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Electrophysiological and Immunofluorescence Characterization of Ca²⁺ Channels of Acutely Isolated Rat Sphenopalatine Ganglion Neurons

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Summary

The sphenopalatine ganglion (SPG) is the main parasympathetic ganglion that is involved in regulating cerebral vascular tone and gland secretion. SPG neurons have been implicated in some types of migraine headaches but their precise role has yet to be determined. In addition, very little information is available regarding ion channel modulation by neurotransmitters that are involved in the parasympathetic drive of SPG neurons. In this study, acute isolation of adult rat SPG neurons was developed in order to begin the electrophysiological characterization of this ganglion. Under our dissociation conditions, the average number of neurons obtained per ganglion was greater than 1200. Immunofluorescence imaging results showed positive labeling with acetylcholinesterase (AChE), confirming the parasympathetic nature of SPG neurons. On the other hand, weak tyrosine hydroxylase immunostaining was observed in these neurons. Whole-cell patch-clamp recordings revealed that most of the Ca²⁺ current is carried by N-type (53%) and SNX-482 resistant R-type (30%) Ca²⁺ channels. In addition, Ca²⁺ currents were inhibited in a voltage-dependent manner following exposure to oxotremorine-M (Oxo-M), norepinephrine and ATP via muscarinic acetylcholine receptor 2 (M₂ AChR) subtype, adrenergic and P2Y purinergic receptors, respectively. The peptides VIP and angiotensin II failed to modulate Ca²⁺ currents, suggesting that these receptors are not present on the SPG soma or do not couple to Ca²⁺ channels. In summary, our data suggest that the Ca²⁺ current inhibition mediated by Oxo-M, NE and ATP in adult rat SPG neurons plays an integral part in maintaining parasympathetic control of cranial functions.

Keywords

sphenopalatine ganglion; parasympathetic; N-type Ca²⁺ channels; whole-cell patch-clamp; immunofluorescence

The sphenopalatine ganglion (SPG) is part of the parasympathetic branch of the autonomic nervous system and is located in the pterygopalatine fossa beneath the maxillary nerve [7]. SPG neurons receive projections from the superior salivatory nucleus and both sympathetic and sensory neurons course through the ganglion as well [7]. SPG neurons innervate the cerebral vasculature, nasal, palatine and lacrimal glands, and thus, regulate cerebral blood flow and secretion from the aforementioned glands [8]. Few electrophysiological studies have been undertaken to examine signaling mechanisms that couple Ca²⁺ channels and G protein-coupled

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receptors (GPCR) in SPG neurons. Neurotransmitter release in autonomic neurons is regulated by Ca^{2+} entry through voltage-gated Ca^{2+} channels. One study found that activation of the M_2 muscarinic acetylcholine receptor subtype (mAChR) lead to inhibition of Ca^{2+} channels in rat SPG neurons kept in culture 1–3 weeks [16]. The purpose of the present study was to begin to characterize the coupling pathways in acutely dissociated adult rat SPG neurons between Ca^{2+} channels and neurotransmitters that may be involved in modulating parasympathetic function. Immunofluorescence staining was initially employed with antibodies targeted against, acetylcholinesterase (AChE) and tyrosine hydroxylase (TH), ACh-degrading enzyme and specific marker for adrenergic neurons, respectively. Thereafter, whole-cell patch-clamp recordings were performed to determine the Ca^{2+} channel subtypes expressed in SPG neurons and the coupling of these channels to several agonists known to modulate Ca^{2+} channel currents. It should be noted that the electrophysiological experiments were performed on the SPG soma and the results may not reflect events at the nerve terminals.

The experiments performed were approved by the Penn State College of Medicine Animal Care and Use Committee (IACUC). SPG neurons were isolated from adult Wistar male rats (175–225 g). Rats were anaesthetized with CO_2 and immediately decapitated. The ganglia were dissected in cold Hanks' balanced salt solution and this was followed by enzymatic dissociation in Earle's balanced salt solution with 0.6 mg/ml collagenase (Roche Diagnostics Corp., Indianapolis, IN) and 0.4 mg/ml trypsin (Worthington Biochemical Corp., Lakewood, NJ), saturated with 95% O_2 /5% CO_2 for 40 min at 35°C . Following the dissociation period, the neurons were mechanically dispersed in a culture flask by vigorous shaking. The dispersed neurons were then centrifuged twice for 6 min at 1000 rpm, resuspended in minimal essential medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% glutamine (all from Invitrogen Corp., Carlsbad, CA) and plated on poly-L-lysine coated 35 mm tissue culture plates. Thereafter, the neurons were stored in a humidified incubator with 5% CO_2 /95% air at 37°C . Rat superior cervical ganglion (SCG) neurons were isolated as previously described [25].

Ca^{2+} channel currents were recorded employing the whole-cell variant of the patch-clamp technique. Patch pipettes were pulled from borosilicate glass (type 8250; Garner Glass Co., Claremont, CA) on a P-97 micropipette puller (Sutter Instrument, Novato, CA) and fire polished on a microforge. Whole-cell currents were acquired with the Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA), analog filtered at 5–10 kHz (–3 dB; 4-pole Bessel) and digitized using custom-designed software (S5) on a Macintosh PowerPC computer equipped with a 16-bit analog-to-digital converter board (Instrutech Corp., Elmont, NY). The pipette internal solution contained (in mM): 120 *N*-methyl-D-glucamine, 20 tetraethylammonium hydroxide (TEA-OH), 20 HCl, 11 EGTA, 10 HEPES, 1 CaCl_2 , 4 MgATP, 0.3 Na_2GTP , 14 tris creatine phosphate, pH 7.2 and osmolality of 286 mOsm/kg. The external solution contained (in mM): 140 $\text{CH}_3\text{SO}_3\text{H}$, 145 TEA-OH, 10 HEPES, 15 Glucose, 10 CaCl_2 , pH was adjusted to 7.4 and osmolality of 324 mOsm/kg. In experiments where the agonist Oxo-M was employed, the external solution contained (in mM): 155 Tris hydroxymethyl aminomethane, 20 HEPES, 10 glucose, 10 CaCl_2 , pH was adjusted to 7.4 and osmolality of 337 mOsm/kg. When the M4-toxin was used, the external solution employed contained the TEA-containing solution. All experiments were performed at room temperature (22 – 24°C). Data analysis was performed with Igor Pro (Wavemetrics, Lake Oswego, OR). Stock solutions of norepinephrine bitartrate salt (NE), angiotensin II (Ang II), α -Conotoxin GVIA (Ctx), Nifedipine (Nif), Na_2 -ATP (ATP), M4-toxin (M4tx), oxotremorine methiodide (Oxo-M; all from Sigma Chemical Co., St. Louis, MO), vasoactive intestinal peptide (VIP; Phoenix Pharmaceuticals, Belmont, CA), α -Agatoxin IVA (Aga) and SNX-482 (both from Alomone Labs, Jerusalem, Israel), were prepared in water and diluted in the external bath solution to their final concentrations just prior to use.

For immunofluorescence, SPG neurons were rinsed five times with 1X phosphate-buffered saline (PBS) and fixed with 2% formaldehyde/2% sucrose in 1X PBS for 20 minutes. Thereafter, the neurons were rinsed five times with 1X PBS and permeabilized in 0.05% TWEEN 20 in 1X PBS/5% goat serum for 10 minutes at 37°C. SPG neurons were rinsed five times with 1X PBS/5% goat serum and then incubated with either the mouse polyclonal antibody targeted against rat acetylcholinesterase enzyme (AChE, 1:2000) or tyrosine hydroxylase (TH, 1:200) (both from Abcam Inc., Cambridge, MA) in 1X PBS/5% goat serum for 60 minutes at room temperature. The neurons were then rinsed five times with 1X PBS and incubated with 1X PBS/5% goat serum for 60 minutes prior to addition of the secondary goat anti-mouse IgG antibody conjugated to Alexa Fluor-488 (Invitrogen) at a final concentration of 3 @g/ml. SPG neurons were incubated with the secondary antibody for 45 minutes and then rinsed three times with 1X PBS. Phase contrast and fluorescence images were acquired with a Nikon TE2000 microscope (Nikon, Japan), an Orca-ER 1394 digital CCD camera (Hamamatsu Photonics, Japan) and IPLab software (Scanalytics Inc., Fairfax, VA). Fluorescence images were obtained with a filter set containing an excitation filter at 480 ± 15 nm, a dichroic beam splitter of 505 nm (LP) and an emission filter at 535 ± 20 nm (B-2E/C, Nikon) and pseudocolored with IPLab software.

In rats, the number of neurons in SPG ranges from 4,900 to 11,000 [4,18]. Under our experimental conditions, the total number of isolated neurons per animal was 1213 ± 134 (SEM; $n=3$). This yield is greater than that reported in a previous study (< 100 neurons/rat) employing SPG [15]. In the first set of experiments, immunofluorescence assays were employed to determine the neurochemical phenotype of the acutely dissociated SPG neurons. The cells were stained for acetylcholinesterase (AChE), a marker for parasympathetic neurons. Figure 1A is a fluorescence image of SPG neurons immunostained with a specific AChE antibody. The inset shows the phase contrast image of the same field of neurons. All SPG neurons showed positive staining for AChE, though the fluorescence intensity was variable. Similar observations were reported to occur in cat SPG neurons [22]. Rat superior cervical ganglion (SCG) neurons were employed as negative controls for AChE staining. As shown in Figure 1B, no fluorescence staining was observed in this sympathetic ganglia. Figures 1E and 1F show the fluorescence images of SPG neurons that were immunostained with the secondary antibody in the absence of primary AChE (E) and TH (F) antibodies. Images of these negative controls show there is minimal non-specific labeling by Alexa Fluor-488 at the concentration employed.

It has been reported that SCG nerve bundles course through the SPG in close proximity to pericaryons [21] and these sympathetic neurons also form synapses with SPG neurons [20]. Thus, SPG neurons were immunostained for the presence of tyrosine hydroxylase, a marker of catecholamine synthesizing neurons. Approximately 80% of cells were TH-positive, however, the fluorescent intensity was weak (Figure 1C). SCG neurons, shown in Figure 1D, were employed as a positive control, and all stained positive for TH.

In the next set of experiments, the whole-cell patch-clamp technique was employed to identify the Ca^{2+} channel subtypes present in acutely dissociated SPG neurons. Previous studies have shown that the majority of Ca^{2+} currents in parasympathetic neurons can be carried by N-type [10], L-type [9] or both channel subtypes [24]. Ca^{2+} currents were typically recorded 24 to 36 hr following cell dissociation. Figure 2A shows the time course of peak Ca^{2+} current amplitude of an SPG neuron before and after external application of channel blockers: 10 @M nifedipine (Nif), 0.2 @M *-agatoxin (Aga), 0.3 @M SNX-482 (SNX) and 10 @M *-conotoxin (Ctx). Ca^{2+} currents were evoked by applying the double-pulse voltage protocol (top, Figure 2B), whereby two identical test pulses (to +10 mV) were separated by a large depolarizing conditioning pulse to +80 mV and then returned to -80 mV. Currents were recorded every 10 sec. Current inhibition (%) was determined as follows: $\text{ICa}^{2+}_{(a)} - \text{ICa}^{2+}_{(b)} / \text{ICa}^{2+}_{(a)} \times 100$, where $\text{ICa}^{2+}_{(a)}$ and $\text{ICa}^{2+}_{(b)}$ are current amplitude measured isochronally 10 ms into the test pulse

(“pre”) before and during agonist application, respectively. Figure 2B shows superimposed Ca^{2+} currents before and during application of channel blockers. Exposure of the cell to the L-type Ca^{2+} channel blocker, Nif, led to an 8% increase of Ca^{2+} currents. Following the washout period, Aga (P/Q-type Ca^{2+} channel toxin) application resulted in a 9% inhibition, while the R-type Ca^{2+} channel blocker had no effect on the peak Ca^{2+} current. External application of Ctx (N-type Ca^{2+} channel toxin) resulted in approximately a 54% inhibition of Ca^{2+} currents. Figure 1C is a summary plot of the Ca^{2+} current inhibition produced by the channel blockers. The results show that in SPG neurons, N-type Ca^{2+} channels carry over 50% of the Ca^{2+} current while P/Q-, L- and R-type channels each contribute less than 15%.

The signaling mechanisms of neurotransmitters known to modulate N-type Ca^{2+} channels were examined next. The double-pulse voltage protocol, described above, was used to determine whether Ca^{2+} currents are modulated in a voltage-dependent (VD) or voltage-independent (VI) manner following exposure to the nonspecific mAChR agonist Oxo-M [25]. Of the five mAChR known (M_{1-5}), M_2 and M_4 mAChR subtypes preferentially couple to PTX-sensitive $\text{G}_{\beta i/o}$ subunits which results in VD inhibition of Ca^{2+} channels, while $\text{M}_{1,3,5}$ receptors couple to $\text{G}_{\beta q/11}$ proteins and produce VI inhibition of Ca^{2+} currents [2,19]. Figure 3Ai shows superimposed Ca^{2+} channel currents evoked with the double-pulse voltage protocol before (bottom trace) and during (upper trace) application of the nonspecific muscarinic acetylcholine receptor (mAChR) agonist, Oxo-M (10 @M). Exposure to Oxo-M lead to a 50% inhibition of the Ca^{2+} currents. Oxo-M also caused a biphasic, or kinetic slowing, of the rising current during the prepulse and a facilitation of the postpulse current, indicative of a VD block. To determine which mAChR subtype (e.g. M_2 or M_4) mediated the VD inhibition of Ca^{2+} currents, SPG neurons were pretreated for five minutes with either a selective M_2 (methoctramine, 5 @M) or M_4 (M_4 -toxin, 0.2 @M) mAChR blocker. Pretreatment with methoctramine abolished the Oxo-M-mediated Ca^{2+} current inhibition to approximately 5% (Figure 3Aii). On the other hand, M_4 -toxin treatment did not have a significant effect on the modulation of Ca^{2+} currents by Oxo-M. Application of Oxo-M resulted in a 53% inhibition of the Ca^{2+} current in the presence of the toxin (Figure 3Aiii). It should be noted that in a different set of experiments, exposure of the neurons to the external solution containing M_4 -toxin alone increased Ca^{2+} currents by 5% (data not shown). Figure 3B summarizes the effect of Oxo-M on Ca^{2+} currents in the absence or presence of the mAChR blockers. Methoctramine significantly ($P < 0.01$) decreased the modulation of Ca^{2+} currents by Oxo-M. These results suggest that the VD modulation of Ca^{2+} currents in SPG neurons is mediated by M_2 mAChR and not through the M_4 subtype.

The modulatory effect of the purine, ATP, on Ca^{2+} currents was tested next. In the cerebral vasculature, ATP has both a vasodilatory and vasoconstrictor effect [14]. Superimposed Ca^{2+} currents are shown in figure 3Aiv before and during external application of ATP (100 @M). As with Oxo-M, ATP exposure resulted in kinetic slowing of the Ca^{2+} current and a VD block of the channels. Next, the effects of the peptides, Ang II and VIP were examined. Under our recording conditions, exposure of the cells to Ang II (Figure 3Av) and VIP (Figure 3B) did not result in Ca^{2+} current inhibition. Finally, SPG neurons were exposed to 10 @M NE. In 7 of 12 neurons tested, Ca^{2+} currents were inhibited in a VD manner following NE application (Figure 3Avi), while the other cells did not respond to the agonist. The mean $\pm$ SEM Ca^{2+} current inhibition in this group of neurons was 29.8 ± 2.2 % ($n = 7$). The graph in Figure 3B also summarizes the Ca^{2+} current inhibition mediated by ATP, VIP, Ang II and NE.

In the present study, rat SPG neurons were acutely isolated in order to begin to characterize the modulation of whole-cell Ca^{2+} currents by activation of several GPCR. The yield of neurons obtained was approximately 10-fold higher than previously reported [15]. Also, the neurons were not kept in culture for long periods (< 24 hr) in order to avoid growth of processes that would affect clamp control in our experiments. Moreover, the immunofluorescence results

showed that all of the dissociated neurons were AChE-positive, consistent with previous immunohistochemical studies [3].

In SPG neurons, the neurotransmitter ACh has been reported to be involved in regulation of cerebral vasodilation [16]. ACh and nitric oxide (NO) are co-released from the same cholinergic-nitergic nerve terminals, through which ACh activates presynaptic mAChR leading to Ca^{2+} channel inhibition, decreased Ca^{2+} entry and consequently inhibition of neurotransmitter release [13,16]. In the present study Oxo-M inhibited the Ca^{2+} currents in a VD manner and likely that the inhibition occurred through $\text{G}_{\text{i/o}}$ -mediated signaling pathways. Exposure to the M_2 mAChR blocker methoctramine abolished the VD block by Oxo-M while blockade of the M_4 receptor subtype with the M_4 -toxin was without effect. These findings are consistent with a previous report that Ca^{2+} currents in SPG neurons are modulated by M_2 mAChR [16]. However, in the present study Oxo-M inhibited Ca^{2+} currents in a robust manner, while it was without effect in the report by Liu et al. [16]. The discrepancy may be due to the period of time that the neurons were kept in culture prior to cell recording (1 day vs 1–3 wks). Whether long-term culture of SPG neurons affects M_2 mAChR affinity for Oxo-M is not known. In any event, in adult rat intracardiac and stellate ganglion neurons, N-type Ca^{2+} channels are also coupled to M_2 mAChR [10,25], while in neonatal rats activation of M_4 receptor subtype inhibits Ca^{2+} currents in the former ganglion [5].

The ATP-mediated Ca^{2+} current inhibition suggests that SPG neurons express purinergic receptors. Of the eight P2Y receptor subtypes known [1], five ($\text{P2Y}_{1,2,4,6,11}$) couple preferentially to $\text{G}_{\text{q/11}}$ subunits and P2Y_{12-14} employ $\text{G}_{\text{i/o}}$ subunits [1]. Since our results show that ATP inhibited Ca^{2+} currents in a VD manner, it is likely that modulation of the channels were mediated by $\text{G}_{\text{i/o}}$ -coupled subunits. However, the purinergic receptor subtype expressed in SPG neurons was not determined in this study.

Both VIP and Ang II aid in regulating cerebral blood flow and SPG neurons have been shown to stain positive for VIP [6,17]. VIP activates vasoactive intestinal peptide/pituitary adenylate cyclase activating polypeptide receptor-1 (VPAC1) and receptor-2 (VPAC2) that are coupled to G_{s} protein subunits [11], while Ang II stimulates angiotensin II types I and 2 (AT1 & AT2) $\text{G}_{\text{q/11}}$ -coupled receptors [17]. In the present study, exposure of SPG neurons to either peptide did not lead to Ca^{2+} channel inhibition, suggesting that these receptors do not modulate Ca^{2+} channels or may not be expressed in SPG soma. Though, it has been shown that VPAC1 receptor mRNA is present in human SPG neurons [23].

SPG like other parasympathetic ganglia does not express TH, the key enzyme in catecholamine synthesis [12]. Nonetheless, projections from sympathetic SCG neurons that traverse through SPG contain clusters of vesicles that may serve to regulate SPG function [21]. Since SPG and SCG ganglion neurons innervate cerebral vasculature and glandular tissue [7], it seems reasonable to suspect that sympathetic adrenergic discharge may modulate parasympathetic activity. In this study, for instance, NE inhibited Ca^{2+} currents in a VD manner in a subpopulation of SPG neurons. It appears that adrenergic receptors may be expressed in a subpopulation of SPG neurons and their presence reflects a neuronal specialization within this ganglion. However, the physiological significance and receptor identification remain to be determined.

In summary, the results presented demonstrate that our isolation protocol of adult rat SPG neurons resulted in an ample yield of cells exhibiting a parasympathetic phenotype. Electrophysiological results showed that the majority of Ca^{2+} current was carried by N-type Ca^{2+} channels. In all neurons tested, Ca^{2+} currents were inhibited by the nonspecific mAChR agonist Oxo-M and the purinergic receptor agonist ATP. Both VIP and Ang II failed to

modulate the Ca^{2+} currents, and NE-activated adrenergic receptors coupled to Ca^{2+} channels in a subpopulation of SPG neurons.

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Fig 1.

Immunofluorescence staining with antibodies targeted against acetylcholinesterase (AChE, A, B) and tyrosine hydroxylase (TH, C, D) in adult rat SPG (A, C) and SCG (B, D) neurons. Mouse antibodies to AChE and TH were employed and was followed by using Alexa Fluor 488-labeled goat anti-mouse secondary antibodies. E and F are images of SPG neurons incubated with the secondary antibody (without the primary antibody) and served as negative controls. Fluorescence images were acquired with a filter set containing an excitation filter at 480 nm and an emission filter at 535 nm at 20X. The images were pseudocolored; scale bar represents 100 μ m. Insets show the phase contrast images for each field. E and F are images of SPG neurons immunostained with the secondary antibody in the absence of primary AChE and TH antibodies, respectively.

Fig 2.

Pharmacological identification of Ca²⁺ channel subtypes present in SPG neurons. (A) Time course of Ca²⁺ current amplitude recorded every 10 sec. Currents were evoked with the double-pulse protocol (B, top) in the absence (traces 1, 2, 5, 6, 9, 10, 13 and 14) and presence of 10 @M Nif (traces 3 and 4), 0.2 @M Aga (traces 7 and 8), 0.3 @M SNX (traces 11 and 12) and 10 @M Ctx (traces 15 and 16). Solid bars indicate the sequential exposure to the channel blockers. (B) Superimposed current traces from time course before (lower traces) and during (upper traces) exposure to Ca²⁺ channel blockers. Numbers accompanying traces correspond to recordings from time course in (A). (C) Summary plot of the Ca²⁺ current inhibition

produced by the channel blockers. Numbers in parenthesis indicate the number of neurons tested.

Fig 3.

Effect of M₂ and M₄ muscarinic receptor blockers on the Oxo-M-mediated Ca²⁺ current inhibition, and of selective G protein-coupled receptor agonists on Ca²⁺ channel currents in rat SPG neurons. (A) Superimposed Ca²⁺ current traces from SPG neurons before (lower traces, Con) and during (upper traces) agonist application. Currents were evoked with the double-pulse voltage protocol (shown on top of 3A). Neurons were exposed to Oxo-M (10 @M, i), Oxo-M + Methoctramine (5 @M, ii), Oxo-M + M4 toxin (0.2 @M, iii), ATP (100 @M, iv), Ang II (10 @M, v) and NE (10 @M, vi). (B) Summary graph showing Ca²⁺ current inhibition (%) ± SEM in the presence of the agonists and muscarinic receptor blockers. Values in

parentheses indicate the number of cells tested. * $P < 0.01$ compared to Oxo-M-exposed control neurons, Student's t-test.