Facilitation of N-Type Calcium Current Is Dependent on the Frequency of Action Potential-Like Depolarizations in Dissociated Cholinergic Basal Forebrain Neurons of the Guinea Pig

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Voltage-dependent inhibition of high voltage-activated (HVA) calcium currents by G-proteins can be transiently relieved (facilitated) by strong depolarizing prepulses. However, with respect to the physiological significance of facilitation, it remains to be established if it can be induced by action potentials (AP) in central neurons. With the use of whole-cell recordings of dissociated cholinergic basal forebrain neurons of the guinea pig, it is shown that the GTPγS-inhibited HVA currents that occur through *N*-ethylmaleimide (NEM)-sensitive G_i–G_o subtypes of G-proteins can be facilitated. Furthermore, although different types of HVA channels are present in these neurons, facilitation occurred mostly through disinhibition of the N-type current. On the basis of data indicating that the recovery from facilitation was relatively slow, we tested if more physiological stimuli that crudely mimicked APs (2 msec long depolarizations to 40 mV from a holding of −50 mV) potentially could induce facilitation of HVA currents inhibited by GTPγS and cholinergic agonists. Indeed, evidence is provided that the extent of facilitation is dependent on both the number and frequency of AP-like depolarizations. These results suggest that firing rates and patterns of discharge of neurons could influence their responsiveness to transmitters acting on N-type HVA calcium channels.

Key words: facilitation; N-type channels; G-protein; action potentials; modulation; mode of discharge

It is now well established that calcium currents can be modulated by a variety of neurotransmitters (Anwyl, 1991; Hille, 1994). Most of these effects are reversible reductions of calcium currents, which may be mediated partly by fast and direct effects of G-proteins on the calcium channel itself (membrane-delimited effects) or indirectly via second messengers (Hille, 1994). Inhibition of calcium currents directly by G-proteins can occur by voltage-dependent and -independent mechanisms (Bean, 1989; Elmslie et al., 1990; Lubke and Dunlap, 1994; Diversé-Pierluissi et al., 1995; Dolphin, 1996). One particularly interesting facet of voltage-dependent reduction of calcium currents is the capacity to display transient disinhibition (also called facilitation), which is produced by strong depolarizing prepulses (Marchetti et al., 1986; Bean, 1989; Ikeda, 1991; Kasai, 1991; Lopez and Brown, 1991).

However, it remains unclear if facilitation evoked after G-protein activation can occur with action potentials. In a study using peripheral neurons (dorsal root ganglion neurons; Womack and McCleskey, 1995), only one-third of the tested cells displayed a weak (up to 20%) facilitation after depolarizations similar to action potentials (AP-like depolarizations). Moreover, in central neurons (raphe neurons; Penington et al., 1991), facilitation could not be elicited using AP-like depolarizations, whereas large depolarizations could restore calcium current inhibited by serotonin. Because it could bear on the physiological significance of facilitation in central neurons, the

aim of the following study was thus to reinvestigate that question using cholinergic basal forebrain (BF) neurons as a model (see Materials and Methods).

These cells, which provide the major cholinergic input to the neocortex (Rye et al., 1984), can discharge either tonically at low frequency (up to a maximum of 15 Hz) or in a high frequency bursting mode, with up to 250 Hz within a burst (Khateb et al., 1992, 1995; Alonso et al., 1994; Griffith et al., 1994). These properties can be ascribed in part to the different types of calcium currents with which they are endowed (Allen et al., 1993; Griffith et al., 1994; S. Williams, M. Serafin, M. Mühlethaler, L. Bernheim, unpublished data). In particular, cholinergic neurons display an important low voltage-activated (LVA) current that underlies the low-threshold spike (LTS), which permits them to fire on hyperpolarization with bursts of action potentials (Khateb et al., 1992; Allen et al., 1993; Griffith et al., 1994; S. Williams, M. Serafin, M. Mühlethaler, L. Bernheim, unpublished data). In addition, these neurons possess at least five subtypes of high voltage-activated (HVA) calcium currents based on the particular pharmacological sensitivity to the L-type blocker nifedipine, the N-type blocker cono-GVIA (Allen et al., 1993), and the P/Q-type blocker AGA-IVA (S. Williams, M. Serafin, M. Mühlethaler, L. Bernheim, unpublished data). It has not yet been determined if facilitation occurs in these cells.

The following study thus was designed to test the hypothesis that action potential-like depolarizations could trigger facilitation in BF cholinergic neurons when applied in a frequency domain corresponding to that which is known to occur for these cells. For that purpose it was necessary to determine first, the presence of G-protein-induced facilitation of calcium currents inhibited by GTPγS; second, the nature of the currents implicated in the facilitation process; third, the kinetics of the facilitation; and,

finally, the ability of "physiological stimuli" such as AP-like depolarizations to promote facilitation.

MATERIALS AND METHODS

Dissociation. Slices from young guinea pigs (80–200 gm) were obtained using standard methods (Khateb et al., 1992, 1993) and dissociated with a slightly modified version of the method developed by Kay and Wong (1986). Briefly, guinea pigs were deeply anesthetized with Nembutal and decapitated. The brain was removed and rapidly transferred in cold (4°C) oxygenated (95% O_2/5% CO_2) physiological saline containing (in mM): 130 NaCl, 20 NaHCO$_3$, 1.25 KH$_2$PO$_4$, 1.3 MgSO$_4$, 5 KCl, 10 glucose, and 2.4 CaCl$_2$, pH 7.35. Using a vibrotome (Campden Instruments, WPI, Berlin, Germany), two to three slices (400 μm thick) containing the basal forebrain were cut and left at room temperature for a period of 1 hr in physiological saline. The region of interest, which included the substantia innominata, the horizontal limb of the diagonal band, and the magnocellular preoptic nucleus [Paxinos and Watson (1986); see Gritti et al. (1993) for a discussion on basal forebrain cholinergic nuclei], then was dissected out (1 piece from each hemisphere) with a small razor blade. The regions of the medial septum and the vertical limb of the diagonal band that make up most of the septo-hippocampal projecting neurons were excluded. In this study, the term basal forebrain cholinergic neurons will be used to characterize those cells located within the above mentioned nuclei. For dissociations, slices first were immersed in a gassed 100% oxygenated PIPES solution containing (in mM): 120 NaCl, 5 KCl, 0.5 CaCl$_2$, 1 MgCl$_2$, 25 glucose, and 20 PIPES (pH-adjusted to 7.0 with NaOH). The tissue was placed in small test tubes containing an oxygenated PIPES solution with the enzyme trypsin (Sigma type XII, 0.8 mg/ml; St. Louis, MO) 2 hr at 30°C. The enzymatic reaction was stopped by rinsing the tissue with 10% goat or horse serum (Life Technologies, Basel, Switzerland) and left to rest in enzyme-free PIPES for at least another hour. Neurons were dispersed by triturating in HEPES (see below) with the help of two different sized fire-polished Pasteur pipettes. The suspension was transferred to a Cell-Tek-coated Petri dish, which was mounted on an inverted microscope (Zeiss, Oberkochen, Germany). Healthy neurons adhered to the bottom of the dish within 15 min.

Whole-cell recordings and solutions. Voltage-clamp recordings from neurons were obtained using the whole-cell version of the patch-clamp technique (Hamill et al., 1981). Patch pipettes were pulled (Sutter Instruments, Novato, CA) from borosilicate glass (1.5 o.d.; 0.86 i.d.; Clark Instruments, Pangbourne, UK) and had resistances typically of 2–4 MΩ in the bath. Signals were recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) and monitored on a 486 PC clone equipped with pClamp software (v. 6.0) and a 125 kHz interface (Digidata 1200, Axon Instruments). Records were low-pass-filtered at 2 or 5 kHz. Typical access resistances ranged from 4–8 MΩ, and compensation of 70–80% was used. Interferences from linear leak current, and capacitive transients were subtracted by using a P/4 protocol or by subtracting raw traces from those in the presence of cadmium (100–200 μM). The internal recording solution contained the following (in mM): 130 Cs acetate, 20 CsCl, 5 MgCl$_2$, 5 HEPES, 3 Na$_2$ATP, 0.2 GTPγS, 0.1 BAPTA, and 14 phosphocreatine (pH-adjusted to 7.3 with CsOH). In some experiments, GTPγS was replaced by 0.2 mM GTP-Na or 1 mM GDPβS. Neurons were perfused continuously (3 ml/min) with a HEPES medium containing (in mM): 150 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 8 glucose, and 10 HEPES (pH-adjusted to 7.3 with NaOH). To record barium currents, calcium was replaced with an equimolar concentration of barium in the HEPES solution. Calcium or barium currents were isolated by adding TTX (1 μM, Latoxan, Rosans, France), TEA (20 mM), and 4-AP (4 mM) to the HEPES medium. The effects of GTPγS could be observed when calcium or barium was used as the charge carrier. The calcium channel blocker conotoxin-GVIA (Alomone Labs, Jerusalem, Israel), muscarine, and carbachol (Sigma) were prepared in single-use aliquots and thawed on the day of the experiments. These agents were applied with a more rapid application system, which consisted of a multibarrel constructed with polyethylene tubings according to the method of Bertrand et al. (1997). All experiments were performed at room temperature (20–22°C). Measurements of the current amplitude were obtained 7–8 msec after the onset of the voltage step. Currents were activated once every 6 or 7 sec, and access resistance was checked periodically. For analysis of current kinetic slow-down, exponential fits were performed on the first 5 msec (excluding the artefactual first few 100 μsec) for the faster phase of activation and until the current reached a more steady-state level of activation for the slower phase. Statistical analysis was performed using a Student's *t* test with $p \leq 0.05$ chosen as statistically significant. All means are expressed as mean $\pm$ SE.

RESULTS

Facilitation of N-type calcium current

There is now strong evidence that G-proteins can block calcium currents by a direct membrane-delimited pathway (Bean, 1989; Elmslie et al., 1990; Bernheim et al., 1991, 1992; Beech et al., 1992; Hille, 1994; Herlitze et al., 1996; Ikeda, 1996). In BF cholinergic neurons, cell dialysis with the poorly hydrolyzable GTP analog GTPγS, in contrast to dialysis with GTP, produced a gradual and irreversible decrease in the HVA, but not the LVA (data not shown), calcium current. Figure 1*A,B* shows the reduction of the HVA barium current (evoked by a 40 msec test pulse to 0 mV from a V_h of -90 mV) from -2.6 to -1.3 nA within 3 min after break-in in whole-cell mode. At the end of the reduction, the HVA current was facilitated by 84% when a 100 msec long prepulse to 100 mV was given 15 msec before the test pulse (to 0 mV from V_h -90 mV). For all neurons sampled ($n = 18$), $53 \pm 5\%$ of the barium current remaining in presence of GTPγS was facilitated after applying a prepulse (such as the one described above) 139 ± 19 sec after break-in. In a similar manner, $28 \pm 2\%$ ($n = 29$) of the calcium current was facilitated 146 ± 18 sec after break-in. Dialysis of the neurons with GTPγS also produced a slowing of the activation phase (also called "kinetic slowing") of the barium current, which seemed relieved (data not shown) with the depolarizing prepulse (to 100 mV for 100 msec). In 10 neurons recorded in the presence of GTPγS, the activation onset of the barium current could be fit accurately with two time constants. The faster constant τ_1 was 0.84 ± 0.05 msec, whereas the slower phase had a τ_2 of 55.7 ± 14.2 msec. After the prepulse, τ_1 was significantly decreased to 0.67 ± 0.05 msec, whereas the slower phase disappeared. Facilitation of the current flowing through calcium channels was never observed when GTPγS (200 μM) was replaced with GTP (200 μM, $n = 15$) or GDPβS (1 mM, $n = 5$). These results suggest that facilitation in BF cholinergic neurons is dependent on GTPγS and that a "tonic" G-protein-mediated inhibition of HVA currents such as that seen in other neuronal types (Ikeda, 1991; Kasai, 1991) is not present here.

Another method to visualize the voltage dependence of facilitation is to perform *I–V* values before and after a prepulse (100 msec long to 100 mV). The averaged *I–V* values for seven neurons obtained before and after a prepulse are shown in Figure 1*C*. Currents elicited at negative test pulses (-50 to -30 mV) were generally smaller when preceded by prepulses (which likely is attributable to LVA inactivation during the prepulse), but the facilitation was most important at the peak current (-10 mV) and decreased gradually thereafter for test potentials up to 50 mV. It is also noticeable for these averaged *I–V* values that the peak current in the presence of GTPγS seems to occur at more positive potentials (~ 10 mV more) than the peak current elicited after the prepulse. As is shown in Figure 1*D*, voltage-dependent facilitation peaked at test potentials of -10 mV and 0 mV ($83 \pm 14\%$ and $52 \pm 10\%$ of facilitation, respectively) and was null for very depolarized test pulses of 40 and 50 mV. These data suggest that the inhibition mediated by G-proteins is strongly voltage-dependent, being maximal when the current is elicited at the peak but minimal at depolarized potentials.

Previously it was determined pharmacologically that BF cholinergic neurons are endowed with an LVA as well as several HVA calcium currents, including L (17%), N (35%), P/Q (30%), and R (18%) types (S. Williams, M. Serafin, M. Müh-
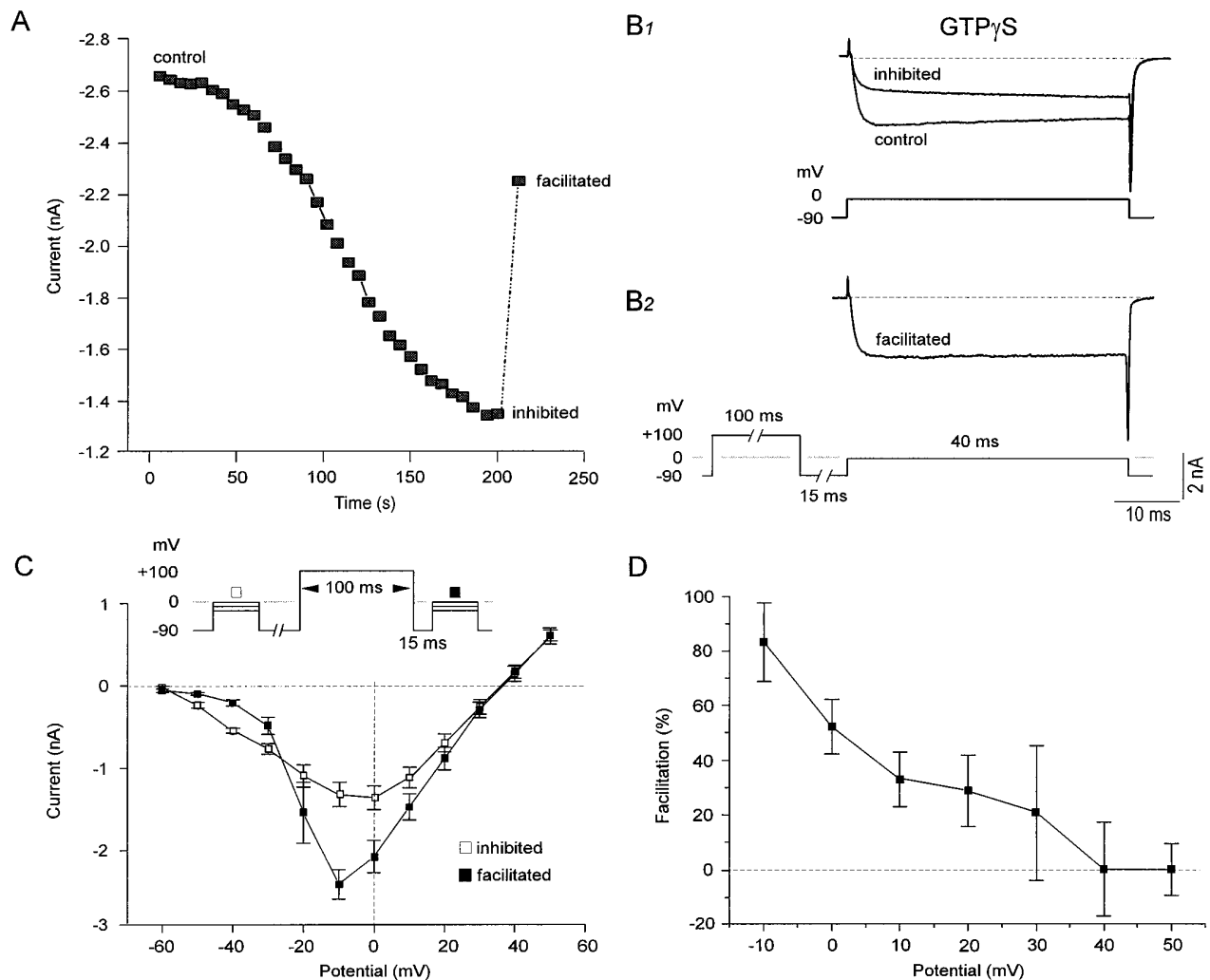
Figure 1. Reduction of inward HVA barium current by GTPγS is voltage-dependent. *A*, Plot of barium current measured at the peak after break-in (*control*), after a maximal reduction (*inhibited*), and during facilitation of the current when it is preceded by a depolarizing prepulse (100 msec long to 100 mV, 15 msec before the test pulse from a V_h of −90 mV). Traces are displayed in *B1* and *B2* to show the extent of inhibition and facilitation. *C*, Averaged *I–V* values for seven neurons obtained before (□) and 15 msec after (■) a 100 msec prepulse to 100 mV. Notice that currents obtained after prepulses of −50 to −30 mV are reduced in size but are increased after those of more depolarized potentials. *D*, Plot of averaged percentage of facilitation expressed as a function of amplitude of the test pulse. Large facilitation was observed at the peak (−10 mV) current but gradually decreased to near null levels at very depolarized potentials of 40 and 50 mV.

lethaler, L. Bernheim, unpublished data). There are indications in the literature that facilitation potentially could occur through disinhibition of most of these calcium current subtypes. For example, it was demonstrated that facilitation can occur through disinhibition of L-type current (Artalejo et al., 1992; Sculptoreanu et al., 1993a,b; Bourinet et al., 1994), N-type current (Elmslie et al., 1990; Boland and Bean, 1993; Swartz, 1993), and also P/Q-type currents (Mintz et al., 1992; Mintz and Bean, 1993; Toselli and Taglietti, 1995; Bourinet et al., 1996). However, as is shown for a representative neuron (Fig. 2*A*), the facilitated current principally flowed through a single type of calcium channel in BF neurons. Indeed, this cell, the current flowing through calcium channels, was facilitated by 88% in control medium, but application of 400 nM cono-GVIA reduced it by 39% and completely abolished the facilitation. In 11 neurons tested, the application of 200–400 nM cono-GVIA reduced the facilitation from 36 ± 7% to 5 ± 2% (Fig. 2*C*). These data suggest that, in BF cholinergic neurons, facilitation

occurred mostly through disinhibition of the N-type calcium current.

Many types of G-proteins are known to be implicated in inhibition of calcium currents; however, not all types have been shown to play a role in facilitation. In fact, pertussis and *N*-ethylmaleimide (NEM)-sensitive G-proteins (G_i and G_o) seem to be of primary importance in mediating the voltage-dependent pathway in both peripheral (Hille, 1994) and central neurons (Foehring, 1996; Yan and Surmeier, 1996). To test if these subtypes of G-proteins also are involved in the facilitation observed in cholinergic basal forebrain neurons, we perfused NEM (50 μM; Shapiro et al., 1994a,b; Foehring, 1996; Yan and Surmeier, 1996) for 2 min before the whole-cell configuration was achieved and GTPγS dialyzed. The amount of facilitation (evoked with a 100 msec long prepulse to 100 mV) after perfusion of NEM was compared with that of control neurons recorded with the same recording solution and in the same Petri dish before NEM perfusion. Facilitation was present in all control neurons recorded
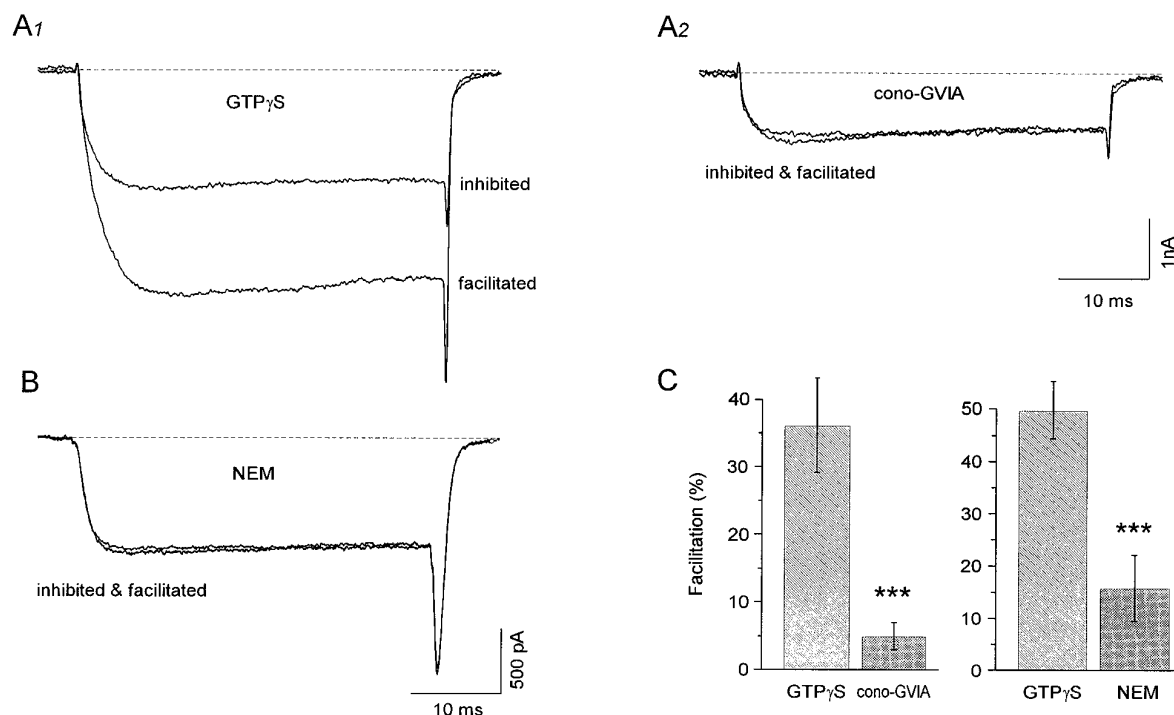
Figure 2. Facilitation of the N-type calcium current through G_i–G_o G-proteins. *A1*, Overlay of current flowing through calcium channels maximally diminished by GTPγS (*inhibited*) and after a prepulse (*facilitated*; 100 msec to 100 mV, from a V_h of −90 mV). *A2*, The inhibited current is reduced further by the application of the N-type inhibitor cono-GVIA (400 nM), and facilitation is abolished. *B*, In another cell, pre-exposure with NEM (50 μM) for 2 min before obtaining whole-cell configuration abolished facilitation (evoked by a 100 msec long prepulse to 80 mV, from a V_h of −50 mV). The mean facilitation obtained after exposure to either cono-GVIA ($n = 11$) or NEM ($n = 8$) is shown in histogram form in *C*. *Asterisks* correspond to a level of significance of $p < 0.01$.

($49.9 \pm 5.5\%$, $n = 15$) but, in contrast, was only $15.9 \pm 6.3\%$ ($n = 8$) when neurons were exposed to NEM (Fig. 2*B,C*).

Kinetics of facilitation

The effects of varying the amplitude or duration of the prepulse, as well as the interval between the pre- and test pulse, were verified on the test current. An example of varying the amplitude of the prepulse from −60 to 100 mV while keeping the values fixed for the duration of the prepulse (100 msec) and duration of interpulse (15 msec) is shown for a neuron in Figure 3*A1*. In the neuron illustrated, the current flowing through the calcium channels was decreased slightly by 6% when compared with control if it was preceded by a prepulse to −60 mV. With further depolarization to 0, 40, and 100 mV, the current was facilitated by 15, 56, and 90%, respectively. Mean results for all neurons sampled ($n = 6$) are shown in Figure 3*A2*. Although some decrease in current amplitude was observed for prepulses between −50 and −15 mV (which may be attributable partly to inactivation of the LVA current), an increase was observed thereafter for prepulses of increasing amplitude potentials up to 100 mV. The most important facilitation ($57.0 \pm 14\%$, $n = 6$) was obtained with prepulses to 100 mV. Increasing the duration of the prepulse also produced an increase in the extent of facilitation (Fig. 3*B*). The onset of facilitation was fast (<10 msec) and increased gradually to a quasi-plateau with prepulse duration of up to 140 msec.

The time for recovery from facilitation was verified by varying the duration of the interval between pre- (100 msec to 100 mV) and test pulse (40 msec to 0 mV). As illustrated in Figure 3*C1* for a representative neuron, facilitation steadily decreased in an exponential manner with larger duration interpulse intervals from

10 to 340 msec (from 84 to 0%). For all neurons recorded (Fig. 3*C2*), facilitation decreased with a τ of 76 msec from $57.6 \pm 8.2\%$ to $3.3 \pm 1.0\%$ for intervals ranging from 10 to 340 msec.

AP-like depolarizations induce facilitation

Because of (1) the characteristics of the facilitation described above and (2) the extensive range of firing frequencies displayed by these neurons *in situ* (up to 250 Hz), we tested whether successive depolarizations more like those occurring during action potentials could elicit facilitation. Hence, series of action potential (AP)-like depolarizations (2 msec long to a test potential of 40 mV from a V_h of −50 mV) were given 15 msec before the test pulse (40 msec to 0 mV from V_h of −50 mV). A test potential of 40 mV was chosen because action potentials in these cells had an amplitude of 113 ± 18 mV (from a resting potential of approximately −60 mV, $n = 57$). Experiments were performed with two distinct facilitation paradigms: first, by increasing the number of AP-like depolarizations (from 2 to 20 events) applied at a frequency of 200 Hz or second, by giving 20 AP-like events at increasing frequencies (from 5 to 200 Hz). Results for a representative neuron are shown in Figure 4. Increasing the number of AP-like events from 2 to 20 increased the facilitation from 12 to 91%, respectively (Fig. 4*A*). In a similar manner (Fig. 4*C*), increasing the frequency also augmented the facilitation from 8 to 84%. For all the neurons sampled (Fig. 4*B,D*), increasing the number of AP-like events from 2 to 20 augmented facilitation from $19 \pm 3\%$ to $66 \pm 8\%$ ($n = 13$), whereas augmenting the frequency from 5 to 200 Hz produced an increase in facilitation from $10 \pm 2\%$ to $61 \pm 4\%$ ($n = 11$). Therefore, when more physiological stimulations crudely mimicking action potentials
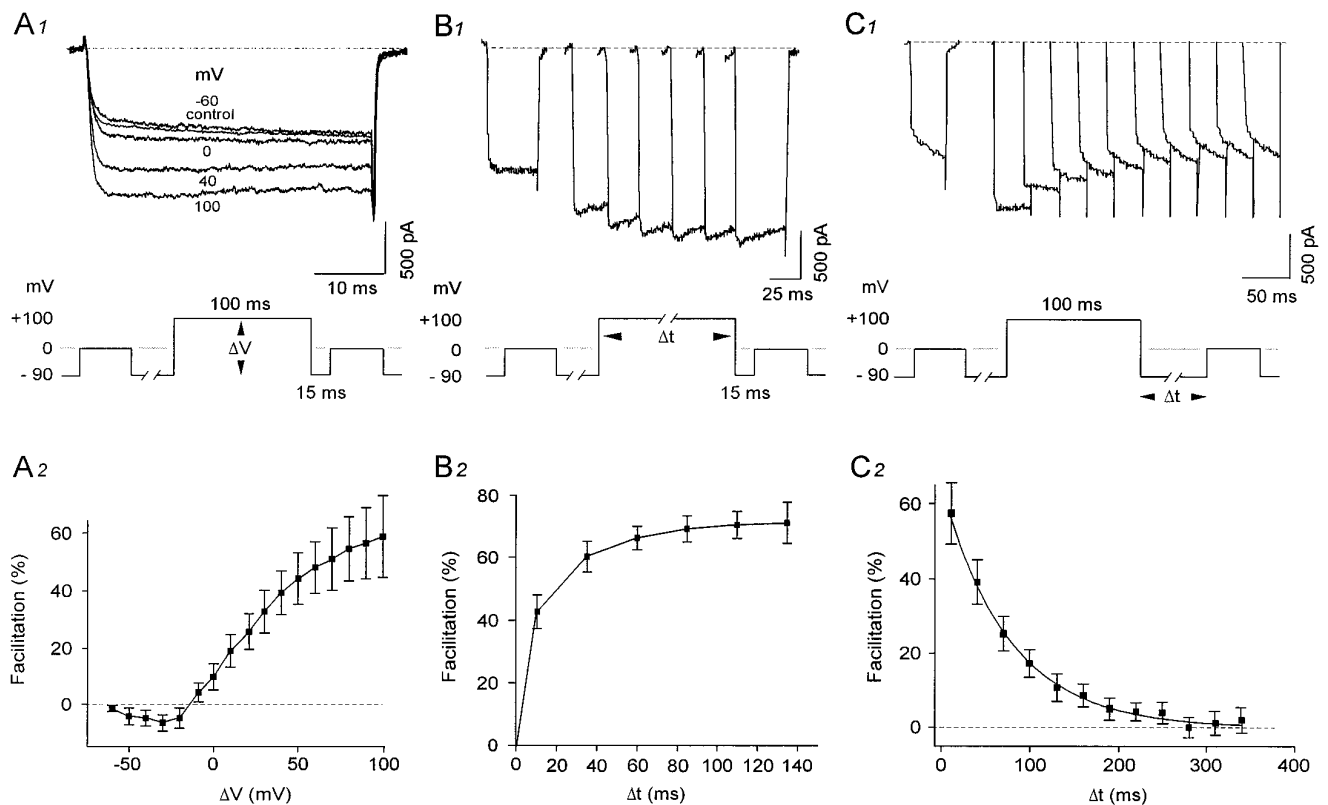
Figure 3. Characteristics of facilitation. *A*, Facilitation is augmented by depolarizing steps of increasing amplitude. *A1*, Example of a barium current elicited 15 msec after a prepulse (100 msec long, from V_h −90 mV) to −60, 0, 40, and 100 mV. Currents were reduced by more negative prepulses such as that at −60 mV but increased steadily in amplitude with respect to more depolarized prepulse steps. *A2*, Plot of averaged facilitation ($n = 6$) as a function of membrane potential showing that the facilitation is augmented with depolarizing prepulses more positive than −10 mV. *B*, Onset of facilitation is fast. *B1*, Current traces in control and after prepulses of increasing duration from 10 to 135 msec (at 100 mV, from a V_h of −90 mV). Currents were facilitated with prepulses as short as 10 msec in duration and increased in size up to a duration of 135 msec ($\Delta t = 10, 35, 60, 85, 110,$ and 135 msec). *B2*, Plot displaying averaged facilitation ($n = 6$) as a function of prepulse duration. Onset of facilitation was fast and augmented until a plateau was reached near 135 msec. *C*, Recovery of facilitation is relatively slow. *C1*, Series of barium current traces in control and after interpulse intervals ranging from 10 to 280 msec ($\Delta t = 10, 40, 70, 100, 130, 160, 190, 220, 250,$ and 280 msec). Currents decreased in size as a function of augmenting time interval durations. *C2*, Graph displays a plot of averaged facilitation in six neurons as a function of duration of the interpulse interval. Facilitation decreased exponentially from 58 to 3% with a τ of 76 msec.

were used, the inhibition of the current could be partially reversed even by as few as two AP-like events.

To verify further the physiological significance of facilitation, we tested whether the inhibition of the current by cholinergic agonists (20 μM carbachol or 10 μM muscarine; Allen and Brown, 1993) could be relieved by using AP-like depolarizations (20 AP-like events at 200 Hz). In the example shown in Figure 5*A*, the application of 10 μM muscarine caused a 70% reduction of the current. After the AP-like train, this inhibition was only 22%. In four neurons tested, the application of carbachol or muscarine reduced the current flowing through calcium channels by 54 ± 7%; the reduction was only 20 ± 4% after AP-like trains (Fig. 5*B*). When the facilitation elicited in the presence of cholinergic agonists was quantified in a similar manner to that obtained with the GTPγS (facilitated current/inhibited current), the facilitation was 83 ± 27%.

DISCUSSION

Three important findings of this study are that, in basal forebrain cholinergic neurons, (1) more than one-half of the G-protein-inhibited HVA calcium current (in presence of GTPγS or a cholinergic agonist) can be disinhibited (facilitated) by action

potential-like prepulses, (2) the facilitation is induced mainly through disinhibition of the N-type current (whereas L- and P/Q-type currents were not significantly involved), and (3) G_i–G_o subtypes of G-proteins are implicated in this coupling.

Cell identification

The cholinergic nature of the vast majority of cells used in this study was estimated on the basis of a parallel study (S. Williams, M. Serafin, M. Mühlethaler, L. Bernheim, unpublished data) in which it was shown that 80% of BF-dissociated neurons with a soma diameter greater than 25 μm were ChAT-positive and thus cholinergic, whereas the remaining 20% were noncholinergic and thus likely GABAergic (Freund and Meskenaite, 1992; Gritti et al., 1993). Furthermore, when these large dissociated neurons were selected for recordings in current-clamp mode, they were shown to have the capacity to fire in low-threshold bursts that were similar in every respect to those of identified ChAT-positive BF neurons in basal forebrain slices (Khateb et al., 1992). Therefore, in this area, selection of neurons based on size offers a reasonably accurate method of choosing their phenotypic identity (cholinergic vs noncholinergic). It must be stressed that the char-
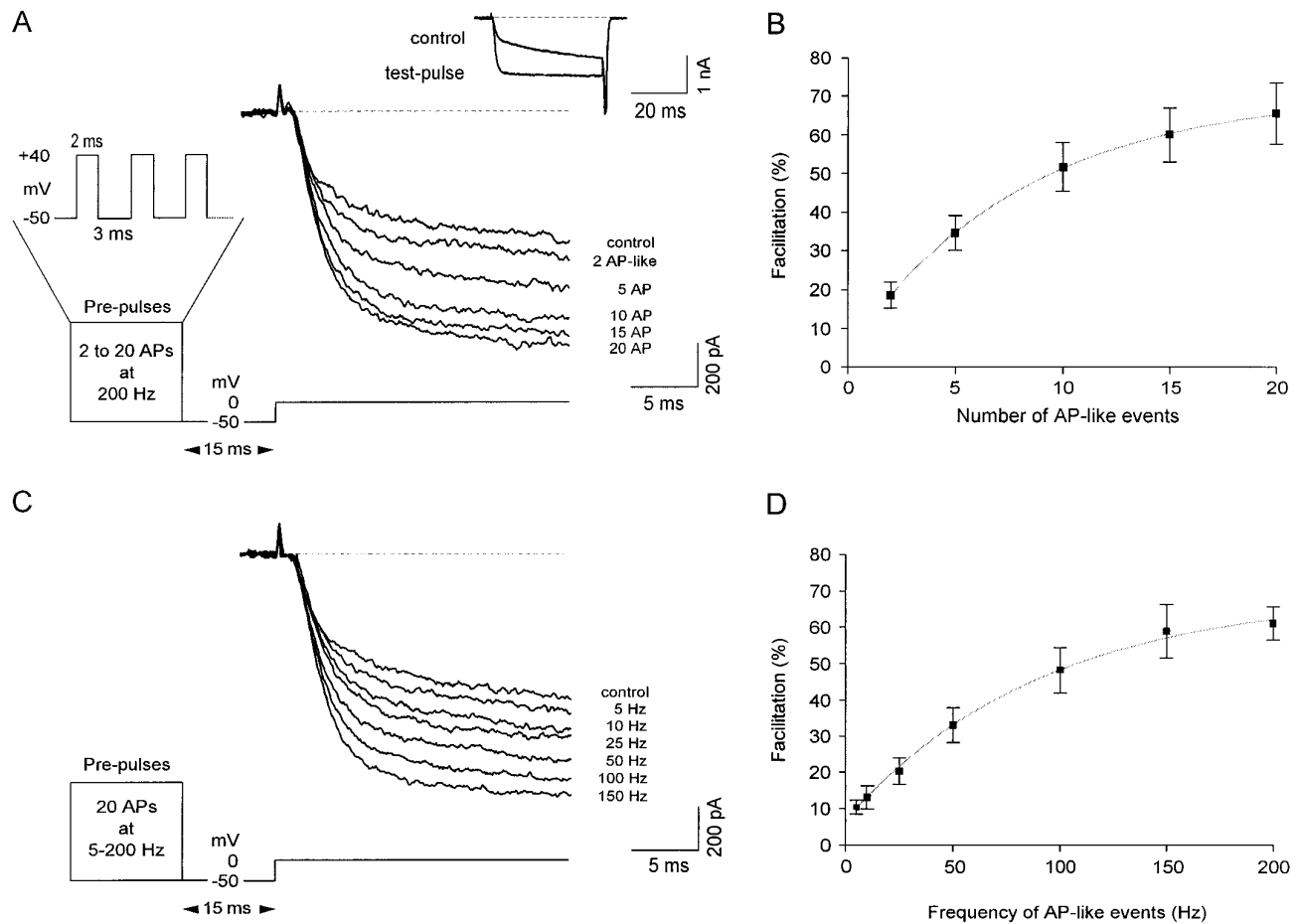
Figure 4. Action potential-like depolarizations can induce facilitation. *A*, Example of barium current traces evoked by a test pulse that was preceded by a series of 2 msec long depolarizing pulses to 40 mV from a V_h of −50 mV. Increasing the number of AP-like depolarizations from 2 to 20 at a frequency of 200 Hz increased facilitation. The *inset* shows that, for the cell in *A* and *C*, applying a prepulse (100 msec duration to 40 mV from a V_h of −50 mV) before the test pulse facilitated the current by 112%. *B*, Graph displays a mean averaged increase in facilitation ($n = 11$) as a function of augmented number of AP-like events. *C*, For the same neuron shown in *A*, facilitation also can be augmented when the frequency of AP-like depolarizations is increased. *D*, The plot displays a mean average increase in facilitation ($n = 11$) when frequency of AP-like events is augmented.

acteristics of facilitation were relatively similar for all neurons recorded. Hence, facilitation that could have been recorded in a small number of large-sized noncholinergic neurons must be similar to that observed in cholinergic neurons.

Mechanisms of facilitation

It is now well established that membrane-delimited G-proteins mediate inhibition of calcium currents in a variety of preparations (Hille, 1994). For BF cholinergic neurons, one study has shown a muscarinic m_2 receptor-mediated reduction of HVA calcium currents via G-protein activation (Allen and Brown, 1993). However, the voltage dependency of this inhibition was not assessed. Here we present evidence that G-protein activation reduces HVA, but not LVA, currents of BF cholinergic neurons. Furthermore, it is shown that only a portion of this inhibition can be reversed or facilitated with depolarizing prepulses, suggesting that the inhibition can be both dependent and independent of voltage. Short pre-exposures of NEM, a sulfhydryl-alkylating agent known to block pertussis-sensitive G_i–G_o (Shapiro et al., 1994a,b; Foehring, 1996; Yan and Surmeier, 1996), significantly reduced facilitation. This suggests that, as in peripheral neurons (Bean, 1989; Elmslie et al., 1990; Beech et al., 1992; Hille, 1994), G_i–G_o subtypes of G-proteins also may play an important role in voltage-dependent
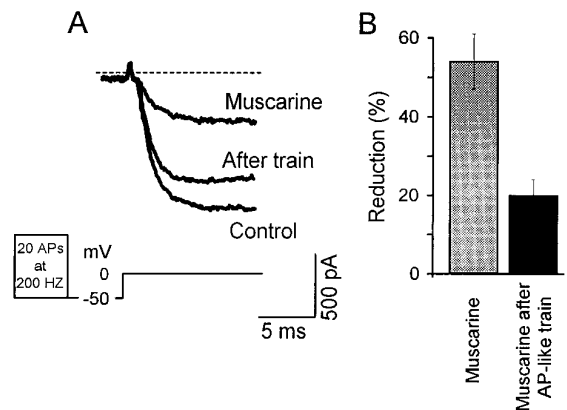

Figure 5. Action potential-like depolarizations induce facilitation of current inhibited by cholinergic agonists. *A*, Example of barium current traces evoked by a test pulse in control conditions, its inhibition during the application of 10 μM muscarine, and the facilitation of the current when 20 AP-like events were given at a frequency of 200 Hz. Recordings were performed using GTP instead of GTPγS in the recording pipette. *B*, Summary of results ($n = 4$) obtained using same protocol as in *A*.

inhibition of central neurons. The inhibition of the HVA current as well as the "kinetic slowing" produced by G-proteins may be the result of a shift in the activation threshold to more depolarized potentials (Bean, 1989; Boland and Bean, 1993; Golard and Siegelbaum, 1993).

Disinhibition of N-type current

The current involved in facilitation in the present study seems to be carried principally through N-type cono-GVIA-sensitive channels and not through L, P, and Q types. There are indications that facilitation potentially can occur through different types of HVA calcium channels. For example, the $\alpha 1A$ (corresponding to P/Q-type currents), and $\alpha 1B$ (N-type) calcium channel subunits (Bourinet et al., 1994, 1996; Herlitze et al., 1996) expressed in cell lines or in oocytes, as well as N- and P-type currents *in situ* (Mintz et al., 1992; Mintz and Bean, 1993; Toselli and Taglietti, 1995), display agonist- or GTPγS-induced inhibition and facilitation. However, in a similar manner to what is observed in BF cholinergic neurons, facilitation in cortical neurons also occurs principally through disinhibition of the N current (Swartz, 1993), although other types of calcium currents (T, L, P, and R) are known to be present (Lorenzon and Foehring, 1995; Markram et al., 1995).

Facilitation by action potential-like depolarizations

Kinetics of facilitation onset was relatively rapid (<10 msec) in BF cholinergic neurons and comparable with other neuronal cell types either with cell dialysis with GTPγS or application of a cholinergic agonist. In contrast, recovery from inhibition was slower ($\tau = 76$ msec) than that obtained for G-protein-dependent facilitation found in peripheral neurons (30–60 msec; Elmslie et al., 1990; Golard and Siegelbaum, 1993). This slower recovery from inhibition is important, because this criterion partly will determine the "summation" of facilitation that is shown to occur during AP-like stimulations. In fact, calcium current facilitation was shown to be substantial when AP-like depolarizations were used as prepulses, thus supporting our original hypothesis that facilitation in central neurons can be induced by "physiological stimuli." Two aspects are of a particular interest in these results. First, the relationship between the frequency of the AP-like prepulses (2 msec in duration and 90 mV in amplitude) with the amount of facilitation indicates that at low frequencies (10–20 Hz) the amount of facilitation is quite small (~15%), whereas at higher frequencies (150–200 Hz) a high degree of facilitation (60%) is attained. This possibly could explain the weak level of facilitation (20%) observed with AP-like depolarizations for a minority of DRG cells (Womack and McCleskey, 1995) and the near absence of facilitation (5%) found in raphe neurons (Penington et al., 1991). In both of these studies, however, only low frequency trains were used (up to 20 Hz), which could explain the weak level of facilitation obtained with respect to the present study (see Fig. 4*D* for comparison). A lack of facilitation also was demonstrated in another study performed on sympathetic neurons using AP waveforms (Toth and Miller, 1995). However, whereas APs were applied at moderate frequencies (40–75 Hz), only a small number of APs (7) were given at this rate, indicating that this parameter is also a contributing factor in facilitation. The second interesting aspect concerns the number of AP-like depolarizations given at a high frequency that permit facilitation. Indeed, when tested at 200 Hz, it is noteworthy that two APs were enough to yield a 20% facilitation, whereas five APs could produce more than one-half (~35%) of the maximal reachable level of facilitation (60%). This latter result could be particularly relevant for cells such as the BF cholinergic neurons, which have the ability to fire in high frequency bursts (see below).

Physiological significance

Identified cholinergic neurons *in vitro* and presumed cholinergic neurons *in vivo* have been shown to fire either tonically at a rather low frequency (up to 15 Hz *in vitro* and 25 Hz *in vivo*) or in high frequency bursts (up to 13 AP/burst at 250 Hz) (Khateb et al., 1993, 1995; Alonso et al., 1994, 1996; Szymusiak, 1995; Nuñez, 1996; A. Khateb, unpublished data). Because of the characteristics of facilitation described here and the implication of the N current in the calcium-dependent potassium conductance that produces the slow after-hyperpolarization (AHP) and accommodation (S. Williams, M. Serafin, M. Mühlethaler, L. Bernheim, unpublished data), it is most probable that voltage-dependent inhibition of the N current (by a transmitter) would greatly reduce the slow AHP when the neuron fires near rest (at a low frequency). One can speculate, however, that when the neuron is hyperpolarized and thus brought at the level where the LTS can trigger high-frequency bursting (Khateb et al., 1992; Alonso et al., 1996), the blocking effect on the consecutive AHP (which also depends importantly on the N current; (S. Williams, M. Serafin, M. Mühlethaler, L. Bernheim, unpublished data) would fade away, thereby allowing the burst to terminate. Such a mechanism could allow neurotransmitters that block AHPs (through the reduction of N-type current) to promote and shape the bursting pattern in BF cholinergic neurons. Thus, because of the mechanism of facilitation, the blocking intensity of the AHP by a transmitter would be dependent on the state of excitability of the neuron.

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