The calcium ion influx through voltage-dependent calcium channels (VDCCs) has a vital role in the control of neurotransmitter release and membrane excitability. Prepulse facilitation is a phenomenon in which a strong depolarizing pulse induces a form of the VDCCs that exhibits an increased opening probability in response to a given test potential; this persists for several seconds after repolarization. It has been reported that prepulse facilitation occurs via dissociation of the guanosine triphosphate (GTP)-binding proteins (G-proteins) from the VDCCs and that recovery from facilitation involves rebinding of the G-proteins.

The heterotrimeric G-proteins act as switches that regulate information processing circuits connecting cell surface G-protein-coupled-receptors to a variety of effectors. In this study, we have studied the characterization of G-protein subtypes in prepulse facilitation of VDCCs currents (ICa) in hamster submandibular ganglion (SMG) neurons, using whole-cell patch clamp recordings.

Under control conditions, with GTP (0.1 mM) in the recording pipette, the rate of prepulse facilitation was 19.0 ± 1.9% (n = 13). Intracellular dialysis with GDP-βS (0.1 mM), G-protein blocker, and pretreatment of neurons with N-ethylmaleimide (NEM) (100 μM for 2 min), Gαi blocker, attenuated the rate of prepulse facilitation. Intracellular dialysis of anti-Gq/11-antibody did not alter it. These results suggest that prepulse facilitation of VDCCs is due to Gαi-type of G-protein, but not to the Gq/11-type, in SMG neurons.

Key words: Hamster submandibular ganglion neurons—Voltage-dependent calcium channels—Prepulse facilitation—GTP-binding protein—Whole-cell patch clamp recordings

INTRODUCTION

Signal transducing guanosine triphosphate (GTP) -binding proteins (G-proteins) occur in two forms, the “small G-proteins” that are generally found as single polypeptides composed of about 200 amino acids and the “heterotrimeric G-proteins” that are made up of α, β and γ subunits. The small G-proteins have two interconvertible forms: guanosine diphosphate
An upstream signal stimulates the dissociation of GDP from the GDP-bound form, which is followed by the binding of GTP, eventually leading to a conformational change of the downstream effector-binding region, so that this region interacts with the downstream effectors. This interaction causes a change in the functions of the downstream effectors. The GTP-bound form is converted by the action of the intrinsic GTPase activity to the GDP-bound form, which then releases the bound downstream effectors. In this way, one cycle of activation and inactivation is achieved, and small G-proteins serve as molecular switches that transduce an upstream signal to a downstream effector.

The heterotrimeric G-proteins are associated with signal transduction from the cell surface G-protein-coupled-receptor (GPCR) and act as switches that lead to a complex series of intracellular events.

G-protein α subunits generally can be classified into G1, G2, G11, G3, and G4, based on the effectors to which they couple and their relative sensitivity to inactivation or down regulation. In particular, G1, G3, and G11 transduce hormone and neurotransmitter receptor-mediated modulation of ion channels.

In neurons, transmembrane Ca2+ entry via voltage-dependent calcium channels (VDCCs) is of major physiological importance, because several neuronal functions such as neuronal excitability, neuronal migration, neurite outgrowth, gene expression, and neurotransmitter release, depend on this event.

Prepulse facilitation is a phenomenon in which a train of depolarizations, or a long and strong depolarizing pulse, induces a form of the VDCCs that exhibits an increased opening probability in response to a given test potential; this persists for several seconds after repolarization. According to these models, prepulse facilitation occurs via dissociation of the G-proteins from the VDCCs, and recovery from facilitation involves rebinding of the G-proteins.

We previously reported that application of a strong depolarizing prepulse caused facilitation of VDCCs currents (ICa) in submandibular ganglion (SMG) neurons. VDCCs are negatively regulated by G-proteins. This response is primarily mediated by specific types of G-protein (Gi/Gi, and/or Gq11); these G-proteins have been shown to inhibit VDCCs. It remains unclear, however, which subtypes of G-proteins preferentially interact in prepulse facilitation of VDCCs. Therefore, the present study was planned to clarify which G-proteins contributed to the prepulse facilitation of VDCCs in SMG neurons.

MATERIALS AND METHODS

Experiments were conducted according to the guidelines for the treatment of experimental animals at Tokyo Dental College. SMG neurons from hamsters were acutely dissociated with a modified version of the method described previously. In brief, SMG neurons were isolated from 4–6-week-old hamsters and maintained in Ca2+-free Krebs solution of the following composition (in mM): 136 NaCl, 5 KCl, 3 MgCl2·6H2O, 10.9 glucose, 11.9 NaHCO3, 1.1 NaH2PO4·2H2O. The neurons were treated with collagenase type I (3 mg/ml in Ca2+-free Krebs solution; Sigma, St. Louis, MO, U.S.A.) for 50 min at 37°C, followed by incubation in trypsin type I (1 mg/ml in Ca2+-free Krebs solution; Sigma, St. Louis, MO, U.S.A.) for an additional 10 min. The supernatant was replaced with normal Krebs solution of the following composition (in mM): 136 NaCl, 5 KCl, 2.5 CaCl2, 0.5 MgCl2·6H2O, 10.9 glucose, 11.9 NaHCO3, and 1.1 NaH2PO4·2H2O. Neurons were then plated onto poly-l-lysine (Sigma)-coated glass coverslips.

Voltage-clamp recordings were conducted using the whole-cell configuration of the patch clamp technique. Fabricated recording pipettes (2–3 MΩ) were filled with an internal solution with the following composition (in mM): 100 CsCl, 1 MgCl2, 10 HEPES, 10 BAPTA, 3.6 MgATP, 14 Tris·CP, 0.1 GTP, and 50 U/ml CPK. The pH was adjusted to 7.2 with CsOH.

After the formation of a giga seal, in order to record ICa, the external Krebs solution was
replaced by solution containing the following (in mM): 67 choline-Cl, 100 tetraethylammonium chloride (TEA-Cl), 5.3 KCl, 5 CaCl₂, and 10 HEPES. The pH was adjusted to 7.4 with Tris base. Command voltage protocols were generated with a computer software pCLAMP version 8 (Axon Instruments, Union City, CA) and transformed to an analogue signal using a DigiData 1200 interface (Axon Instruments, Union City, CA). The command pulses were applied to the cell through an L/M-EPC7 amplifier (HEKA Elektronik, Lambrecht, Germany). The currents were recorded with the amplifier and a computer software pCLAMP 8 acquisition system.

Guanosin 5′-O-(2-thiodiphosphate) (GDP-β-S) and N-ethylmaleimide (NEM) were purchased from Sigma Chemical Co. Anti-Gq/11 antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). The anti-Gq/11 antibodies were derived from rabbits immunized with a synthetic peptide corresponding to the COOH-terminal sequence of the human Gq/11 subunit.

All data analyses were performed using pCLAMP 8 acquisition system. Statistical analysis was made by student t-test. Probability (p) values of less than 0.05 were considered significant.

RESULTS

An example of prepulse facilitation of I_ca in the presence of GTP (0.1 mM) in the pipette solution is shown in Fig. 1A. To evoke this facilitation, we used a double-pulse voltage protocol, as shown in Fig. 1. In this and subsequent descriptions, we refer to I_ca before and after the strong depolarizing voltage pulse as ‘I_ca − pp’ and ‘I_ca + pp’, respectively.

Scott and Dolphin[35] have suggested that tonic inhibition of the VDCCs by G-protein was responsible for the prepulse facilitation. Therefore, experiments were performed with pipette solutions containing GDP-β-S (0.1 mM), a non-hydrolyzable analogue of GDP and competitive inhibitor of G-protein to G α-subunits[7]. In this experiment, the tip of the recording pipette was filled with standard internal solution, and the pipette was then backfilled with solution substitution of GDP-β-S for GTP. An example of prepulse facilitation of I_ca in the presence of GDP-β-S (0.1 mM) in the pipette solution is shown in Fig. 1B. As shown, GDP-β-S in the pipette solution attenuated the prepulse facilitation of I_ca.

It has been shown that low concentrations of the sulfhydryl alkylating agent, NEM, can preferentially inactivate VDCCs modulation by Gᵦᵣᵦ subunit-containing, PTX-sensitive G-proteins[36]. This is presumably due to direct alkylation of the G-protein subunit because NEM can prevent ADP-ribosylation of purified Gᵦᵣᵦ subunits and modify specific amino acid residues, one of which is the substrate for ADP-ribosylation, on purified Gᵦᵦ[17]. Thus, experiments were performed in neurons pretreated with NEM (100μM for 2 min). An example of prepulse facilitation of I_ca in a neuron treated with NEM is shown in Fig. 1C. As shown, pretreatment with NEM attenuated the prepulse facilitation of I_ca.

We next attempt to test the effects of Gq/11 subunit in the prepulse facilitation of I_ca. Experiments were performed with pipette solutions containing the anti-Gq/11 antibody (1:50 dilution; final concentration was approximately 0.5 mg/ml), which was dissolved in the internal solution. In this experiment, the tip of the recording pipette was filled with standard internal solution, and the pipette was then backfilled with solution containing anti-Gq/11 antibody. An example of prepulse facilitation of I_ca in the presence of anti-Gq/11 antibody in the pipette solution is shown in Fig. 1D.

In Fig 2, the bar graphs summarize the rate of facilitation under the various conditions. In 13 neurons, the rate of prepulse facilitation of I_ca in the presence of GTP was 19.0 ± 1.9%. In 5 neurons, the rate of prepulse facilitation of I_ca in the presence of GDP-β-S was 4.4 ± 1.8%. In 5 neurons, the rate of prepulse facilitation of I_ca in neurons pretreated with NEM was 5.0 ± 1.6%. In 4 neurons, the rate of prepulse facilitation of I_ca in the presence of anti-Gq/11 antibody was 15.2 ± 2.9% (mean ± SEM).
Fig. 1

(A) Representative current tracing of ICa recorded in the presence of GTP contained in the recording pipette. Paired ICa were evoked from a holding potential of −80 mV by a 100 ms voltage step to +10 mV. An intervening strong depolarizing prepulse (100 mV, 30 ms) ended 5 ms prior to the second ICa activation. Current calibration, 1,000 pA; Time calibration, 100 ms. In this and subsequent descriptions, we refer to ICa before and after the strong depolarizing voltage pulse as ‘ICa−pp’ and ‘ICa+pp’, respectively.

(B) Representative current tracing of ICa recorded in the presence of GDP-β-S contained in the recording pipette.

(C) Representative current tracing of ICa in neuron pretreated with NEM (100 μM for 2 min).

(D) Representative current tracing of ICa recorded in the presence of anti-Gq/11 antibody contained in the recording pipette (1:50 dilution for 7 min).
As shown above, pretreatment of neurons with Gi/o block attenuated the rate of prepulse facilitation, but intracellular dialysis with anti-Gq/11 antibody did not alter it. These results suggest that prepulse facilitation of VDCCs is due to Gi/o-types of G-protein, but not to the Gq/11-type, in SMG neurons. Artalejo et al. concluded that the prepulse facilitation does not involve G-protein activation\(^1\). In contrast, Doupnik et al. concluded that a block of Gi/o-proteins abolished prepulse facilitation\(^6\). Our data support the thesis that a block of G\(_{\alpha}\)-proteins, but not of Gq/11-proteins, abolished the prepulse facilitation in SMG neurons.

It has also been proposed that G-protein molecules act simultaneously at the VDCCs complex to promote inhibition\(^24\). Distinct G-protein subtypes serve to transduce activation of various GPCRs. For instance, G\(_{\alpha}\) transduces the norepinephrine receptor\(^4,11\), \(\gamma\)-aminobutyric acid receptor\(^8\), opioid receptor\(^35\), serotonin receptor\(^10\), and somatostatin receptor-modulation\(^11,25\) of VDCCs. Gq/11 transduces the muscarinic receptor\(^23\), neurokinin receptor\(^23\), and angiotensin II receptor-modulation\(^40\) of VDCCs. The ligand-bound GPCR initiates two processes; one leads to desensitization and occurs through receptor modification, and the other is a signal generating process that begins with the activation of the heterotrimeric G-protein. Interaction of the G-protein with the activated receptor promotes the exchange of GDP, bound to the \(\alpha\) subunit, for GTP and the subsequent dissociation of the \(\alpha\)-GTP complex from the \(\beta\gamma\) heterodimer. A single receptor can activate multiple G-protein molecules, thus amplifying the ligand binding event. The \(\alpha\) subunit with GTP bound and the free \(\beta\gamma\) subunit may interact with effector proteins that further amplify the signal. Such effectors include ion channels and enzymes that generate regulatory molecules or second messengers. Termination of the signal occurs when GTP bound by the \(\alpha\) subunit of the G-protein is hydrolysed to GDP. The \(\alpha\) subunit then reassociates with the \(\beta\gamma\) complex.

There are at least two distinct forms of prepulse facilitation of VDCCs. One type, prepulse facilitation of N- and P/Q-type VDCCs, is mediated by direct interactions between \(\beta\gamma\) subunits and VDCCs\(^16,39\). In this model, VDCCs are tonically inhibited by \(\beta\gamma\) subunits\(^14,19\). Application of a prepulse may release \(\beta\gamma\) subunits from VDCCs. Another type, prepulse facilita-
tion of L-type VDCCs of thalamic neurons, is independent of G-proteins and phosphorylation\(^2\). Similar to previous suggestions that direct conformational changes in the L-type VDCCs protein underlie the mechanism for the voltage-dependent facilitation in smooth muscle L-type VDCCs\(^2\), these authors concluded that no chemical modification was necessary to produce the facilitated state of the VDCCs.

In a previous study, we reported that SMG possessed T-, L-, N-, P/Q- and R-type VDCCs\(^8\). These prepulse facilitations of L-type VDCCs in SMG neurons may be interpreted as a shift in the gating of the VDCCs toward a new mode in which an additional voltage-dependent gating (modulation gating) functions. A sufficiently depolarized prepulse transiently in the number of channels in the willing mode of gating increases the number of channels opened by a depolarizing step into the voltage ranges of willing gating in SMG neurons. Furthermore, we also suggest that G-protein-dependent tonically inhibition of N- and P-type VDCCs can be reversed by application of a prepulse in SMG neurons.

In SMG neurons, we previously demonstrated that several different neurotransmitters produce modulation of VDCCs via a pathway using G-proteins\(^20,38\). GPCR activated by neurotransmitters couple to the carboxy-terminals of G-protein \(\alpha\) subunits, and polyclonal antibodies raised against C-terminal peptide sequences of different \(G\) subunits have been shown to functionally antagonize neurotransmitter modulation of VDCCs (e.g. anti-G\(\alpha\), anti-G\(\alpha\(\gamma\))\(^25\)).

The results in this paper thus provide a possible mechanism for effects of G\(\alpha\)\(\gamma\), but not G\(\alpha\)\(\gamma\) protein, in the inhibitory regulation of VDCCs and dissociation of \(\beta\gamma\) subunits from heterotrimeric G-protein to serve in signal transduction.

**REFERENCES**


