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Neurotransmitter Modulation of Neuronal Calcium Channels

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There are many different calcium channels expressed in the mammalian nervous system, but N-type and P/Q-type calcium channels appear to dominate the presynaptic terminals of central and peripheral neurons. The neurotransmitter-induced modulation of these channels can result in alteration of synaptic transmission. This review highlights the mechanisms by which neurotransmitters affect the activity of N-type and P/Q-type calcium channels. The inhibition of these channels by voltage-dependent and voltage-independent mechanisms is emphasized because of the wealth of information available on the intracellular mediators and on the effect of these pathways on the single-channel gating.

KEY WORDS: N-type; P/Q-type; G protein; betagamma subunit; inhibition; facilitation; potentiation.

INTRODUCTION

Increases in cytoplasmic Ca^{2+} can arise from several sources. One source is Ca^{2+} influx across the plasma membrane via voltage-dependent VD calcium channels. There are two roles for Ca^{2+} permeating these channels. The first is electrical where Ca^{2+} ions act to depolarize the plasma membrane in a manner similar to Na^{+}. The second role is to act as an intracellular messenger. A transient rise in intracellular Ca^{2+} concentration can trigger a host of events such as changes in gene transcription, cellular migration, membrane excitability, and neurotransmitter release. Thus, activation of VD calcium channels serves to link cellular action to membrane depolarization.

Given the importance of Ca^{2+} to cellular function, the intracellular concentration is tightly controlled at $\sim$100 nM. The Ca^{2+} influx pathways are also highly controlled by neurotransmitters and hormones, which can alter the gating of VD calcium channels to affect neuronal function. For example, neurotransmitter-induced inhibition of calcium channel activity in presynaptic terminal can reduce synaptic release to alter information transfer in the nervous system (Allen, 1999; Harkins and Fox, 2000; Koh and Hille, 1997; Pfrieger *et al.*, 1994; Toth *et al.*, 1993; Umemiya and Berger, 1994; Yawo and Chuhma, 1993). There are several intracellular pathways that can inhibit these channels, but one pathway has received the majority of attention in the literature. This pathway induces an inhibition that can be temporarily reversed by depolarization (the VD pathway). One reason for the intensity of investigation is that this inhibitory pathway has been observed in the vast majority of neurons studied. Another reason is that it can be induced by a large number of neurotransmitters that activate metabotropic receptors. One final reason for the interest is that this pathway inhibits the two calcium channel types that dominate presynaptic terminals, N-type and P/Q-type (Meir *et al.*, 1999). One section of this review will focus on the mechanisms involved in VD inhibition. Another section will cover additional pathways that induce calcium current inhibition that is not affected by voltage (voltage-independent (VI) inhibition). Finally, a few examples of neurotransmitter potentiation of N-type and P/Q-type calcium-channel activity will be examined.

TYPES OF CALCIUM CHANNELS

Voltage-dependent calcium channels (CaV) have been divided into the three gene families on the basis of sequence homology. The *CaV1* family (also called L-type channels) comprises channels sensitive to dihydropyridines (DHPs), which is a group of compounds that

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can either inhibit (called antagonists) or enhance (called agonists) the activity of these channels. Neurons express three members of the *CaV1* family, *CaV1.2*, *CaV1.3*, and *CaV1.4*. *CaV1.4* appears to be exclusively expressed in retina and mutations of this calcium channel are associated with a congenital form of night blindness (Bech-Hansen *et al.*, 1998; Pietrobon, 2002; Strom *et al.*, 1998). *CaV1.2* and *CaV1.3* channels are more widely expressed in the Central nervous system (CNS) (Catterall, 2000). When these two L-channel types are expressed in Xenopus öocytes they activate over different voltage ranges and have differential sensitivity to DHP antagonists (Xu and Lipscombe, 2001). Unfortunately, these differences are not sufficient to allow an unambiguous separation of these channels in native neurons. Neurotransmitters can either inhibit or enhance L-type channel activity. One of the first well-studied examples of calcium current modulation is the potentiation of cardiac L-current by β-adrenergic receptor agonists (McDonald *et al.*, 1994). This enhancement involves increases in intracellular cAMP and the subsequent activation of cAMP-dependent protein kinase (PKA). Enhancement of L-current by this pathway has also been observed in hippocampal pyramidal cells (Gray and Johnston, 1987) and chromaffin cells (Carabelli *et al.*, 2001). Inhibition of neuronal L-current is mediated by a second-messenger pathway that is sensitive to levels of intracellular Ca^{2+} (Hille, 1994). This pathway can be activated by acetylcholine binding to M1 muscarinic receptors and will be discussed further below (see *Slow voltage-independent inhibition*).

The second family of calcium channels (*CaV2*) includes P/Q-type (*CaV2.1*), N-type (*CaV2.2*), and R-type (*CaV2.3*) calcium channels. N-type and P/Q-type channels have been extensively studied because of their importance to synaptic transmission and their sensitivity to specific toxins that allow them to be identified in neurons. N-type channels are specifically blocked by a toxin called ω-conotoxin GVIA (ωCGVIA) (Aosaki and Kasai, 1989; Jones and Marks, 1989; Plummer *et al.*, 1989). The name of the toxin is derived from the name of the marine snail *Conus geographus* (G) from which it is isolated and from fraction number (VIA) that contains the toxin when crude venom is separated on an HPLC column. Omega is the code for toxins that block calcium channels (Kerr and Yoshikami, 1984; Olivera *et al.*, 1985). N-channels are also blocked by another cone snail toxin isolated from *Conus magus*, ω-conotoxin MVIIC (ωCMVIIC), but this toxin also blocks P/Q-channels (Hillyard *et al.*, 1992; Sather *et al.*, 1993). Thus, when ωCMVIIC is used to determine the component of neuronal calcium current contributed by P/Q-channels, ωCGVIA is applied prior to ωCMVIIC to block N-channels. P/Q-channels are also blocked by a spider toxin called ωAgaIVA (Mintz *et al.*, 1992; Sather *et al.*, 1993), which is isolated from *Agelenopsis aperta*. Both P-type and Q-type channels are derived from the same gene (*CaV2.1*) through alternative splicing (Bourinet *et al.*, 1999) and are differentiated in neurons by their differential sensitivity to ωCMVIIC and ωAgaIVA. P-channels are more sensitive to ωAgaIVA and Q-channels are more sensitive to ωCMVIIC (Randall and Tsien, 1995; Zhang *et al.*, 1993). R-type channels are resistant to these toxins and to DHPs (Randall and Tsien, 1995; Zhang *et al.*, 1993). However, some R-channels have been shown to be sensitive to toxin isolated from the tarantula *Histerocrates gigas* called SNX-482 (Tottene *et al.*, 2000; Wilson *et al.*, 2000). The idea that R-current in neurons (defined by resistant to ωCGVIA, ωCMVIIC, and DHPs) results from a single class of channel has been questioned. Xu and Lipscombe (2001) found that CaV1.3 channels expressed in Xenopus öocytes were incompletely blocked by concentrations of DHP antagonists typically used in studies of native calcium channels. In addition, Wilson *et al.* (2000) demonstrated toxin and DHP resistant channels in neurons isolated from *CaV2.3* knockout mice. Thus, it is difficult to unambiguously identify CaV2.3 channels in neurons naturally expressing many calcium channel types. In a few studies, heterologously expressed CaV2.3 channels have been shown to be inhibited by neurotransmitters (Mehrke *et al.*, 1997; Meza and Adams, 1998; Qin *et al.*, 1997), but little or no inhibition was observed in other studies (Page *et al.*, 1997; Toth *et al.*, 1996). This variability may result from splice variants used in the different studies (Dolphin, 1998). When neurotransmitter-induced inhibition of CaV2.3 channels was observed it was shown to be voltage-dependent. This demonstrates that all members of the *CaV2* family can be inhibited by this mechanism. The *CaV2* family appears to be the only calcium channel family that is inhibited by the VD pathway.

The third family of calcium channels (*CaV3*) comprise T-type channels. All three members of this family are widely expressed in the CNS (Talley *et al.*, 1999). These channels are characterized by a low threshold voltage for activation and fast inactivation (Perez-Reyes *et al.*, 1998). T-channels play a role in setting neuronal excitability and in pacemaker activities (Bertolino and Llinas, 1992; Huguenard *et al.*, 1996). In addition, these channels appear to be involved in some forms of epilepsy (Huguenard, 1999). A few studies have shown T-channels to be resistant to modulation by neurotransmitters (Bayliss *et al.*, 1995; Marchetti *et al.*, 1986; Park *et al.*, 2001), while other studies demonstrate that they can be modulated (Abdulla and Smith, 1997; Chemin *et al.*, 2001; Drolet *et al.*, 1997).

CALCIUM CHANNEL MODULATION

The first report that VD calcium channels could be modulated by neurotransmitters came from studies on chick sensory neurons (Dunlap and Fischbach, 1978). These researchers demonstrated an inhibition induced by norepinephrine (NE). Calcium currents in rat sympathetic neurons were also found to be inhibited by NE (Galvan and Adams, 1982). In a later study, Marchetti *et al.* (1986) separated whole-cell calcium current in chick sensory neurons into low voltage-activated (LVA, T-type) and high voltage-activated (HVA, primarily N-type) calcium current. This separation revealed that dopamine (DA) and NE could inhibit HVA current, but had no effect on the LVA current. Marchetti *et al.* (1986) also noted that HVA current was only partially inhibited by saturating concentrations of either DA or NE, and that the activation kinetics of the inhibited current was slower than control. They hypothesized that this "slow-activation" could result from the inhibition being VD. In other words, the calcium current slowly recovered from inhibition during the voltage step used to activate the current. Unfortunately, the nonspecific loss of current with time (rundown) was too severe in these experiments to allow this hypothesis to be tested.

Around this same period, the Tsien lab demonstrated that HVA current in chick sensory neurons was comprised of multiple channel types, L-type, and N-type (Nowycky *et al.*, 1985). On the basis of this finding, it was proposed that the partial inhibition of HVA current resulted from the complete inhibition of N-type calcium current without an affect on L-type current (Wanke *et al.*, 1987). The slowed activation was explained as normal L-current activation kinetics (Wanke *et al.*, 1987). Under control conditions L-current activation was thought to be obscured by N-current inactivation. This hypothesis was based on the separation of N- and L-current by holding potential changes, a technique that was later shown to be invalid (Jones and Marks, 1989; Plummer *et al.*, 1989).

The identification of the mechanism underlying neurotransmitter-induced slow activation of HVA calcium current was facilitated by the demonstration that ωCGVIA was a specific blocker of N-type calcium current (Aosaki and Kasai, 1989; Jones and Marks, 1989; Plummer *et al.*, 1989), which permitted the unambiguous separation of N-type from L-type calcium current. These papers demonstrated that the majority of HVA calcium current in sympathetic neurons from rat and frog, and chick sensory neurons, was comprised of N-type current. One implication was that the L-current component was too small to account for the slowly-activating current during neurotransmitter-induced inhibition (Jones and Marks, 1989; Kasai and Aosaki, 1989; Plummer *et al.*, 1989). In addition, it was demonstrated in rat and bullfrog sensory neurons that the inhibition of HVA current decreased with depolarization (Bean, 1989). These findings supported the hypothesis that neurotransmitter inhibition of N-type calcium current was VD.

Voltage-Dependent Inhibition

The link between slow activation of neurotransmitter-inhibited current and VD inhibition was confirmed when it was shown that brief strong depolarizations could temporarily reverse inhibition and the accompanying slow activation kinetics (Grassi and Lux, 1989; Elmslie *et al.*, 1990). This effect could easily be observed using a protocol composed of three voltage steps (Fig. 1). Current during the prepulse (prior to the depolarizing conditioning pulse) was inhibited by neurotransmitters, but current generated during the postpulse (following the conditioning pulse) was almost completely recovered to control levels. This increase in current induced by the conditioning pulse was termed facilitation (Elmslie *et al.*, 1990), which has become an important test for identifying VD inhibition (Jones and Elmslie, 1997). There is a wide range of neurotransmitters that can induce VD inhibition (Table I).

All these neurotransmitters induce inhibition by binding to G protein-coupled receptors, which implied that G proteins were intracellular mediators of the inhibition. This was verified by the demonstration that intracellular application of the direct G protein activator GTPγS could mimic the effects of neurotransmitter inhibition, including decreased current amplitude, slowed activation kinetics, and increased facilitation (Elmslie *et al.*, 1990; Grassi and Lux, 1989; Ikeda, 1991). Further support for the involvement of G proteins came from the observation that neurotransmitter-induced VD inhibition of N-current was blocked by the broad-spectrum G protein blocker, GDPβS (Grassi and Lux, 1989; Ikeda, 1991; Schofield, 1991).

Clues as to which G protein was involved in VD inhibition came from experiments showing that pertussis toxin (PTX), a blocker of G protein subtypes G_o and G_i, could block VD inhibition induced by somatostatin and NE in rat sympathetic neurons (Ikeda, 1991; Schofield, 1991). G_o appears to be the primary G protein activated by NE in these neurons (Caulfield *et al.*, 1994). However, it was found that PTX insensitive G proteins could also induce VD inhibition. In frog sympathetic neurons, NE induced an inhibition that was PTX sensitive, but the luteinizing hormone-releasing hormone (LHRH)-induced inhibition was PTX insensitive (Elmslie, 1992). It was later demonstrated in rat sympathetic neurons that NE

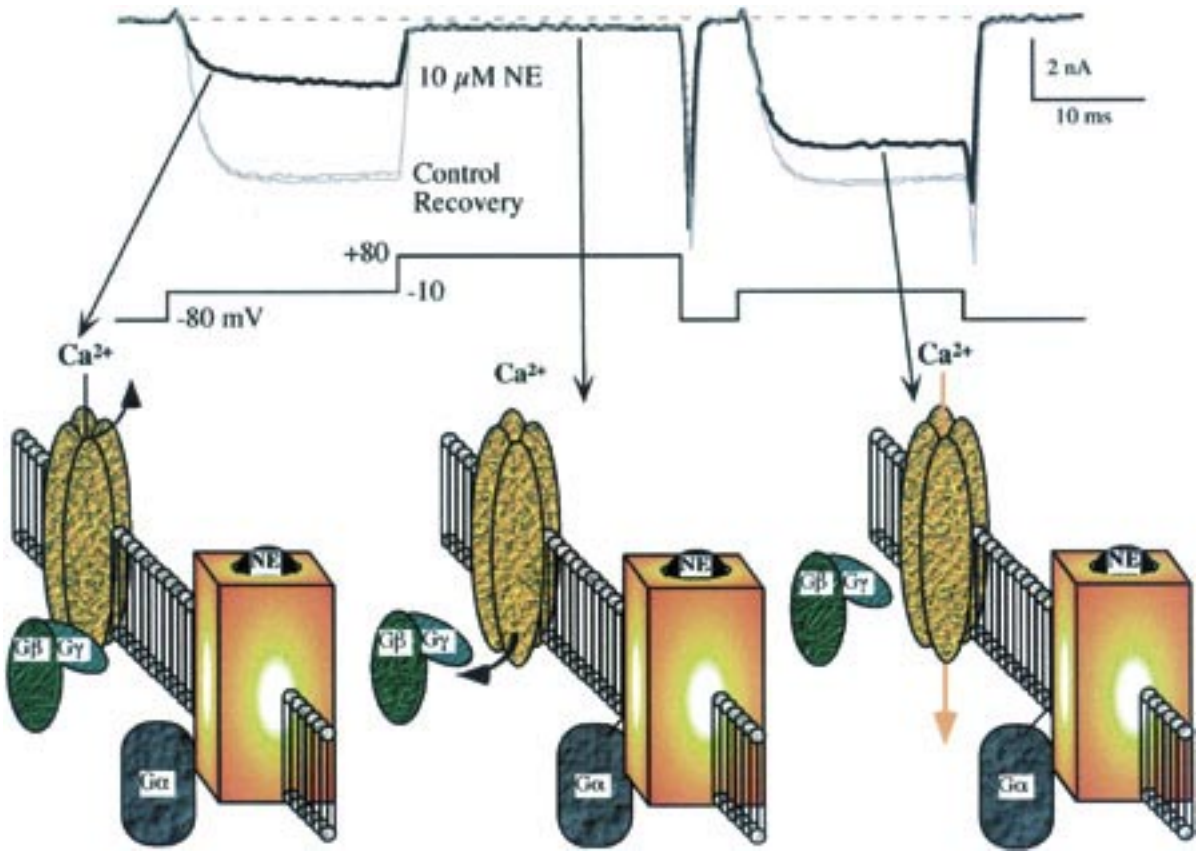

Fig. 1. The facilitation of inhibited N-type calcium current is mediated by the disruption of G$\beta\gamma$-channel coupling during a depolarizing conditioning pulse. Three current traces are shown before, during and after application of 10 μM NE. Current is inhibited during the 20-ms prepulse to -10 mV and a portion of the inhibited current activates slowly. This slow activation results from the slow unbinding of G$\beta\gamma$ from some N-channels during the voltage step, but the majority of channels are still inhibited at the end of the prepulse. Very little current is generated during the conditioning step to $+80$ mV, but G$\beta\gamma$ dissociates from most of the channels during this 25-ms step. The effect of the conditioning step is observed during the postpulse to -10 mV, which shows fast activation kinetics and nearly a complete recovery of current amplitude. Note that the conditioning step has no effect on control current. The cartoon depicts the G$\beta\gamma$-binding state of the majority of channels during each of the three-voltage steps.

could induce VD inhibition via both PTX sensitive and insensitive G proteins in some rat strains (Shapiro *et al.*, 1994a). However, in other strains the NE-induced inhibition is completely blocked by PTX (Caulfield *et al.*, 1994; Zhu and Ikeda, 1994a,b). The PTX insensitive, but cholera-toxin sensitive, G protein G_s was found to mediate VD inhibition in rat sympathetic neurons induced by vasoactive intestinal peptide (Zhu and Ikeda, 1994b). The VD properties of the inhibition were shown to be identical whether the inhibition was generated by activated G_o or G_s, which supported the idea that both G proteins activated a common intracellular pathway (Ehrlich and Elmslie, 1995). This was interpreted as evidence against the involvement of Gα subunits in VD inhibition, since it appeared unlikely that different Gα subunits would activate a common intracellular pathway (Ehrlich and Elmslie, 1995).

While the above experiments demonstrate the involvement of G proteins, a large body of evidence showed that diffusible second messengers (e.g. cAMP or IP_3) were not mediators of VD inhibition (Hille, 1994). Some of this evidence came from experiments using fast agonist application systems. These experiments demonstrated that maximal inhibition could be observed within 1–2 s of agonist application (Bernheim *et al.*, 1991; Jones, 1991), which appeared to be too fast for a diffusible second messenger. In addition, cell-attached patch clamp studies demonstrated that bath applied NE could not inhibit calcium channels isolated by the patch pipette (Bernheim *et al.*, 1991), but NE included in the pipette solution could

Table I. Neurotransmitters That Activate the VD Inhibitory Pathway

Acetylcholine	Beech *et al.*, 1992; Elmslie, 1992
Adenosine	Mogul *et al.*, 1993; Zhu and Ikeda, 1993
Adenosine triphosphate	Elmslie, 1992
Cannabinoids	Mackie and Hille, 1992
Dopamine	Cardozo and Bean, 1995; Marchetti *et al.*, 1986
γ-Aminobutyric acid (GABA)	Cox and Dunlap, 1992; Menon-Johansson *et al.*, 1993; Mintz and Bean, 1993
Glutamate	Ikeda *et al.*, 1995; Swartz and Bean, 1992
Luteinizing hormone-releasing hormone (LHRH)	Boland and Bean, 1993; Elmslie *et al.*, 1990
Neuropeptide Y	Cohen *et al.*, 1996; Foucart *et al.*, 1993; Schofield and Ikeda, 1988
Norepinephrine (NE)	Elmslie, 1992; Schofield, 1990
Opiates	Carabelli *et al.*, 1996; Eckert and Trautwein, 1991; Womack and McCleskey, 1995
Pancreatic polypeptide	Wollmuth *et al.*, 1995
Prostaglandin E2	Ikeda, 1992
Serotonin	Bayliss *et al.*, 1997; Foehring, 1996; Penington *et al.*, 1991
Somatostatin (SOM)	Ikeda, 1991; Ikeda and Schofield, 1989
Substance P	Elmslie, 1992; Golard *et al.*, 1994
Vasoactive intestinal peptide (VIP)	Zhu and Ikeda, 1994b

induce VD inhibition (Elmslie *et al.*, 1994). The rapid onset and membrane-delimited nature of this pathway provided support for the idea that VD inhibition was mediated by direct G protein-channel coupling (Hille, 1994).

The first report to examine the effect of separate G protein subunits (i.e. Gα or G$\beta\gamma$) on N-current inhibition concluded that Gα-mediated VD inhibition, since the intracellular application of G$\beta\gamma$ induced VI inhibition in chick sensory neurons (Diverse-Pierluissi *et al.*, 1995). However, experiments overexpressing G protein subunits in rat sympathetic neurons showed VD inhibition when G$\beta\gamma$ was expressed, whereas expression of a constitutively active Gα had little or no effect (Herlitze *et al.*, 1996; Ikeda, 1996). In addition, the expression of nonactivated GDP-bound Gα (GDP-Gα) blocked the NE-induced inhibition, which was thought to result from GDP-Gα binding the G$\beta\gamma$ released from endogenous G proteins activated by NE (Ikeda, 1996). Additional experiments have verified that G$\beta\gamma$ mediates VD inhibition of N-type and P/Q-type calcium currents by binding directly to these channels (Meza and Adams, 1998; Page *et al.*, 1997; Qin *et al.*, 1997; Simen *et al.*, 2001; Simen and Miller, 1998, 2000; Zamponi *et al.*, 1997; Zhang *et al.*, 1996).

The Mechanism of Voltage-Dependence Inhibition

One hypothesis for the voltage-dependence of inhibition was the disruption of G protein-channel coupling by depolarization. An alternative idea was that G protein-bound channels could gate in either an "inhibited" gating mode or normal gating mode with balance between the two modes determined by voltage. The former hypothesis predicts that the reinhibition of calcium current following strong depolarization will depend on the concentration of active G$\beta\gamma$ near the channel. In other words, if G$\beta\gamma$-channel coupling is disrupted by depolarization the speed by which the inhibition is reestablished will depend on the G$\beta\gamma$ subunits available to bind to the channel. If G$\beta\gamma$ concentration is low, reinhibition will be slow, but reinhibition will be rapid if the G$\beta\gamma$ concentration is high. This hypothesis was initially tested in chick sympathetic neurons using different concentrations of NE to indirectly alter the concentration of active G protein (Golard and Siegelbaum, 1993). Increasing the NE concentration resulted in larger inhibition of N-type current, which was assumed to result from a larger number of activated G proteins. The higher NE concentrations also resulted in faster reinhibition following strong depolarization, which supported the idea that G protein-channel coupling was disrupted by strong depolarization. This result was later confirmed in both frog and rat sympathetic neurons (Ehrlich and Elmslie, 1995; Elmslie and Jones, 1994). The concentration dependence of reinhibition was also investigated by expressing low concentrations of β-adrenergic receptor kinase 1 (βARK1), a protein that could bind G$\beta\gamma$ to reduce its concentration (Delmas *et al.*, 1998a). As predicted reinhibition was slower when the G$\beta\gamma$ concentration was reduced by the expression of low levels of βARK1.

A second prediction of hypothesis that G$\beta\gamma$-channel coupling is disrupted by depolarization is that the time course of facilitation is independent of G$\beta\gamma$ concentration. This prediction was supported by showing that the increase in postpulse current amplitude following conditioning steps (+80 mV) of increasing duration was not affected by neurotransmitter concentration (Ehrlich and Elmslie, 1995; Elmslie and Jones, 1994). Thus, the time constant of the development of facilitation and reinhibition can simplistically be described by

$$1/\tau = [G\beta\gamma]K_{on} + K_{off} \quad (1)$$

where K_{on} is the fundamental-binding rate constant for the binding of G$\beta\gamma$ to the channel, K_{off} is the off-rate constant, and [G$\beta\gamma$] is the G$\beta\gamma$ concentration. At negative holding potentials where reinhibition is measured, K_{off} is negligible so $1/\tau$ is dominated by [G$\beta\gamma$] K_{on}. At depolarized voltages where the time course of facilitation

is measured, K_{on} is negligible, so $1/\tau$ is dominated by the concentration-independent K_{off}. The time course of facilitation (slow activation) at voltages that yield peak current is also not concentration-dependent (Elmslie and Jones, 1994), which suggests that K_{off} also dominates at these voltages. This is supported by the observation that rapid application of neurotransmitter during a voltage step induces minimal N-current inhibition (Boland and Bean, 1993).

Figure 1 depicts the interaction between G$\beta\gamma$ and the channel thought to produce VD inhibition. When the membrane is hyperpolarized and the channels are closed, G$\beta\gamma$ binds to the channel to inhibit channel opening. Current is inhibited during the prepulse (−10 mV in this case) because the majority of channels are G$\beta\gamma$ bound. The slow activation results from the dissociation of G$\beta\gamma$ from some of the channels, which allows them to open normally. During the conditioning pulse (+80 mV) G$\beta\gamma$-channel coupling is disrupted from the majority of inhibited channels, but the effect of this disruption cannot be observed because of the small currents (which results from a small driving force on ion movement). Thus, the effect of the strong depolarization is observed in the postpulse current, which is facilitated because it takes time for G$\beta\gamma$ to rebind to the channel. Note that in this example the conditioning pulse has little effect on control currents. In some neurons, N-current facilitation can be observed under control conditions (without overt G protein activation (Ikeda, 1991)). This facilitation is blocked by introducing GDPβS into the cell to block G protein activation, which supports the idea that G proteins can be active under basal conditions to modulate N-current (Ikeda, 1991).

The Effect of G$\beta\gamma$ on Channel Gating

The mechanism depicted in Fig. 1 predicts that channels gate normally after G$\beta\gamma$-channel coupling has been disrupted by strong depolarization. This prediction has been verified by recording single N-channel activity in the presence of a neurotransmitter before and after a strong depolarizing conditioning pulse. Inhibition resulted in longer latency to channel opening (called first latency), which is the basis for slow activation (Carabelli *et al.*, 1996; Colecraft *et al.*, 2001; Lee and Elmslie, 2000; Patil *et al.*, 1996). Following a conditioning pulse, first latency was normal and channel gating was indistinguishable from that of control (uninhibited) channels (Carabelli *et al.*, 1996; Colecraft *et al.*, 2001; Lee and Elmslie, 2000; Patil *et al.*, 1996). However, whole-cell results generated an additional prediction that G$\beta\gamma$ bound N-channels could open, but this was more difficult to verify in single-channel recordings. This idea was based on the observation that inhibited N-current activated at voltages ∼20 mV depolarized to control current (Bean, 1989), which suggested that inhibited channels could open if the membrane was strongly depolarized. These inhibited channels were termed reluctant (to open) and the normal channels were termed willing (Bean, 1989). This willing–reluctant model was slightly modified when it was observed that the time course of facilitation reached an maximum rate at large depolarizations (Colecraft *et al.*, 2000; Elmslie *et al.*, 1990; Jones and Elmslie, 1997). This observation was interpreted to support the idea that open N-channels could convert from reluctant to willing (Fig. 2). While the model has been embellished over the years (Boland and Bean, 1993; Colecraft *et al.*, 2000), the simple four-state willing–reluctant model is still useful in that it explains the essential features of VD inhibition. The model predicts that reluctant channels will open, but they will have a low open probability and the openings will be brief relative to willing channels (Elmslie *et al.*, 1990). However, the first recordings from single N-type channels were unable to observe a change in channel open times during inhibition (Carabelli *et al.*, 1996; Patil *et al.*, 1996). One problem was that single N-channel currents are small (<1.5 pA), which makes them difficult to observe against background

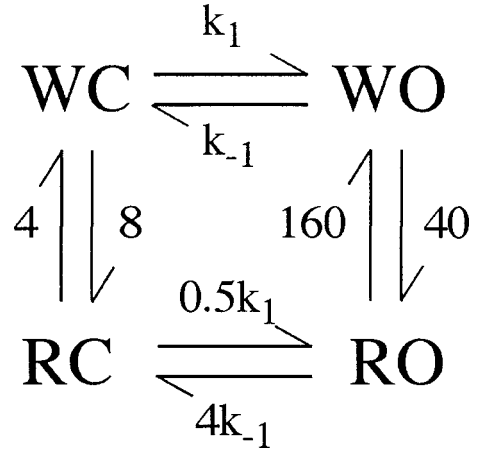

Fig. 2. The willing–reluctant model of N-channel inhibition. The model and rate constants are from Elmslie *et al.* (1990). Under control conditions the majority of channels are in the willing mode. k_1 and k_{-1} are VD rate constants governing the transition between willing closed (WC) and willing open (WO) states, where $k_1 = 200e^{0.06(V+5)}$ and $k_{-1} = 200e^{-0.06(V+5)}$. In the presence of an inhibitory neurotransmitter channels will enter the reluctant closed state (RC) from WC. Upon depolarization the channels in the RC state can move to the reluctant open (RO) state, but the smaller k_1 and larger k_{-1} dictate that RO will have a lower P_o and the shorter open time than WO. Note that the vertical rate constants do not explicitly include dependence on G$\beta\gamma$ concentration, which requires a more complex model.

electrical noise that contaminate all recordings. This problem is combated by using 100 mM Ba^{2+} to maximize the current amplitude and by recording currents at relatively hyperpolarized voltages were driving force is high. Thus, one explanation for the failure to observe reluctant openings was that the voltage steps used to characterize channel opening were too hyperpolarized to activate reluctant channels. This proved to be the case as subsequent single N-channel recordings with improved resolution observed reluctant openings at voltages 10–30 mV depolarized to those used previously (Colecraft *et al.*, 2001 Lee and Elmslie, 2000). Reluctant channels were shown to have a low open probability relative to willing (0.1 vs. 0.8 at +40 mV respectively) and reluctant open times were approximatily 10-fold shorter that willing openings (0.3 vs. 3 ms at +40 mV; Fig. 3) (Lee and Elmslie, 2000; 1999). It is important to note that the single-channel currents were recorded in 100 mM Ba^{2+}, which results in a 30–40 mV depolarizing shift in channel activation relative to typical whole-cell recordings conditions (2–5 mM Ca^{2+} or Ba^{2+}) (Elmslie *et al.*, 1994). This shift results from the surface charge effect of divalent cations on channel gating (Zhou and Jones, 1995). Thus, single N-channel activity (100 mM Ba^{2+}) recorded at +40 mV corresponds with whole-cell currents (2 mM Ba^{2+}) recorded at 0 mV (Elmslie *et al.*, 1994). The implication is that N-channels can open reluctantly at physiologically relevant voltages (Colecraft *et al.*, 2001; Lee and Elmslie, 2000).

The mechanism by which inhibition shifts N-channel activation to more depolarized voltages appears to involve $G\beta\gamma$ retarding voltage sensor movement. This was demonstrated by examining the effect of VD inhibition on gating currents of N-type channels expressed in HEK 293 cells (Jones *et al.*, 1997). The gating charge moved

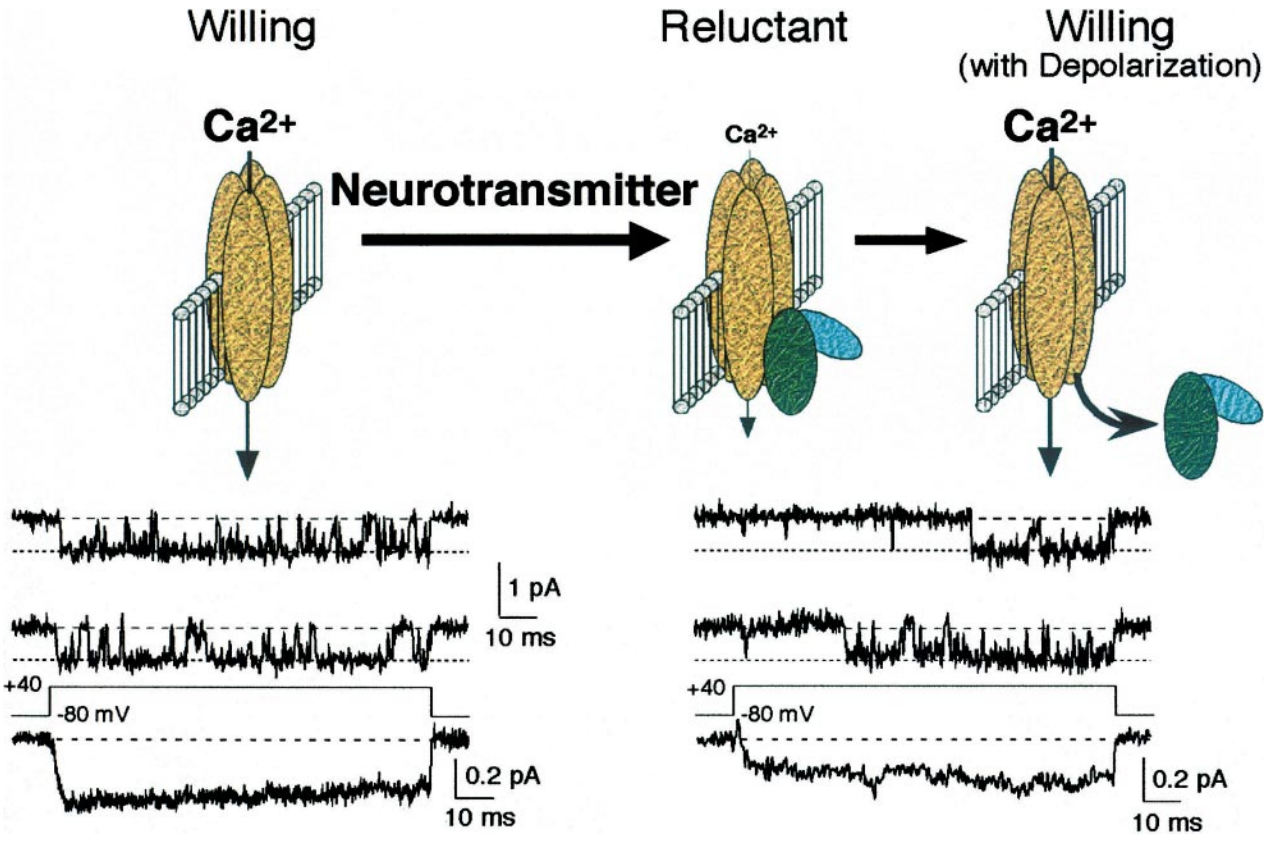

Fig. 3. The effect of VD inhibition on single N-channel gating. Single N-channel activity is shown during 100-ms voltage steps to +40 mV for two different patches. Under control conditions (left) N-channels gating in the willing (high P_o) mode, which is characterized by high P_o, long open times and short latency to channel opening. The addition of 100 μM NE to the patch pipette solution induces VD inhibition of N-channel activity (right). This inhibition is characterized by a long latency to willing opening and brief reluctant openings during this latency period. Once the channel does open to the willing state the gating is indistinguishable from control. The cartoon depicts the molecular events thought to contribute to the different types of gating. The high NE concentration (100 μM) is required to overcome an apparent competitive block of the α-adrenergic receptor by the high Ba^{2+} concentration (100 mM) used in these single channels recordings (Lee *et al.*, 2000).

during a prepulse to moderate voltages (generating peak current) was reduced during inhibition, but was largely recovered in the postpulse (following a depolarizing conditioning step), which indicated normal gating charge movement for facilitated (G$\beta\gamma$ unbound) channels. As prepulse voltage became more depolarized, gating current of inhibited channels (G$\beta\gamma$ bound) recovered to control levels. Thus, voltage sensors can move in reluctant channels if the depolarization is sufficiently strong (Jones *et al.*, 1997).

Differences in VD Inhibition Between N-type and P/Q-type Channels

As stated above, all *CaV2* channels can be inhibited in a VD manner. However, it has recently been recognized that the properties of the inhibition differ between N-type and P/Q-type channels. In general, the inhibition of P/Q-channels is smaller than that of N-type channels (Colecraft *et al.*, 2000; Currie and Fox, 1997; Zhang *et al.*, 1996). In addition, it appears that P/Q-channels require significantly stronger depolarization to open reluctantly (Colecraft *et al.*, 2000, 2001). This was initially demonstrated using a clever technique that compares the time course of facilitation in postpulse currents with the time course of tail current increase induced by increasing the duration of a conditioning step (e.g., +50 mV). For N-channels the increase in tail current amplitude was fast relative to the time course of facilitation (Colecraft *et al.*, 2000). The interpretation was that G$\beta\gamma$ bound N-channels (reluctant) could open. In contrast, the increase in tail current amplitude had the same time course as facilitation for P/Q-channels when the conditioning pulse was +50 mV, which suggested that these channels could not open reluctantly. This conclusion was supported by examining the speed of tail current deactivation, which had previously been shown be faster for inhibited N-channels and was interpreted as evidence that reluctant channels could open (Boland and Bean, 1993; Elmslie *et al.*, 1990). Colecraft *et al.* (2000) found that tail current deactivation was significantly faster for inhibited N-type channels than control, but P/Q-type current deactivation was not altered by inhibition. On the basis of these results, it was concluded that N-channels would open reluctantly at voltages that could be reached by an action potential (AP), but P/Q-channels would not open reluctantly within a physiological range (Colecraft *et al.*, 2000). These conclusions were confirmed by single-channel recording, which found no evidence for reluctant opening of P/Q-channels at voltages where reluctant N-channel gating was observed (Colecraft *et al.*, 2001).

Physiological Consequences of VD Inhibition

The inhibition of N-type and P/Q-type channels can reduce synaptic neurotransmitter release (Meir *et al.*, 1999). The voltage-dependence of inhibition could be important in controlling this reduction of neurotransmitter release. Thus, synaptic release may be strongly inhibited for a single AP, but inhibition relieved by a train of APs. However, an early study found that APs could not reverse VD inhibition (Penington *et al.*, 1991). Later studies demonstrated that AP trains could facilitate inhibited channels (Brody *et al.*, 1997, 2000; Currie and Fox, 2002; Park and Dunlap, 1998; Williams *et al.*, 1997; 1998). This reversal of inhibition by AP trains appears to be a mechanism by which vesicle release can be facilitated at hippocampal synapses (Brody and Yue, 2000).

The other aspect of VD inhibition is reluctant opening of N-type channels. Whole-cell and single-channel recordings predict that N-channels will open reluctantly at voltages $\geq$0 mV (Colecraft *et al.*, 2000, 2001; Lee and Elmslie, 2000), which implied reluctant N-channels could open during an AP. However, the brief open times and relatively depolarized activation voltages have made it difficult to use single-channel recording to observe reluctant openings during an AP. Therefore, Colecraft *et al.* (2001) used whole-cell recording to look for the effect of reluctant openings on currents generated by AP waveforms. They observed inhibited N-current peaked slightly earlier in the AP waveform than control, while there was no difference in between inhibited and control P/Q-current. Thus, reluctant gating appears to shift peak N-current to an earlier point in the AP. The reason for the speeding of the AP-induced currents is that reluctant channels can open rapidly at depolarized voltages (Colecraft *et al.*, 2000, 2001; Jones and Elmslie, 1997), but remain open only briefly relative to willing openings (Colecraft *et al.*, 2001; Lee and Elmslie, 2000).

Voltage-Independent Inhibition

While there appears to be only a single-intracellular pathway that contributes to VD inhibition, there are several different pathways that can induce VI inhibition of neuronal calcium current. This type of inhibition is characterized by a reduction in current amplitude with no change in activation kinetics. Two types of VI inhibition can be differentiated in rat sympathetic neurons by the speed of inhibition and by the involvement of a diffusible second messenger (Beech *et al.*, 1992). Activation of M1 muscarinic receptors and Angiotensin II receptors induces an inhibition characterized by slow development (tens of seconds) and by the involvement of an unidentified

BAPTA-sensitive diffusible second messenger (Bernheim *et al.*, 1992; Delmas *et al.*, 1998a; Shapiro *et al.*, 1994b). The second type of VI inhibition can be induced by substance P (Shapiro and Hille, 1993). This type of inhibition is characterized by rapid development (~1 s) and the involvement of a membrane-delimited pathway, two features that also describe VD inhibition (Hille, 1994). As detailed below $G\beta\gamma$ may mediate fast VI inhibition in addition to VD inhibition.

Other forms of VI inhibition has been studied in chick sensory neurons (Diverse-Pierluissi *et al.*, 1997). These neurons appear to possess at least two pathways that induce VI inhibition of N-type channels. One pathway is induced by NE and involves the activation of protein kinase C (Diverse-Pierluissi and Dunlap, 1993; Rane *et al.*, 1989). As with other forms of N-channel inhibition, this pathway appears to be mediated by $G\beta\gamma$ (Diverse-Pierluissi *et al.*, 1995, 2000; Lu *et al.*, 2001). The other pathway involves γ-aminobutyric acid activation of a tyrosine kinase to induce VI inhibition (Diverse-Pierluissi *et al.*, 1997). This pathway appears to be mediated by the $G\alpha$ subunit instead of $G\beta\gamma$.

Fast Voltage-Independent Inhibition

The mediator of fast VI inhibition of N-type channels observed by the Hille lab is unknown (Hille, 1994), but several labs have provided evidence that it could be $G\beta\gamma$ (Kammermeier *et al.*, 2000; Delmas *et al.*, 1999). The fast VI inhibition induced by NE and the muscarinic agonist oxotremorine-M (Oxo-M) is blocked by treatments that reduce the concentration of free cytoplasmic $G\beta\gamma$ (Delmas *et al.*, 1999; Kammermeier *et al.*, 2000). The $G\beta\gamma$ subunit involved in the NE-induced VI inhibition was preferentially coupled to G_i, whereas the $G\beta\gamma$ subunit involved in NE-induced VD inhibition was coupled to G_o (Delmas *et al.*, 1999). Delmas *et al.* (1999) proposed that different $G\beta\gamma$ subunits mediated VD inhibition versus VI inhibition. They speculated that $G\beta\gamma$ subunit involved in VI inhibition either had a stronger affinity for the N-channel (so that it did not dissociate from the open channel) or that it bound to a different site on the N-channel (to induce voltage-independent inhibition). In support of the idea that different $G\beta\gamma$ subunits could have different effects on N-channels, Garcia *et al.* (1998) found that VD inhibition of N-current in rat sympathetic neurons was induced by the expression of $G\beta1$ and $G\beta2$ subunits, but not $G\beta3$ and $G\beta4$ subunits. However, no differential selectivity between these same four $G\beta$ subunits was found in a separate study also using rat sympathetic neurons (Ruiz-Velasco and Ikeda, 2000). It was speculated that poor expression of $G\beta3$ and $G\beta4$ in the Garcia *et al.* (1998) study resulted in the absence of an effect (Ruiz-Velasco and Ikeda, 2000). Thus, the reason for the differential effects of $G\beta\gamma$ subunits associated with G_o versus G_i is unclear. However, one should be cautious with interpretation of results from exogenously expressed $G\beta\gamma$, since the $G\beta\gamma$ concentrations may be unphysiologically high. The concentration difference between expressed and physiologically released $G\beta\gamma$ could result in different inhibitory pathways dominating calcium channel gating.

In what appears to be a separate pathway, Kammermeier *et al.* (2000) demonstrated a requirement for both $G\beta\gamma$ and $G\alpha_{q/11}$ subunits in fast VI inhibition of N-current induced by activation of M1 muscarinic receptors in rat sympathetic neurons. Kammermeier *et al.* (2000) proposed that the $G\alpha$ subunit somehow 'locks' the $G\beta\gamma$ subunit to the N-channel, preventing dissociation during strong depolarization. This idea is similar to the high affinity $G\beta\gamma$ binding idea proposed by Delmas *et al.* (1999), except the 'high affinity' is mediated by a separate molecule (possibly $G\alpha_{q/11}$) binding to the N-channel to prevent $G\beta\gamma$ unbinding.

In chick sensory neurons a separate pathway is involved in VI inhibition. This pathway is activated by NE and appears to be mediated by a second messenger (Diverse-Pierluissi *et al.*, 1997). Surprisingly, activation this VI inhibition of N-current is as fast as the activation of VD inhibition in these cells with a delay of 1–2 s and maximum inhibition in 3–4 s after onset of agonist application (Luebke and Dunlap, 1994). A large body of evidence supports the idea that protein kinase C (PKC) is involved in this inhibition (Diverse-Pierluissi and Dunlap, 1993; Rane *et al.*, 1989). In looking for the G protein subunit involved in the activation of PKC, the Dunlap lab became the first to show the effect on N-current of directly introducing $G\beta\gamma$ to the cell via the patch pipette (Diverse-Pierluissi *et al.*, 1995). They showed that the addition of $G\beta\gamma$ (20 nM) to the cell-induced VI inhibition that was blocked by PKC inhibitors. The $G\beta\gamma$ mediating VI inhibition appears to be preferentially linked to G_i, while the VD inhibition of N-current in chick sensory neurons is mediated by Go (Diverse-Pierluissi *et al.*, 1995). The VI inhibitory pathway involves $G\beta\gamma$ activating phospholipase $C\beta$ ($PLC\beta$), which generates diacylglycerol to activate PKC. $PLC\beta$ shows specificity for $G\beta$ subunits since it is activated by $G\beta_1$, but is insensitive to $G\beta_2$ (Diverse-Pierluissi *et al.*, 2000). It is interesting that this specificity does not appear to be shared by the VD inhibitory pathway when the different $G\beta\gamma$ subunits are overexpressed (Garcia *et al.*, 1998; Ruiz-Velasco and Ikeda, 2000).

One question that arises is why are avian N-type channels inhibited via a PKC mechanism when

mammalian N-channels are not inhibited by activators of PKC (Abrahams and Schofield, 1992; Plummer *et al.*, 1991). One idea was that the avian N-channel differs from its mammalian counterpart. However, the same G$\beta\gamma$ treatment that induced in VI inhibition in chick sensory neurons induced VD inhibition when each of four avian N-channel isoforms was heterologously expressed in tsA-201 cells (Lu *et al.*, 2001), which disproved the idea that avian N-channels were not sensitive to VD inhibition. This result triggered an alternative hypothesis that auxiliary proteins were affecting the inhibition. It had been shown that syntaxin 1A, a protein involved in synaptic release that also binds to N-channels, could modulate the strength of VD inhibition (Jarvis *et al.*, 2000). However, chick sensory neurons contain syntaxin 1B, which was shown to interfere with VD inhibition (Lu *et al.*, 2001). Exogenous expression of syntaxin 1A in chick sensory neurons permitted VD inhibition by G$\beta\gamma$ (Lu *et al.*, 2001). Thus, accessory proteins that interact with the channel may determine which inhibitory pathway affects N-channel gating.

The Voltage-Independent Portion of VD Inhibition

One common observation is that strong depolarization incompletely recovers N-current inhibited via a VD pathway. This incomplete recovery has been explained as either a naturally occurring component of VD inhibition (Jones and Elmslie, 1997) or as evidence for coactivation of VD and fast VI pathways (Delmas *et al.*, 1999; Kammermeier and Ikeda, 1999). One observation in favor of the latter hypothesis is that depolarization can completely recover inhibited current in some preparations (Kasai, 1992). In addition, for neurotransmitters that have been shown to induce both VD and VI inhibition, blocking the VI component increases facilitation of the remaining inhibition by strong depolarization (Delmas *et al.*, 1999; Kammermeier and Ikeda, 1999). Thus, it is clear that coactivation of VD and VI inhibitory pathways is one mechanism that can limit the depolarization-induced recovery of inhibited current. However, other results support the idea that incomplete facilitation of inhibited current may be an intrinsic property of VD inhibition. When the VI inhibitory pathway is blocked in systems with coactivation of VI and VD inhibition, the recovery of inhibited current by strong depolarization is still incomplete (Delmas *et al.*, 1999; Kammermeier *et al.*, 2000; Kammermeier and Ikeda, 1999). In addition, single-channel recordings show that neurotransmitter-inhibited N-channels can occasionally gating reluctantly during the postpulse (Fig. 1), which indicates that the G$\beta\gamma$ subunit does not always dissociate during the conditioning step (Lee and Elmslie, 2000). Thus, it appears that both of the proposed mechanisms can coexist to limit the recovery of inhibited current by strong depolarization.

Slow Voltage-Independent Inhibition

The majority of whole-cell calcium current recordings utilize high concentrations of Ca^{2+} chelators (ETGA or BAPTA) to control the levels of intracellular divalent cations that can negatively impact the recording (Belles *et al.*, 1988). However, the high concentrations of EGTA and BAPTA used in these studies can block a diffusible second messenger pathway linking M1 muscarinic receptors to calcium channel inhibition in rat sympathetic neurons (Bernheim *et al.*, 1992; Beech *et al.*, 1991; Delmas *et al.*, 1998b). This BAPTA-sensitive pathway may affect a number of different ion channels since in rat sympathetic neurons the inhibition of both N-type and L-type calcium channels (Mathie *et al.*, 1992), as well as M-type potassium current (Beech *et al.*, 1991), has been shown to be sensitive to the concentration of intracellular Ca^{2+} chelators. However, in rat striatal neurons and sensorimotor neurons increases in intracellular BAPTA concentration block the muscarinic inhibition of L-type channels, but the inhibition of N-type and P/Q-type is not affected (Howe and Surmeier, 1995; Stewart *et al.*, 1999). In rat sympathetic neurons, angiotensin II also activates the BAPTA-sensitive pathway to inhibit N-type calcium current (Shapiro *et al.*, 1994b). G_q appears to be the G protein activated by M1 muscarinic receptors to induce slow VI inhibition in rat sympathetic neurons (Delmas *et al.*, 1998b). The mechanism by which BAPTA blocks the pathway was hypothesized to result from its ability to lower resting Ca^{2+} levels (Beech *et al.*, 1991). In rat sympathetic neurons, this was found to be true for the muscarinic inhibition of M-type potassium current, but not for N-type calcium current (Beech *et al.*, 1991). Treatments that maintained resting Ca^{2+} levels in the presence of high-intracellular BATPA concentrations restored M-current inhibition, but not the muscarinic inhibition of N-type calcium current (Beech *et al.*, 1991). Thus, it appears that multiple BAPTA-sensitive pathways may exist. It is interesting that these same treatments can restore the muscarinic inhibition of L-current in both rat striatal neurons and sensorimotor neurons (Howe and Surmeier, 1995; Stewart *et al.*, 1999), which supports the idea that L-current inhibition may be mediated by the same pathway that inhibits M-type potassium current.

The BAPTA-sensitive pathway appears to involve an diffusible intracellular messenger (Bernheim *et al.*, 1991; Mathie *et al.*, 1992), but the identity of that messenger

is unknown (Hille, 1994). A slow voltage-independent inhibition of N-type and L-type calcium current in rat sympathetic neurons can be induced by application of arachidonic acid (Liu *et al.*, 2001; Liu and Rittenhouse, 2000). However, the inhibition by arachidonic acid requires nearly 10 min to complete as opposed to 30 s for slow muscarinic inhibition of calcium current (Bernheim *et al.*, 1991; Liu *et al.*, 2001). So the relationship between these two inhibitions is unclear.

Potentiation of N-type and P/Q-Type Channel Activity

The vast majority of publications on modulation of N-type calcium current describe inhibition, with relatively few showing potentiation of this current by neurotransmitters. When enhancement is reported the magnitude is typically small and the time course is slow. For example, angiotensin II enhances N-current in rat subfornical neurons by 12% with a maximal effect 1–2 min after onset of application (Washburn and Ferguson, 2001). Morikawa *et al.* (1999) showed that opioid agonists could both inhibit and potentiate N-current in NG108-15 cells. The inhibition developed rapidly, but the potentiation developed over several minutes and required up to 20 min to recover. Thus, an over recovery of N-current was observed upon removal of the opioid agonist (Morikawa *et al.*, 1999). Again the enhancement in NG108-15 cells was small (15%). The slow enhancement of N-current in these studies supports idea that a second messenger is involved. One candidate could be nitric oxide (NO), which has been shown to enhance N-current in rat sympathetic neurons (Chen and Schofield, 1993, 1995), but N-current inhibition by NO has been observed in other neurons (Yoshimura *et al.*, 2001).

One explanation for the relatively small enhancement of N-type current appears to be the primary mode in which the channels gate (Carabelli *et al.*, 1996; Colecraft *et al.*, 2001; Lee and Elmslie, 1999, 2000). The willing mode (also called the high P_o mode) of N-channel gating is characterized by high open probability (Lee and Elmslie, 1999). Since one mechanism of increasing calcium current is to enhance P_o (Carbone *et al.*, 2001; Yue *et al.*, 1990), the high P_o gating of willing N-channels strongly limits such increases. However, the small N-current enhancement appears to be sufficient to potentiate neurotransmitter release (Keren *et al.*, 1997, 1999).

In a few studies P/Q-current was shown to be enhanced by neurotransmitters (Fukuda *et al.*, 1996; Mogul *et al.*, 1993). Mogul *et al.* (1993) showed that activation of adenosine 2B receptors potentiated P-current and shifted activation to more hyperpolarized voltages. This enhancement had a slow time course with a peak effect occurring after 100–200 s of agonist application, which suggested the involvement of an intracellular second messenger. Indeed, intracellular application of a peptide blocker of PKA inhibited the enhancement of P-current, which supports the involvement of the cAMP-PKA pathway (Mogul *et al.*, 1993). This pathway may be involved in the potentiation of glycine release from brainstem neurons by activation of adenosine type 2 receptors (Umemiya and Berger, 1994). Increases in intracellular cAMP were also shown to enhance the activity of Q-channels expressed in Xenopus öocytes (Fukuda *et al.*, 1996). However, this enhancement was not accompanied by a shift in the voltage-dependence of channel activation.

SUMMARY

The N-type and P/Q-type channels are inhibited by a large number of neurotransmitters. One effect of this inhibition is to reduce the synaptic release of neurotransmitter. In some cases, the inhibition is induced by the neurotransmitter released by the nerve terminal as part of a negative feedback pathway. The VD and fast VI pathways induce two prominent types of inhibition that show a rapid onset and recovery so that the inhibition is closely timed with presence of the neurotransmitter at the nerve terminal. The removal of the neurotransmitter rapidly terminates the inhibition. However, the VD pathway provides the added feature that AP trains temporarily reduce inhibition by disrupting $G\beta\gamma$-channel coupling. This feature may aid the transmission of information carried by high-frequency AP trains, while blocking that carried the low-frequency trains. In addition, the Ca^{2+} contributed by the brief openings of reluctant channels may sum during the train to induce transmitter release or facilitate transmitter release once $G\beta\gamma$-channel coupling has been disrupted.

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