voltage-activated (HVA) Ca^{2+} channels in a voltage-dependent and voltage-independent manner. Local superfusion of chromaffin cells with normal bath solution produced a rapid and reversible increase (~50%) in I_{Ba} amplitudes that also abolished prepulse facilitation. Together, these results demonstrate that tonic facilitation of HVA Ca^{2+} channels in bovine chromaffin cells involves the voltage-dependent relief of a G-protein-mediated suppression, imposed by chromaffin cell secretory products that feedback and activate G-protein-coupled autoreceptors.

Key words: Ca channel — Facilitation — G protein — Chromaffin cell — Bovine — Whole-cell patch clamp

Introduction

HVA Ca^{2+} channels are facilitated by brief predepolarizations to positive membrane potentials in several excitable secretory cells (Fenwick, Marty & Neher, 1982; Grassi & Lux, 1989; Jones & Marks, 1989; Scott & Dolphin, 1990; Aicardi et al., 1991; Kasai, 1991; Ammala et al., 1992). The physiological consequences of Ca^{2+} channel facilitation are not well established, although it is generally thought that repetitive action-potential firing could facilitate Ca^{2+} channel activity and thereby potentiate Ca^{2+}-dependent processes such as secretion (Hoshi, Rothlein & Smith, 1984; Elmslie, Zhou & Jones, 1990). In support of this hypothesis, high frequency step depolarizations can manifest Ca^{2+} channel facilitation in adrenal medullary chromaffin cells, an excitable secretory cell of neuronal lineage (Hoshi et al., 1984).

Recently, the molecular mechanisms underlying Ca^{2+} channel facilitation in adrenal chromaffin cells were reported to differ from the mechanisms purported for neuronal cells (Artalejo et al., 1992*b*). In neurons, prepulse facilitation is expressed when HVA Ca^{2+} channels are suppressed by neurotransmitter activation of G-protein-coupled membrane receptors, or intracellular dialysis with the nonhydrolyzable GTP analogue guanosine-5′-*O*-[3-thiotriphosphate] (GTP-γS) (*see reviews by* Carbone & Swandulla, 1989; Dolphin, 1990; Schultz et al., 1990; Anwyl, 1991). These observations, together with the findings that neurotransmitter inhibition of HVA Ca^{2+} channels is rapid and voltage dependent (Marchetti, Carbone & Lux, 1986; Bean, 1989), have led to the hypothesis that activated G proteins directly interact with and block HVA Ca^{2+} channels in a voltage-dependent manner. Depolarizing prepulses to very positive membrane potentials "relieve" the G-protein block, thereby facilitating subsequently evoked Ca^{2+} channel currents.

* *Present address:* Division of Biology 156-29, California Institute of Technology, Pasadena, California 91125
Correspondence to: C.A. Doupnik
Reprint requests to: R.Y.K. Pun

In contrast to neuronal cells, prepulse facilitation of HVA Ca^{2+} channels in bovine chromaffin cells has been observed as a "tonic" phenomenon, not involving neurotransmitter-mediated channel inhibition (Fenwick et al., 1982; Hoshi et al., 1984; Artalejo et al., 1991). Tonic prepulse facilitation in chromaffin cells is unaffected by the presence or absence of GTP in the intracellular patch pipette during whole-cell recording (Artalejo et al., 1992*b*). This observation led Artalejo and colleagues to conclude that the underlying mechanism of Ca^{2+} channel facilitation in chromaffin cells does not involve G-protein activation as in neurons. The inclusion of GTP in whole-cell patch pipettes, however, has not been a requirement for G-protein-coupled receptor modulation of Ca^{2+} channels in neuronal cells (Holz, Rane & Dunlap, 1986; Lewis, Weight & Luini, 1986; Hescheler et al., 1987). Indeed, endogenous cytoplasmic GTP concentrations are sufficient for receptor activation of G proteins even during long-term whole-cell recording (Hescheler et al., 1987). Given the similar voltage-dependent features of Ca^{2+} channel facilitation in adrenal chromaffin cells and neuronal cells, we further investigated the role of G-protein activation on tonic Ca^{2+} channel facilitation in bovine adrenal chromaffin cells. Our results indicate that as in neuronal cells, G-protein activation mediates prepulse facilitation of HVA Ca^{2+} channels in chromaffin cells and is tonically induced by chromaffin cell secretory products (e.g., catecholamines, ATP, and enkephalins) that feedback and activate G-protein coupled autoreceptors.

Materials and Methods

Cell Culture

Chromaffin cells were enzymatically dissociated (0.15% type I collagenase) from isolated adult bovine adrenal medullae as previously described (Doupnik & Pun, 1992). Petri dishes (35 mm) coated with calf skin collagen were seeded with 150,000–250,000 chromaffin cells identified by neutral red staining. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 200 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.04 mg/ml gentamicin. On day 1 of culture, cells were washed and replenished with fresh culture medium. Electrophysiological experiments were performed from day 3 to day 7 on 10–20 μm diameter "spherical" chromaffin cells devoid of any outgrowths.

Electrophysiological Recordings

Whole-cell patch clamp recording (Hamill et al., 1981) was performed at room temperature (22–24°C) using borosilicate glass electrodes having tip resistances of 2–4 MΩ. Capacitive transients were cancelled using the analog circuitry of the amplifier (Axopatch 1A, Axon Instruments, Foster City, CA) from which the whole-cell membrane capacitance was directly determined (range, 8–20 pF). For comparisons between cells under different experimental conditions, whole-cell currents were normalized to membrane capacitance and expressed as "current density" (pA/pF). Series resistance (R_s) was typically 3–4 MΩ following 50–70% compensation. The voltage errors resulting from the IR_s drop, less than 5 mV in all cases, were corrected for in the analysis of voltage-dependent relationships. Voltage clamp protocols were executed using pCLAMP 5.5 software (Axon Instruments) and a microcomputer. The analog current signals were filtered ½ to ¼ the sampling rate (−3 dB, 4-pole low pass Bessel-type filter), digitized, and stored on the hard disk of the computer for later analysis.

Solutions and Drugs

Whole-cell HVA Ca^{2+} channel currents were recorded using Ba^{2+} as the charge carrier (I_{Ba}). The extracellular bath solution (1 ml/35 mm dish) consisted of 5 mM $BaCl_2$, 130 mM tetraethylammonium (TEA)-Cl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 10 mM D-glucose, 2 mM 4-aminopyridine, 1 mM $MgCl_2$, 3 μM tetrodotoxin, and 10 mg/liter phenol red at pH 7.4 (TEA-OH). The osmolarity was raised to 320–325 mOsm by addition of sucrose (~30 mM). Patch electrodes were filled with a high-Cs^+ intracellular solution that consisted of (mM) 120 CsCl, 20 TEA-Cl, 10 HEPES, 11 ethyleneglycol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 $CaCl_2$, 2 MgATP, 0.2 Na_2GTP, and 10 mg/liter phenol red at pH 7.2 (CsOH). Purine nucleotides MgATP, Na_2GTP, Li_4GTP-γS, and Li_3GDP-βS were added to the intracellular pipette solution from aliquoted concentrated stock solutions dissolved in distilled H_2O and stored at −23°C. Intracellular solutions were made up fresh the day of the experiment and kept on ice. The potential influence of a recently identified GTP-activated Cl^- conductance (Doroshenko, Penner & Neher, 1991) was minimized using a P/−4 on-line subtraction protocol. The effectiveness of this procedure was evident in that the I_{Ba} reversal potential (+60 mV) was not significantly altered under the various conditions of G-protein activation tested.

For PTX experiments, aliquots of a PTX-concentrated stock solution were directly added to culture dishes to a final concentration of 500 ng PTX/ml of culture medium. Chromaffin cells were then incubated in the PTX-containing culture medium for 20–24 hr. The concentrated PTX stock solution (50 μg/ml distilled H_2O) was stored at 4°C.

MgATP, Na_2GTP, PTX (from *Bordetella pertussis*), type I collagenase, calf-skin collagen, and antibiotics were obtained from Sigma Chemical (St. Louis, MO). Li_4GTP-γS and Li_3GDP-βS were from Boehringer-Mannheim (Indianapolis, IN). DMEM and fetal calf serum were from GIBCO Laboratories (Grand Island, NY), and tetrodotoxin was obtained from Calbiochem (LaJolla, CA).

Data Analysis

I_{Ba} records were analyzed using pCLAMP analysis programs. Statistical comparisons between experimental groups were performed using Student's unpaired *t*-test, with $P < 0.05$ considered significant. Nonlinear curve fitting was performed using the software program NFIT (Island Products, Galveston, TX), which utilizes the Marquardt-Levenberg algorithm for nonlinear least-squares curve fitting.

Results

G-Protein Activation Mediates Prepulse Facilitation of I_{Ba}

Tonic facilitation of Ca^{2+} channel currents was examined in bovine chromaffin cells using a double voltage-pulse protocol. From a holding membrane potential of −90 mV, a 50 msec depolarizing prepulse to varying membrane potentials preceded a 25 msec test pulse to

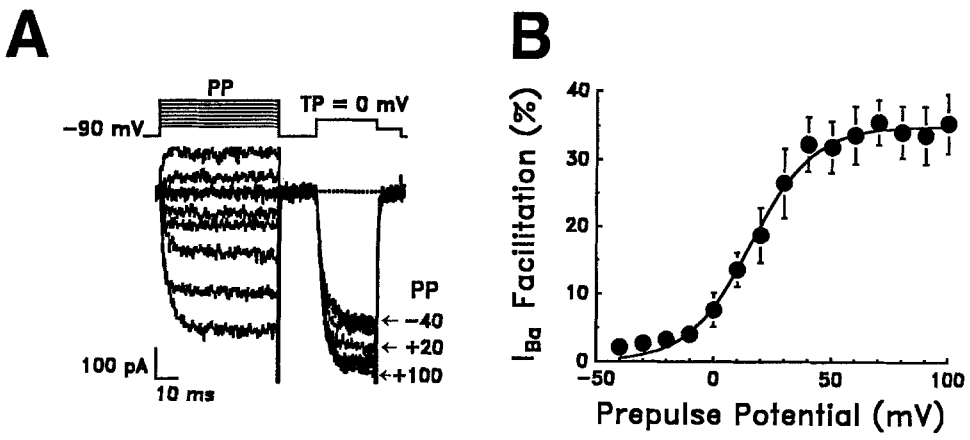

Fig. 1. Tonic prepulse facilitation of adrenal chromaffin cell Ca^{2+} channel currents. (*A*) Superimposed Ca^{2+} channel currents elicited by the double-pulse voltage protocol shown above current traces (not all traces are shown for clarity). Incremental increases in the prepulse potential (*PP*) increased the amplitude of Ba^{2+} currents evoked by test pulses (*TP*) to 0 mV. Double-pulse sequences were evoked every 15 sec from a holding membrane potential of −90 mV. Currents were filtered (−3 dB) at 3 kHz and sampled at 10 kHz. (*B*) Voltage-dependent activation properties of facilitated HVA Ca^{2+} channels. Test pulse currents were normalized to the I_{Ba} amplitude elicited by prepulses to −40 mV within each cell. Data represent the mean ± SEM values from six chromaffin cells. The unbroken curve is a least-squares fit of the mean data points and represents a first-order Boltzmann function of the following form. Percent I_{Ba} facilitation = 35% maximal I_{Ba} facilitation/{1 + exp [1.9 e^- (+16 mV − V_m)/kT]}, where 1.9 e^- is the limiting valence for I_{Ba} facilitation, +16 mV is the membrane potential for 50% facilitation, k is Boltzmann's constant, and T is the absolute temperature.

0 mV. The prepulse and test pulse were separated by a 15 msec step return to −90 mV. These pulse parameters have previously been demonstrated to evoke optimal Ca^{2+} channel facilitation in chromaffin cells (Hoshi et al., 1984; Artalejo et al., 1991). Shown in Fig. 1*A* are superimposed I_{Ba} recordings from a chromaffin cell in which the prepulse potential was varied from −40 to +100 mV in 10 mV depolarizing increments. Depolarizing prepulses potentiated the test pulse current in a voltage-dependent manner. Figure 1*B* shows the relationship between prepulse potential and the relative change in I_{Ba} amplitude evoked by the test pulse. The voltage dependence of facilitated I_{Ba} followed a sigmoidal relationship that could be well described by a Boltzmann distribution where the membrane potential for 50% facilitation occurred at +16 ± 1 mV (mean ± SD, n = 6). The limiting valence for activation of facilitated I_{Ba}, reflected by the slope of the relation, was 1.9 ± 0.2 charges (e^-). Prepulse facilitation of I_{Ba} saturated between +70 and +100 mV, resulting in a 35 ± 4% increase in I_{Ba} amplitude (mean ± SEM, n = 6, range: 23 to 50%).

To examine the effects of G-protein activation on I_{Ba} facilitation, intracellular GTP (200 μM) was replaced with either GTP-γS to chronically activate G proteins, or guanosine-5′-*O*-[2-thiodiphosphate] (GDP-βS) to inactivate G proteins. The role of PTX-sensitive G proteins was determined by examining I_{Ba} facilitation in chromaffin cells pretreated 20–24 hr with PTX. Shown in Fig. 2*A* are representative I_{Ba} recordings from different chromaffin cells under the varying conditions of G-protein activation tested. The I_{Ba} amplitude from each test pulse to 0 mV was compared isochronally (at 15 msec into the step pulse) to the I_{Ba} amplitude during the 0 mV prepulse, which effectively represents the I_{Ba} amplitude at 0 mV without a depolarizing prepulse. In chromaffin cells dialyzed with GTP-γS or GTP (control), prepulses to both 0 and +90 mV evoked prominent I_{Ba} facilitation (Fig. 2*B*). The magnitude of facilitation for the control group was comparable and not statistically different to that shown in Fig. 1. Conversely, tonic I_{Ba} facilitation was completely abolished in cells dialyzed with GDP-βS. I_{Ba} was in fact reduced at prepulses to 0 mV, due to the time-dependent I_{Ba} inactivation observed with GDP-βS dialysis (*see* Fig. 5). For PTX-treated chromaffin cells, I_{Ba} facilitation by prepulses to +90 mV was reduced 68% ($P < 0.05$) compared to untreated cells. Facilitation of I_{Ba} by prepulses to 0 mV was completely abolished by PTX treatment. The effects of intracellular GDP-βS and PTX treatment on I_{Ba} facilitation demonstrate that tonic facilitation in bovine chromaffin cells is mediated by activation of G proteins, and especially PTX-sensitive G proteins.

Voltage-Dependent and Voltage-Independent Suppression by Activated G Proteins

Evident in Fig. 2*A* is an increase in I_{Ba} amplitude with intracellular GDP-βS dialysis. In Fig. 3 are comparisons of normalized current-voltage relations from chromaffin cells under the various conditions of G-protein activation. Intracellular dialysis with GDP-βS resulted in

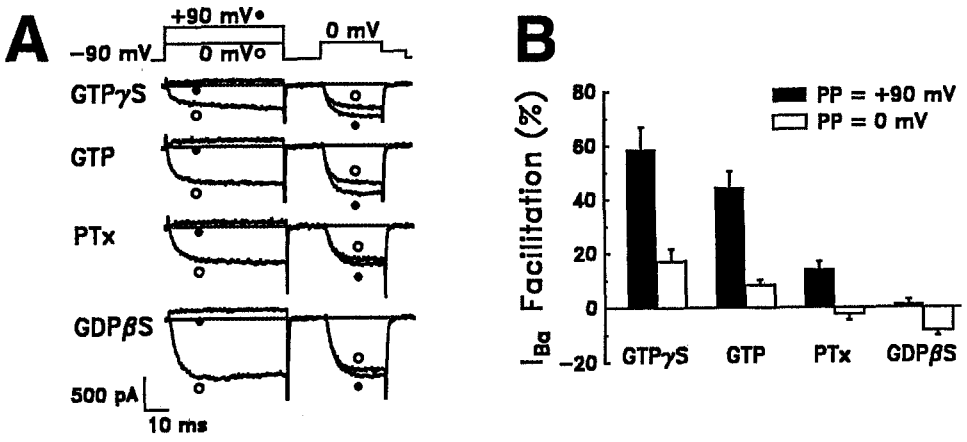

Fig. 2. Inactivation of G proteins by GDP-βS and PTX abolishes tonic Ca^{2+} channel facilitation in bovine chromaffin cells. (*A*) Effects of intracellular GTP-γS, GDP-βS, and pretreatment with PTX on prepulse I_{Ba} facilitation. Superimposed Ba^{2+} currents were elicited by double-pulse protocols at two prepulse potentials, 0 mV (open circles) and +90 mV (filled circles). For GTP-γS and GDP-βS dialyzed cells, GTP in the intracellular pipette solution was replaced on an equimolar basis (200 μM) with each respective nucleotide. PTX-treated cells (20–24 hr with 500 ng/ml) were recorded with normal intracellular solution which contained 200 μM GTP. (*B*) PTX treatment significantly attenuated and intracellular GDP-βS abolished tonic prepulse facilitation of HVA Ca^{2+} channels. Facilitation of I_{Ba} by depolarizing prepulses to 0 mV (open bar) or +90 mV (filled bar) is expressed as the percent change in the current amplitude elicited by the test pulses compared to the current amplitude evoked by the 0 mV prepulse, which represents I_{Ba} without a depolarizing prepulse. All I_{Ba} amplitudes were isochronally measured 15 msec after the start of the voltage step. Values are the mean ± SEM (GTP-γS, n = 9; GTP, n = 11; PTX, n = 13; GDP-βS, n = 9).

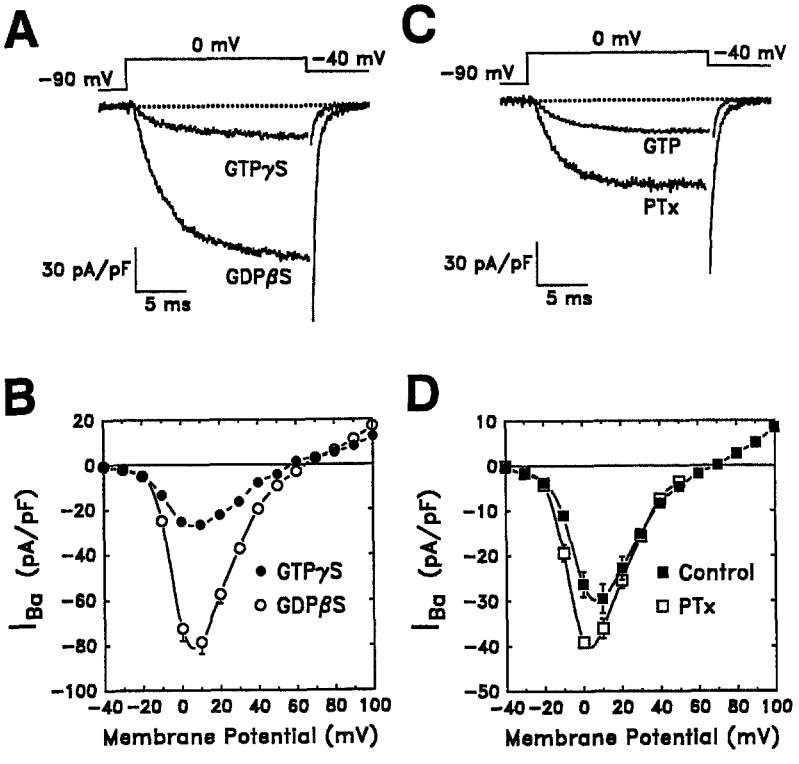

Fig. 3. Intracellular GDP-βS and PTX pretreatment increase whole-cell Ba^{2+} conductances in bovine chromaffin cells. (*A*) Ba^{2+} currents from two different chromaffin cells dialyzed with either 200 μM GTP-γS or 200 μM GDP-βS. Currents are normalized to the membrane capacitance of each cell (13.1 pF, GTP-γS; 9.8 pF, GDP-βS), and superimposed for comparison. Currents were evoked by 15 msec voltage steps to 0 mV, filtered at 10 kHz (−3 dB) and sampled at 40 kHz. (*B*) Current/voltage relations for cells dialyzed with GTP-γS (filled circles) and GDP-βS (open circles). The peak current amplitude during 15 msec test pulses to varying membrane potentials was normalized to cell membrane capacitance. Values represent the mean ± SEM from nine cells under each condition. (*C*) Comparison of Ba^{2+} currents from a PTX-treated and untreated chromaffin cell. The membrane capacitance of the PTX-treated cell was 15.3 pF, and 18.9 pF for the untreated control cell. (*D*) Current/voltage relations for PTX-treated (open squares) and untreated (filled squares) chromaffin cells. Note that the y-axis range is reduced 50% compared to Fig. 3*B*. Data are the mean ± SEM, n = 8 (control) and n = 9 (PTX treated).

nearly a threefold increase in I_{Ba} density at 0 mV when compared to control cells or cells dialyzed with GTP-γS (GDP-βS, −72.6 ± 5.5 pA/pF, n = 9; GTP-γS, −25.3 ± 1.5 pA/pF, n = 9). The current density at 0 mV in cells dialyzed with GTP-γS was not significantly different from control (GTP, −26.3 ± 2.5 pA/pF, n = 8). In chromaffin cells pretreated with PTX, I_{Ba} density at 0 mV was increased 49% (−39.1 ± 1.5 pA/pF,

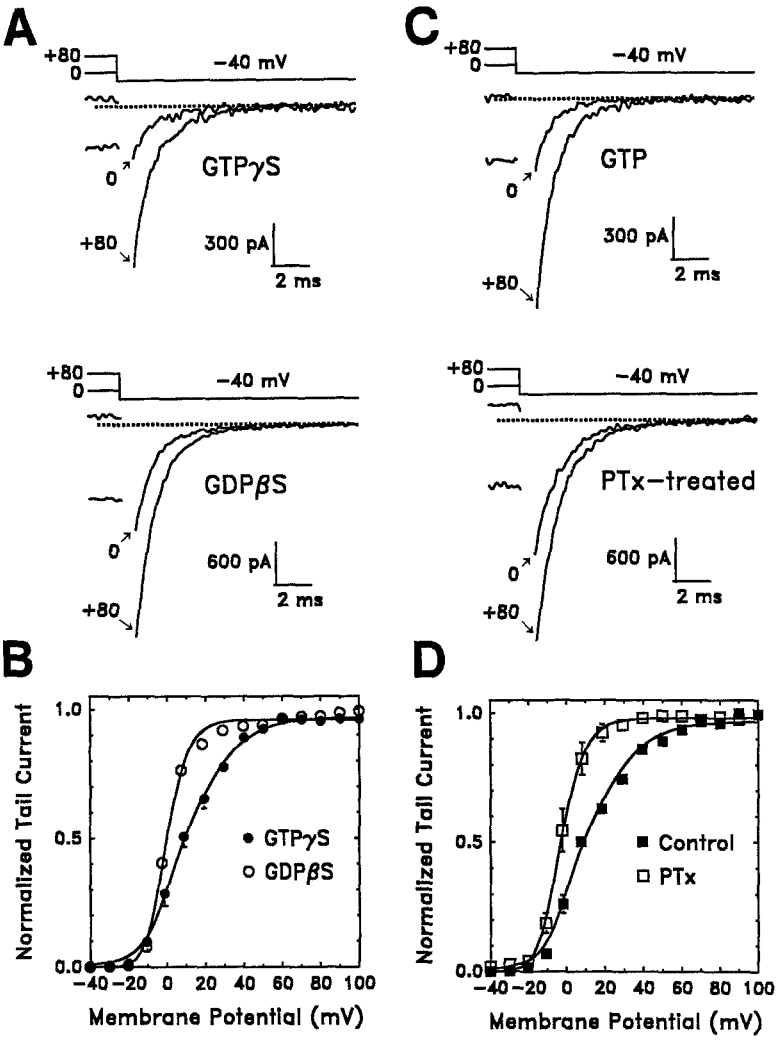

Fig. 4. Modulation of voltage-dependent Ca^{2+} channel activation by PTX-sensitive G-proteins. (*A*) Ba^{2+} tail currents elicited by repolarization to −40 mV following 15 msec depolarizing test pulses to 0 and +80 mV in two different chromaffin cells dialyzed with either GTP-γS (upper traces) or GDP-βS (lower traces). Currents were filtered at 10 kHz (−3 dB) and sampled at 40 kHz. Points 300 μsec after the step to −40 mV were "blanked" to accommodate clamp settling. (*B*) Voltage-dependent I_{Ba} activation curves were constructed from tail current amplitudes measured 300–500 μsec after the end of the depolarizing test pulse. Tail currents were normalized to the maximal tail current amplitude within each cell. Data points are the mean ± SEM from GTP-γS (filled circles) and GDP-βS (open circles) dialyzed chromaffin cells (n = 9 for each condition). Unbroken curves through the data points represent least-squares fits to the meaned values. For GDP-βS containing cells, the fitted curve is a second-order Boltzmann function of the form: $I_{tail}/I_{max} = 0.98/\{1 + \exp[3.8\ e^{-}\ (-7\text{ mV} - V_m)/25.5]\}^2$. For GTP-γS dialyzed cells, the fitted curve represents the sum of two voltage-dependent processes: $I_{tail}/I_{max} = 0.32/\{1 + \exp[3.8\ e^{-}\ (-7\text{ mV} - V_m/25.5]\}^2 + 0.65/\{1 + \exp[1.9\ e^{-}\ (+16\text{ mV} - V_m)/25.5]\}$. (*C*) Representative tail current records from a chromaffin cell under control conditions (upper traces), and from another chromaffin cell pretreated with PTX (500 ng/ml) (lower traces). (*D*) Activation curves as in *B* from normalized tail current amplitudes measured from eight control cells and five cells pretreated with PTX. The unbroken curves fit to the mean values are as follows: control, $I_{tail}/I_{max} = 0.31/\{1 + \exp[3.4\ e^{-}\ (-10\text{ mV} - V_m)/25.5]\}^2 + 0.65/\{1 + \exp[1.9\ e^{-}\ (+16\text{ mV} - V_m)/25.5]\}$; PTX, $I_{tail}/I_{max} = 0.98/\{1 + \exp[3.4\ e^{-}\ (-10\text{ mV} - V_m)/25.5]\}^2$.

n = 9) when compared to untreated cells, yet still considerably suppressed when compared to chromaffin cells dialyzed with GDP-βS. The degree of I_{Ba} inhibition that was relieved by PTX treatment was similar to the suppression relieved by large depolarizing prepulses in untreated cells, 49 *vs.* 45% (from Fig. 2*B*), respectively. Thus, accompanying the voltage-dependent and largely PTX-sensitive I_{Ba} suppression was a significant G-protein inhibitory process unveiled by intracellular GDP-βS that was voltage independent and PTX insensitive.

The voltage dependent relief of I_{Ba} inhibition at positive membrane potentials (i.e., I_{Ba} facilitation) was also reflected in the voltage-dependent I_{Ba} activation relation (Fig. 4). Isochronal I_{Ba} activation curves were constructed from Ba^{2+} tail currents under the different conditions of G-protein activation. Tail currents were elicited by repolarization to −40 mV following 15 msec depolarizing test pulses to varying membrane potentials. The tail currents decayed monoexponentially and had time constants that were not significantly different with intracellular GTP-γS (τ = 436 ± 24 μsec at 0 mV, n = 9) or GDP-βS (τ = 482 ± 23 μsec at 0 mV, n = 9). In chromaffin cells dialyzed with GDP-βS, the voltage-dependent I_{Ba} activation curve was well described by a second-order Boltzmann relation in accord with a Hodgkin and Huxley (1952) m^2 gating process (Ceña, Stutzin & Rojas, 1989; Doupnik & Pun, 1992). The membrane potential for half activation ($V_{1/2}$) was −7 mV, and the equivalent gating charge for each "m-gate" was equal to 3.8 e^{-}. In GTP and GTP-γS dialyzed cells, the activation curves were significantly less steep

and slightly shifted toward more positive membrane potentials. The features of these relations could be described by the combined influence of both the voltage-dependent properties of Ca^{2+} channel activation, and the voltage-dependent properties of G-protein-mediated suppression (as shown in Fig. 1*B*). The activation curves for GTP and GTP-γS dialyzed cells were well described by the sum of both of these processes, where the fractional current due to voltage-dependent suppression was 65% for both GTP and GTP-γS containing cells. Pretreatment with PTX abolished the influence of the voltage-dependent component as expected from the I_{Ba} facilitation results. This description for Ca^{2+} channel gating is analogous to the voltage-dependent gating model proposed by Bean (1989), where the voltage-dependent properties of facilitated I_{Ba} correspond to activation of Ca^{2+} channels in a "reluctant" gating mode. The intrinsic voltage-dependent properties for I_{Ba} activation correspond to Ca^{2+} channels in a "willing" gating mode.

Inactivating I_{Ba} Induced by Depolarizing Prepulses and GDP-βS

In chromaffin cells containing GTP or GTP-γS, I_{Ba} evoked by 500 msec depolarizing step pulses displayed virtually no time-dependent inactivation (Fig. 5). However, when preceded by a large depolarizing prepulse, the facilitated I_{Ba} slowly inactivated as previously reported (Hoshi et al., 1984; Artalejo et al., 1991). This inactivating I_{Ba} component was also observed in cells intracellularly dialyzed with GDP-βS (n = 8) in the absence of a depolarizing prepulse (Fig. 5*B*). The slow I_{Ba} inactivation was also observed in PTX-treated cells, but to an extent less than that observed with GDP-βS (*not shown*).

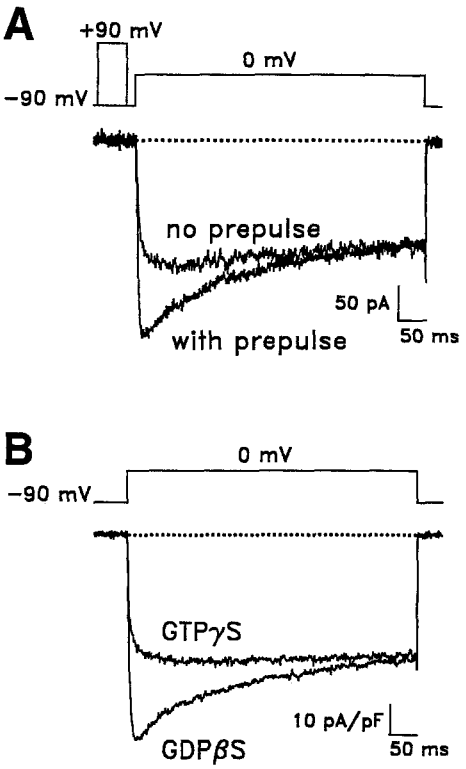

Fig. 5. I_{Ba} induced by depolarizing prepulses and intracellular GDP-βS similarly inactivate. (*A*) Ba^{2+} current facilitated by a large depolarizing prepulse slowly inactivates during prolonged test pulses. Currents were elicited by 500 msec voltage steps, with or without a 50 msec prepulse to +90 mV. The test pulse was evoked 15 msec after the prepulse step. Currents were filtered at 500 Hz and sampled at 1,667 Hz. (*B*) Ba^{2+} current elicited during prolonged voltage steps in a chromaffin cell dialyzed with 200 μM GTP-γS, and in a different cell dialyzed with 200 μM GDP-βS. The currents from the two different cells were normalized to each individual cell membrane capacitance (GTP-γS, C_m = 11.7 pF; GDP-βS, C_m = 15.1 pF), and superimposed for comparison. Currents were evoked by 500 msec step pulses to 0 mV from a holding potential of −90 mV.

Voltage-dependent I_{Ba} Suppression by Chromaffin Cell Secretory Products

Since voltage-dependent suppression of neuronal HVA Ca^{2+} channels is induced by agonist activation of G proteins via G-protein-coupled membrane receptors (Marchetti et al., 1986; Bean, 1989), the potential autocrine/paracrine influence of chromaffin cell secretory products was examined. Among the products secreted from chromaffin cells are catecholamines, adenosine nucleotides, and opioids, which are known inhibitors of HVA Ca^{2+} channels in neurons. In the previous experiments, chromaffin cells were recorded in a static flow environment where cells were first washed three times with the extracellular solution then voltage clamped within approximately 10 min. To test whether secretory products released during this period were causing I_{Ba} suppression, cells were locally superfused with control bath solution applied via a large bore (25–50 μm) pipette positioned 10–20 μm from the cell. The flow of solution from the pipette was gravity driven and readily verified under the microscope. Figure 6 shows the time course for the effects of superfusing normal bath solution, where there was a very rapid 60% increase in I_{Ba} amplitude upon cell superfusion. The enhancement of I_{Ba} was quickly reversed when the superfusion pipette was moved away from the cell. The I_{Ba} increase was not attributable to a response to solution flow, since reversing the flow with negative pressure (i.e., sucking bath solution in the dish across the cell) did not effect I_{Ba} amplitudes. In an additional test, bath solution incubated in a separate dish of cells for 10 min was applied via a second superfusion pipette to examine the effects of products secreted during the experimental period. "Conditioned" bath solution sup-

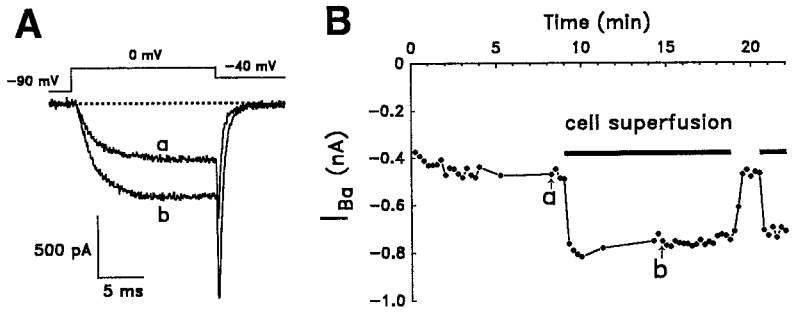

Fig. 6. Local superfusion rapidly enhances I_{Ba} in bovine chromaffin cells. (*A*) Superimposed I_{Ba} recordings before (*a*) and during (*b*) superfusion of a chromaffin cell with normal extracellular bath solution. Ba^{2+} currents were evoked by the voltage protocol shown above the current traces. (*B*) Time course for effects of cell superfusion. Currents were evoked every 15 sec, and the peak I_{Ba} amplitude was plotted *vs.* time after establishing whole-cell recording. The horizontal bars indicate periods when the cell was superfused with standard bath solution via a large bore pipette ~10 μm from the cell.

pressed I_{Ba} to an extent similar to that observed without cell superfusion (*data not shown*).

Associated with the I_{Ba} increase by local cellular superfusion was the abolishment of tonic I_{Ba} facilitation by large depolarizing prepulses. Figure 7 shows the effects of cell superfusion on currents evoked using the double-pulse protocol. Prior to cell superfusion, Ba^{2+} currents were tonically facilitated by large depolarizing prepulses. During cell superfusion, depolarizing prepulses to various membrane potentials produced little change in the currents evoked by subsequent test pulses to 0 mV. The maximal amplitude of tonic facilitation was equivalent to the level of current enhancement produced by cell superfusion. These findings indicate that paracrine and/or autocrine secretory products suppress HVA Ca^{2+} channels in chromaffin cells, and can be greatly relieved by large predepolarizations.

Discussion

G-Protein Activation Mediates Tonic Prepulse Facilitation

The results from our studies demonstrate that large depolarization-induced facilitation of HVA Ca^{2+} channel currents in bovine chromaffin cells is mediated in a manner analogous to that observed in neuronal cells. That is, large depolarizations relieve voltage-dependent suppression of HVA Ca^{2+} channels induced by agonist activation of G-protein-coupled membrane receptors (Anwyl, 1991). Agonist activation of dopamine D_2 receptors (Bigornia et al., 1990; Sontag et al., 1990), $GABA_B$ receptors (Doroshenko & Neher, 1991), purinergic P_{2Y} receptors (Diverse-Pierluissi, Dunlap & Westhead, 1991; Doupnik & Pun, 1993), α_2-adrenergic receptors, and opioid receptors (Kleppisch et al., 1992), are all reported to produce inhibitory effects on HVA Ca^{2+} channel currents in bovine chromaffin cells (*but see also* Callewaert, Johnson & Morad, 1991). Since agonists for all of these G-protein-coupled receptors are synthesized and secreted from bovine chromaffin cells, it is to no surprise that bath medium likely to contain these secretory products would exert suppressive effects on HVA Ca^{2+} channel currents. The voltage dependency of Ca^{2+} channel suppression by select receptor activation remains unknown with the exception of purinergic P_{2Y} receptors, which we recently found to suppress chromaffin Ca^{2+} channel currents in a voltage-dependent manner that is indistinguishable from the voltage-dependent suppression reported here (Doupnik & Pun, 1993). Our findings underscore the necessity for effective control of the local extracellular environment when recording from cells with high secretory capacities.

Voltage-dependent Inhibition of Chromaffin Cell Ca^{2+} Channels

Tonic voltage-dependent suppression of HVA Ca^{2+} channels in bovine chromaffin cells was relieved by large depolarizing prepulses in a manner well described by a single Boltzmann relationship. In the kinetic scheme we adopted from Bean (1989), this Boltzmann relation defines the voltage-dependent activation properties of Ca^{2+} channels in a G-protein-suppressed gating mode, and is analogous to the "reluctant" gating mode ascribed to neurotransmitter-suppressed HVA Ca^{2+} channels in bullfrog dorsal root ganglion (DRG) neurons (Bean, 1989; Elmslie et al., 1990). The activation of reluctant Ca^{2+} channels was assumed to be first order (Artalejo et al., 1992*b*), where the equivalent gating charge for activation of "reluctant" chromaffin cell Ca^{2+} channels (z_r = 1.9 e^-) was similar to the z_r values (1.5–2.0 e^-) reported in different peripheral neurons (Bean, 1989; Ikeda, 1991; Beech, Bernheim & Hille, 1992). However, the membrane potential for half-activation of the facilitated I_{Ba} was considerably more negative in chromaffin cells (+16 mV) compared to neurons under similar recording conditions (range +38 to +62 mV). Using the prepulse paradigm described previously, the voltage-dependent activation properties of the facilitated current should be at steady-state; however, we did not characterize the voltage dependency using varying prepulse durations. This caveat

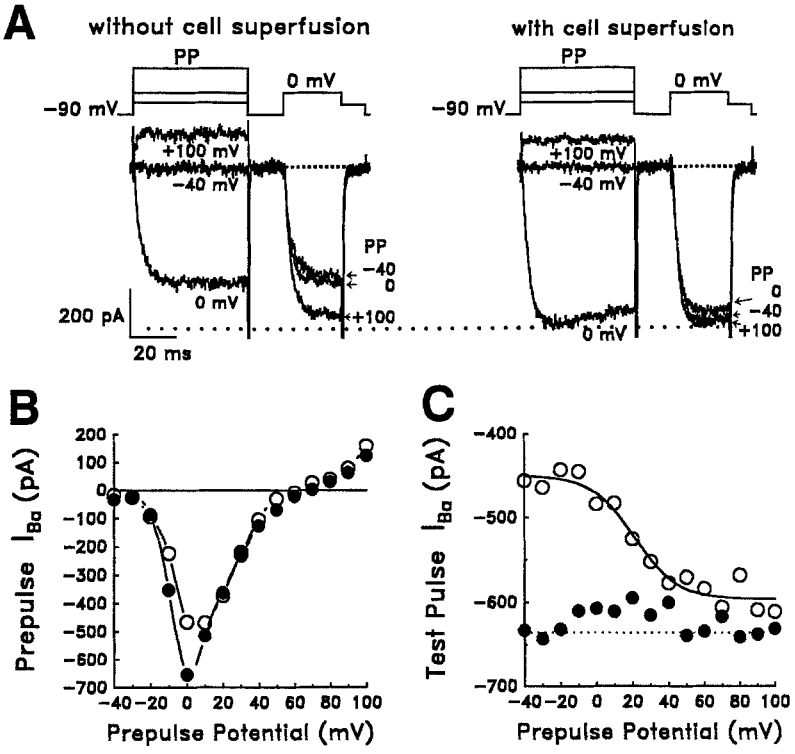

Fig. 7. Tonic prepulse facilitation of Ca^{2+} channels is abolished during local cell superfusion. (*A*) Selected current traces evoked by double pulse voltage steps before (left traces) and during (right traces) local cell superfusion with extracellular bath solution. (*B*) Current/voltage relations derived from the currents evoked by depolarizing prepulse voltage steps, before (open circle) and during (filled circle) cell superfusion. (*C*) Current amplitudes evoked by test pulses to 0 mV when preceded by prepulses to varying membrane potentials. Open circles are before, and filled circles are during, local cell superfusion with standard bath solution. The horizontal dotted line indicates the current amplitude at 0 mV without a depolarizing prepulse. The unbroken curve is a least-squares fit of the open circles and is defined by: $I_{Ba} - (I_{max} - I_{min})/\{1 + \exp[2.0\, e^{-}\, (+20\text{ mV} - V_m)/25.5]\}$.

aside, the difference in the voltage dependency for activation of G-protein-suppressed channels may reflect variations in the types of HVA Ca^{2+} channels being suppressed.

In rat superior cervical ganglion neurons, I_{Ba} facilitation is abolished by ω-conotoxin GVIA (ω-CgTx), indicating that large depolarizations primarily relieve G-protein-suppressed N-type Ca^{2+} channels (Ikeda, 1991). In bovine chromaffin cells, tonic I_{Ba} facilitation is abolished by dihydropyridines, thus indicating the involvement of an L-type Ca^{2+} channel (Artalejo et al., 1991). Bovine chromaffin cells, however, do possess a large ω-CgTx-sensitive current (Hans, Illes & Takeda, 1990; Artalejo et al., 1992*a*). Kleppisch and coworkers (1992) found that α_2-adrenergic inhibition of chromaffin Ca^{2+} channels involved a current component that could be blocked by either dihydropyridines or ω-CgTx. Their observations are consistent with the observations of Bossu and colleagues (1991*a,b*) who showed that dihydropyridine-sensitive Ca^{2+} channels in chromaffin cells are also sensitive to ω-CgTx. Thus, the voltage-dependent G-protein-suppressed Ca^{2+} current in bovine chromaffin cells is expected to have a mixed L/N-type pharmacology, and is currently being investigated. An alternative explanation for the different voltage dependencies for activation of suppressed HVA Ca^{2+} channels in chromaffin cells *vs.* neurons may be due to differences in the PTX-sensitive G-protein species underlying Ca^{2+} channel inhibition (Taussig et al., 1992).

Voltage-Independent Suppression of HVA Ca^{2+} Channels

Inhibition of Ca^{2+} channel currents elicited by multiple signal transduction pathways can be distinguished by their differing time courses of action and voltage dependencies (Beech et al., 1992). In bovine chromaffin cells, intracellular dialysis with GTP or GTP-γS elicited a substantial voltage-independent and PTX-insensitive inhibition that was revealed by intracellular dialysis with GDP-βS. The underlying mechanism for this inhibition, which was 3–4 times greater than the voltage-dependent inhibition, is not known. Since chromaffin cells were dialyzed with an intracellular solution of high Ca^{2+} buffering capacity and low free Ca^{2+} concentration (10–20 nM), the voltage-independent suppression is not likely to be Ca^{2+} mediated. However, the effects of Ca^{2+}-dependent processes activated by cell secretory products prior to establishing whole-cell dialysis cannot be excluded. Yet since cell superfusion enhanced currents in a voltage-dependent manner and to a level consistent with the PTX-sensitive component, the voltage-independent suppression appears to be agonist independent. Protein kinase C (PKC) activation by phorbol ester did not significantly reduce I_{Ba} in GDP-βS dialyzed cells (*data not shown*), indicating that a PKC-mediated inhibitory process is not likely to be causing the voltage-independent inhibition. Other possibilities to consider include the interaction of small

molecular weight G proteins directly or indirectly with chromaffin cell Ca^{2+} channels (Sudhof & Jahn, 1991).

Reinterpretation of cAMP-dependent Phosphorylation Mechanisms

Our observations that PTX treatment increased I_{Ba} and significantly reduced I_{Ba} facilitation could be initially interpreted in the context of a direct cAMP-dependent phosphorylation mechanism as proposed by Artalejo and colleagues (1990). In accord with this hypothesis, PTX-mediated ADP-ribosylation of $G_{i\alpha}$ subunits would allow uninhibited adenylate cyclase activity causing elevated cAMP production and enhanced protein kinase A (PKA) activation, and finally the phosphorylation and activation of a distinct facilitation Ca^{2+} channel. The elimination of I_{Ba} facilitation and dramatic increase in current density by GDP-βS, however, is inconsistent with the phosphorylation hypothesis. With "global" inactivation of G proteins by GDP-βS, dephosphorylation would be favored due to the loss of basal G-protein-activated kinase activity (e.g., PKA and PKC) and continued phosphatase activity independent of G-protein activation. Moreover, GTP-γS did not activate the facilitated Ca^{2+} channel as is expected by the phosphorylation hypothesis.

The observations of PKA-activated Ca^{2+} channels, which are also facilitated by large depolarizations, can be reinterpreted such that cAMP-dependent phosphorylation interferes at some level with direct G-protein-mediated Ca^{2+} channel suppression. A phosphorylation-induced decoupling could occur at multiple levels, for example, altered G protein/Ca^{2+} channel interaction through the phosphorylation of a channel protein, G protein, or both. Support for such a mechanism has recently been suggested for HVA Ca^{2+} channel currents in cultured rat DRG neurons (Dolphin, 1992). We previously found PKA activation to modify the voltage-dependent gating properties of Ca^{2+} channel currents in bovine chromaffin cells in a manner consistent with this interpretation (Doupnik & Pun, 1992). PKA activation shifted the Ca^{2+} channel activation curve ~6 mV towards more negative membrane potentials and increased the limiting valence of the gating charge ~1.0 e^-. Ceña and colleagues (1991) observed a similar shift in the $V_{1/2}$ value of Ca^{2+} current activation after PTX treatment (~8 mV more negative); however, they did not observe a change in the gating charge valence or an increase in membrane conductance. Our present findings with PTX treatment and intracellular GDP-βS are consistent with the shift in the activation curve and share the characteristics we previously observed with 8-bromo cAMP (i.e., also an increased slope). These features also can be explained by the presence of two channel gating modes, the second being identified from the voltage-dependent activation properties of facilitated I_{Ba}. It should be noted that in our previous study, 8-bromo cAMP was applied via local application. Thus due to our findings on the suppressive effects of chromaffin cell secretory products, we must consider the potential inhibitory effects of cell secretory products. Although sham drug application had no effect on I_{Ba} when GTP was absent from the intracellular pipette solution, a more rigorous examination of the effects of PKA activation under defined states of G-protein activation is warranted.

In conclusion, the mechanism of Ca^{2+} channel facilitation by large depolarizing prepulses in bovine chromaffin cells is consistent with the voltage-dependent G-protein block hypothesis proposed in neuronal cell types. Tonic G-protein activation via autoreceptor stimulation by cell secretory products results in tonically suppressed Ca^{2+} channels that can be activated by large depolarizations. The G-protein-coupled autoreceptors underlying tonic Ca^{2+} channel facilitation remain to be determined, as well as the signaling mechanisms underlying the large G-protein-mediated voltage-independent suppression of chromaffin cell Ca^{2+} channels.

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