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VIP AND PACAP INHIBIT L-, N- AND P/Q-TYPE Ca\textsuperscript{2+} CHANNELS OF PARASYMPATHETIC NEURONS IN A VOLTAGE INDEPENDENT MANNER

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Abstract

In this study, we investigated the effects of vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide 1-38 (PACAP) on the voltage-gated calcium currents in hamster submandibular ganglion neurons. VIP and PACAP inhibited the high threshold voltage-gated calcium current in a voltage-independent and a concentration-dependent manner via the G protein-mediated pathway. L-, N- and P/Q-type components of the total maximum voltage-gated calcium current accounted for 48.0 ± 3.1% (n = 4), 35.1 ± 4.7% (n = 4), and 13.5 ± 2.3% (n = 3) of the total peak amplitude, respectively. VIP at a concentration of 1μM inhibited the L-type calcium current by 33.2% ± 1.4% (n = 4), the N-type current by 31.0 ± 3.6%, and the P/Q-type current by 3.2 ± 1.1% (n = 3). PACAP at a concentration of 1μM inhibited the L-type current by 35.6 ± 5.7%, the N-type current by 34.4 ± 3.1% (n = 4), and the P/Q-type current by 6.4 ± 2.1% (n = 2). However, VIP and PACAP did not inhibit the low threshold voltage-gated (T-type) calcium current. The rank order of potency was PACAP>VIP. In experiments replacing GTP with GDP-β-S, the inhibitory effects of VIP and PACAP were prevented. In experiments of double-pulse protocol, depolarizing conditioning pulses could not relieve the inhibition of total high threshold voltage-gated calcium currents produced by VIP and PACAP. Therefore, the inhibition of the high threshold voltage-gated calcium channels produced by VIP and PACAP in hamster parasympathetic neurons differed in its mechanisms from that of N-type calcium channels in rat sympathetic neurons.

Key words: VIP—PACAP—Voltage-independent inhibition—Calcium currents—Submandibular ganglion

INTRODUCTION

The submandibular ganglion (SMG) belongs to the parasympathetic system and innervates the submandibular salivary gland to control saliva secretion. Noncholinergic, nonadrenergic neurotransmitters have been found in cell bodies and nerve fibers within mammalian parasympathetic ganglia\textsuperscript{12}. In the SMG, strong vasoactive intestinal polypeptide (VIP) immu-
noreactivity is observed in most cell bodies\(^2\). Following stimulation of the chorda tympani nerve, VIP is released from the cat submandibular gland together with acetylcholine (ACh)\(^3\). VIP, injected intravenously, evokes a small flow of saliva from three major glands of the rat in the presence of atropine and adrenergic agonists; of these three, the submandibular gland secretes the most saliva\(^4\). In experiments on salivary glands of the cat, when VIP had been infused during ongoing secretion elicited by parasympathetic stimulation (in the absence of atropine) or administration of a parasympathomimetic drug, both fluid and protein secretion increased\(^5\).

Pituitary adenylate cyclase-activating polypeptide (PACAP) belongs to a family of structurally and biologically related peptides including VIP, secretin, glucagon, peptide histidine isoleucine, and growth hormone-releasing hormone\(^15,23\), and has an N-terminal sequence that exhibits 68% homology with VIP\(^1,3\). It possesses 1,000 times the potency of VIP in activating adenylate cyclase in the rat pituitary cells in culture\(^8\). Intravenous injection of PACAP (PACAP 1-38) in the rat in the presence of adrenoceptor blockers and atropine also evokes secretion of saliva from the major salivary glands\(^3\). When comparing the secretory responses of VIP with those of PACAP in the rat, the effectiveness of PACAP was the same or less than that of VIP. However, when comparing the effect of these peptides on the vascular response of the submandibular glands, PACAP was more effective than VIP in reducing the vascular resistance and in increasing the blood flow\(^3\).

VIP was previously shown to induce depolarizations primarily by a decrease in \(K^+\) conductance (\(g_K\)) in 80% of hamster SMG neurons\(^26\). Co-application of VIP and ACh potentiated muscarinic ACh-evoked potentials in this study. PACAP also induced slow depolarizations accompanied by both a decrease and increase in membrane conductance (\(g_m\)) in hamster SMG neurons (Suzuki, unpublished). VIP and PACAP have recently been shown to potentiate nicotinic ACh-evoked currents via mediation of the guanine nucleotide binding protein (G protein) activation in SMG neurons and intracardiac ganglion neurons of rats\(^13\). VIP and PACAP exert tissue-specific effects through the activation of specific isoforms of the G-protein-coupled VIP- and PACAP-selective receptors; VPAC\(_1\), VPAC\(_2\), and PAC\(_3\) receptors\(^28\). Elevation of intracellular cAMP levels has been proposed as the mechanism by which VIP and PACAP potentiated ACh-evoked currents in chick ciliary ganglion neurons\(^14\).

VIP and PACAP play neurotransmitter, secretagogue, neuroprotective, neurotrophic, mitogenic and differentiation roles in neurons\(^1\). Effects of VIP on the modulation of calcium channels in neurons are not well characterized. A recent study in sympathetic neurons dissociated from rat superior cervical ganglia showed that VIP primarily inhibited N-type calcium currents in a voltage-dependent manner\(^25\). In this study, we investigated the effects of VIP and PACAP on low and high threshold voltage-gated calcium currents in hamster SMG (parasympathetic) neurons. In addition, the involvement of G protein in the inhibition of these calcium channels was investigated. A brief preliminary report on the effects of VIP has been published previously\(^6\).

**MATERIALS AND METHODS**

1. **Cell preparation**

Experiments were conducted according to the Guiding Principles for the Care and Use of Animals approved by the Council of the Physiology Society of Japan. The neurons of hamster SMG were freshly dissociated as reported previously\(^22\). Briefly, male hamster 4–6 weeks old were anesthetized using pentobarbital sodium (30 mg/kg, i.p.), and SMG neurons were isolated. SMG neurons were identified as neuron clusters located along the duct in the hilus of the submandibular gland. Isolated SMG neurons were maintained in \(Ca^{2+}\)-free Krebs solution. SMG neurons were treated with collagenase type I (3 mg/ml in \(Ca^{2+}\)-free Krebs solution; Sigma) for 50 min at 37°C, followed by incubation in trypsin type I
solution (1 mg/ml in Ca²⁺-free solution; Sigma) for an additional 10 min. The supernatant was replaced with normal Krebs solution with the following composition (in mM): 136 NaCl, 5 KCl, 2.5 CaCl₂, 0.5 MgCl₂, 6 H₂O, 10.9 glucose, 11.9 NaHCO₃, and 1.1 NaH₂PO₄·2 H₂O. Neurons were planted on poly-L-lysine-coated coverslips.

2. Whole-cell patch-clamp recordings

Voltage-clamp recordings were conducted using the whole-cell configuration of the patch-clamp technique. Neurons with clear cytoplasm and a well-defined shape without granules were selected for recording. The neurons were planted on the recording chamber bottom of the glass coverslip, placed on the stage of an upright microscope (BH-2, Olympus), and was viewed under differential interference-contrast optics. The neurons were perfused at a rate of 2 ml/min. The bath solution was connected to the ground via a Krebs-agar bridge and an Ag-AgCl electrode. Patch pipettes were pulled from glass capillary tubes (1 mm o.d.; Mercer Glass Works, Inc.) using a Flaming/Brown micropipette puller (model P-80/PC; Sutter Instrument Co.) and polished by a heat polisher (model MF-83; Narishige Co.) before use; they were filled with an internal solution of the following composition (in mM): 100 CsCl, 1 MgCl₂, 10 HEPES, 10 BAPTA, 3.6 MgATP, 14 phosphocreatine-Tris₂, 0.1 GTP or 5’-O-(2-thiodiphosphate) (GDP-S) and 50 U/ml phosphocreatine kinase. The pH was adjusted to 7.2 with CsOH. After the formation of a giga seal, in order to record calcium current, the external solution was exchanged from Krebs solution for a 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. Under these conditions, the calcium current was isolated by eliminating the Na⁺ current by the replacement of external Na⁺ with choline (67 mM) and by minimizing the K⁺ current with a combination of cesium (130 mM) in the internal solution and TEA-Cl (100 mM) in the external solution. Command voltage protocols were generated with a computer software pCLAMP version 6 (Axon Instruments) and transformed to an analogue signal using a DigiData 1200 interface (Axon Instruments). The command pulses were applied to the neuron through an L/M-EPSC 7 amplifier (HEKA Electronik). The currents were recorded with the amplifier and computer software pCLAMP 6 acquisition system. All experiments were performed at room temperature (24–27°C).

3. Solutions and reagents

VIP, PACAP1-38, ω-conotoxin GVIA (ω-CgTx), and ω-agatoxinIVA (ω-AgTx) were purchased from Peptide Institute, Inc. All drugs except nifedipine were dissolved in distilled water. Nifedipine was dissolved in DMSO as a stock solution at a concentration of 100μM. All drugs were diluted to the desired final concentration in the external solution immediately before use. The final concentration of DMSO was 0.01%, which had no effect on the calcium current.

4. Data analysis and statistics

All data analysis was performed using the pCLAMP 6 acquisition system. Values in the text and figures are expressed as mean ± S.E. of the number (n) of tested neurons, unless otherwise indicated. Statistical analysis was made by paired Student’s t-test to evaluate the statistical significance. Probability (P) values of less than 0.05 were considered significant.

RESULTS

1. Effects of VIP and PACAP on high threshold voltage-gated calcium currents

VIP reduced the amplitude of the total voltage-gated calcium current and slowed the rising phase of the current. The effects of VIP and PACAP on total voltage-gated calcium currents are shown in Fig. 1. Concentration-response relationships for VIP and PACAP inhibitions of total voltage-gated calcium currents are shown in Fig. 2. The maximum effective concentrations of VIP and PACAP for the total calcium current
were 0.1μM (Fig. 2). VIP at a concentration of 0.1μM reduced the peak amplitude of the total maximum voltage-gated calcium current (I_{Ca}) by 39.4 ± 0.3% (n = 6). PACAP at a concentration of 0.1μM also inhibited the peak amplitude of the total maximum voltage-gated calcium current by 49.6 ± 4.2% (n = 6).

2. Effects of VIP and PACAP on the low threshold voltage-gated calcium current

VIP at a concentration of 1μM did not inhibit low threshold voltage-gated calcium currents (T type; n = 5) (Fig. 3). PACAP (1μM) followed a similar pattern (n = 5, data not shown). Therefore, neither VIP nor PACAP affected the T type calcium current in hamster SMG neurons.

![Fig. 1 VIP and PACAP effects on high threshold voltage-gated calcium currents](image1)

(A) Current traces: Calcium currents (I_{Ca}) were evoked by applying a depolarizing test pulse from a holding potential = −80mV to a test potential = −10mV. VIP (1μM) decreased the peak I_{Ca} by 37.2% from −2,635.0pA (control I_{Ca}) to −1,655.0pA.

(B) Current traces: PACAP (1μM) decreased the peak I_{Ca} by 30.9% from −3,060.0pA (control I_{Ca}) to −2,115.0pA.

![Fig. 2 Concentration-response relationships for VIP and PACAP inhibitions of the total calcium currents](image2)

Ordinate, percentages of inhibition of I_{Ca}; abscissa, concentration of VIP or PACAP. Bars are presented as mean ± S.E., and the number of the tested neurons is indicated in the parentheses.

![Fig. 3 VIP effect on low threshold voltage-gated calcium (T-type) current](image3)

Current traces: I_{Ca} were evoked by applying a depolarizing test pulse from a holding potential = −80mV to a test potential = −40mV in a neuron. VIP (1μM) did not inhibit the peak of low threshold voltage-gated calcium (T-type) current.

![Fig. 4 Current-voltage relationships of VIP and PACAP effects](image4)

Current-voltage (I-V) relationship in control (below line) as well as after application of 1μM VIP (central line) and 1μM PACAP (upper line). Data are presented as mean ± S.E., and the number of the tested neurons is indicated in the parentheses.
3. Effects of VIP and PACAP on the L-, N- and P/Q-type calcium currents

Current-voltage (I-V) relationships for high threshold voltage-gated calcium currents in the control as well as after application of 1 μM VIP and 1 μM PACAP are shown in Fig. 4. VIP and PACAP inhibited the high threshold voltage-gated I_{Ca}. The rank order of potency for the inhibition was PACAP > VIP. Effects of VIP on L-, N- and P/Q-type calcium currents are shown in Fig. 5A, B, and C, respectively. The percentage inhibition of each type of calcium current by VIP is shown in Fig. 6A. L-, N- and P/Q-type components of total maximum voltage-gated I_{Ca}, accounted for 48.0 ± 3.1% (n = 4), 55.1 ± 4.7% (n = 4), and 13.5 ± 2.5% (n = 3) of the total peak amplitude, respectively.

L-type calcium currents were evoked by a command pulse from a holding potential = −80 mV to −10 mV in neurons treated with 1 μM ω-CgTx and 1 μM ω-AgTx. VIP at a concentration of 1 μM inhibited L-type calcium currents by 33.2 ± 1.4% (n = 4) (shown in Fig. 6A).
N-type calcium currents were evoked by a command pulse from a holding potential = −80 to −10 mV in neurons treated with 1 μM ω-AgTx and 1 μM nifedipine. VIP at a concentration of 1 μM inhibited N-type calcium currents by 31.0 ± 3.6% (n = 4) (shown in Fig. 6A).

P/Q-type calcium currents were evoked by a command pulse from a holding potential = −80 mV to −10 mV in neurons treated with 1 μM ω-CgTx and 1 μM nifedipine. VIP at a concentration of 1 μM inhibited P/Q-type calcium currents by 3.2 ± 1.1% (n = 3) (shown in Fig. 6A).

Percentage inhibitions of L- and N-types of calcium currents by PACAP, compared with those of VIP, are shown in Fig. 6B. PACAP at a concentration of 1 μM inhibited N-type calcium currents by 34.4 ± 3.1% (n = 4), L-type calcium currents by 35.6 ± 5.7% (n = 4) (shown in Fig. 6B), and P/Q-type calcium currents by 6.4 ± 2.1% (n = 2, data not shown).

4. The participations of G protein in inhibition of VIP on the total high threshold voltage-gated calcium current and the effect of depolarizing conditioning pulse on it

In experiments replacing GTP with GDP-β-S, the effect of VIP on the total high voltage-gated calcium current was investigated when 0.1 mM GDP-β-S was present in the recording pipette. VIP at a concentration of 1 μM inhibited the control peak ICa only by 7.7 ± 2.4% (n = 3) (shown in Fig. 7A). PACAP at a concentration of 1 μM inhibited the control peak ICa only by 6.9 ± 2.9% (n = 2, data not shown).

The effect of a double-pulse protocol on the total high threshold voltage-gated calcium current was also investigated. The calcium currents were evoked using a double-pulse protocol consisting of two identical test pulses with durations of 100 msec to −10 mV from a holding potential = −80 mV. A large depolarizing conditioning pulse (to +100 mV) was followed by the latter command pulse. The conditioning pulse did not relieve the effect of PACAP (1 μM).

The inhibition of high threshold voltage-gated peak ICa by VIP(1 μM) in the first command pulse was 36.9 ± 5.7% (n = 4) of the control ICa, while the inhibition of the peak ICa in the second command pulse was 36.6 ± 6.5% of the control peak ICa. The inhibition of high threshold voltage-gated ICa by PACAP in the first command pulse was 46.3 ± 1.8% of the
control peak $I_{Ca}$, while the inhibition of the peak $I_{Ca}$ in the second command pulse was $48.6 \pm 4.1\%$ of the control peak $I_{Ca}$.

**DISCUSSION**

In this study, we investigated the effects of VIP and PACAP on the voltage-gated calcium currents in hamster submandibular ganglion neurons. VIP and PACAP inhibited the high threshold voltage-gated calcium current in a voltage-independent and a concentration-dependent manner via the G protein-mediated pathway. The rank order of potency was PACAP > VIP. However, VIP and PACAP did not inhibit the low threshold voltage-gated (T-type) calcium current.

The L- and N-type calcium currents were inhibited by VIP and PACAP, but these molecules hardly inhibited the P/Q-type calcium currents. We proved by the double-pulse protocol that the effects of VIP and PACAP were voltage-independent. The G protein is composed of $\alpha$, $\beta$, and $\gamma$-subunits. The $\beta$- and $\gamma$-subunits directly modulate $Ca^{2+}$ channels in voltage-dependent manner. At this step of this study, we have no evidence relevant to the subunits. Therefore, we will have to elucidate an associated subunit of the G protein in the near future.

The best characterized calcium channel modulation in mammalian sympathetic neurons is an inhibition of N-type channels via a pertussis toxin (PTX)-sensitive G protein. In rat sympathetic neurons, VIP inhibited N-type calcium channels in a voltage-dependent and membrane-delimited manner. The effect of VIP was insensitive to PTX but was attenuated by cholera toxin or anti-$G_{sa}$ antibodies. The evidence indicated that the N-type calcium channel modulation required activation of $G_{sa}$ but was independent of protein kinase A (PKA)-mediated phosphorylation. The result by using a double pulse protocol indicated that the effect of VIP on the N-type calcium channels in rat superior cervical ganglion neurons was voltage-dependent.

Although VIP has been shown to increase levels of cAMP in various tissues, including the rat superior cervical ganglion, it is still unclear whether the calcium channel modulation in hamster SMG (parasympathetic ganglion) neurons is dependent on or independent of an elevation in intracellular cAMP and an activation of PKA.

Receptors for VIP and related peptides belong to a subfamily of seven transmembrane-spanning proteins that couple to the heterotrimeric G protein, $G_{i/o}$. The G protein, activated $G_{s}$, directly stimulates cardiac L-type calcium channels via its $\alpha$ subunit. The $\alpha$ subunit generally mediates effects of target effectors through the cAMP signal transducer.

Using parasympathetic neurons (SMG neurons), we performed experiments to determine the requirement for a G protein in the transduction pathway, because GDP-$\beta$-S is a useful tool for the identification of G protein-mediated responses. Although, we still have not identified the subtype of G protein involved in the inhibition of the L-, N-, and P/Q-type calcium channels, we proved a voltage-independency in the inhibition of the high threshold voltage-gated calcium current of hamster SMG neurons using double-pulse protocol (Fig. 7).

VIP and PACAP potentiation of nicotinic ACh-evoked currents in rat parasympathetic neurons (SMG and intracardiac ganglia) was mediated by G protein activation. The G protein subtype modulating the neuronal nicotinic ACh receptors (nAChRs) was examined by intracellular dialysis with antibodies. Only the anti-$G_{o}$ and anti-$G_{\beta}$ antibodies significantly inhibited the effect of VIP and PACAP on ACh-evoked currents. VPAC1 receptors probably mediated effects of VIP. PACAP-induced potentiation of the ACh-induced current was not additive, suggesting that activation of VPAC1 and PAC1 receptors converged to modulate nAChRs in rat parasympathetic neurons. The potentiation of ACh-evoked currents by VIP and PACAP might be mediated by a membrane-delimited signal transduction cascade involving the PTX-sensitive $G_{o}$ protein in rat intracardiac and submandibular ganglion neurons.
Members of the PACAP/VIP receptor family have been shown to couple to a variety of G proteins, including G_s, G_q/11, G_o and G_{i1–2}.

VIP (10 μM) induced slow depolarizations in 80% of tested neurons in hamster SMG. The g_m during the slow depolarization decreased by 18–20% of the control value. In 7% of the neurons, the g_m was accompanied by both a decrease and increase. PACAP (10 μM) also induced slow depolarizations accompanied by both a decrease and increase in g_m in approximately 80% of tested neurons (Suzuki, unpublished observation). Studies in neuronal tissue have shown that VIP activates a Na⁺-selective cation current and inhibits three distinct g_Ks: a background g_K, a g_K(Ca), and an inward rectifier g_K. An inhibition of slow g_K(Ca) was induced via stimulation of adenylate cyclase, while g_Cation was activated. In addition, an inhibition of high threshold voltage-gated I_Ca by VIP or PACAP may have potentiated the inhibition of slow g_K(Ca), through a decrease in Ca²⁺ entry.

The identity of the receptor subtype that mediates VIP- and PACAP-induced calcium channel modulation in hamster SMG neurons is unclear. However, VIP and PACAP induced slow depolarizations in hamster SMG neurons, and the rank order of the potency was PACAP>VIP (Suzuki, unpublished observation). Therefore, hamster SMG neurons may possess PAC1 receptors as well as VPAC1 receptors, taking into account of the VIP- and PACAP-potentiation of nicotinic ACh-evoked in parasympathetic neurons.

The physiological relevance of VIP- and PACAP-mediated calcium channel modulation in hamster SMG neurons is yet to be defined. VIP-like immunoreactivity is observed in most of somata of postganglionic parasympathetic neurons such as the submandibular, otic, and sphenopalatine ganglia, but is unclear on pre-ganglionic parasympathetic neuronal somata and preganglionic nerve fibers surrounding the postganglionic somata. The presence and potential origin of PACAP was determined in the cardiac ganglia of the mudpuppy. PACAP-immunoreactivity was found primarily in visceral afferent fibers, originating from neuronal somata in either the dorsal root ganglia or vagal sensory ganglia. VIP- and PACAP-containing nerve fibers may serve as local reflex inputs that modulate postganglionic neuronal output within the hamster SMG.

REFERENCES


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