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Role of pituitary adenylyl cyclase-activating polypeptide in intracellular calcium dynamics of neurons and satellite cells in rat superior cervical ganglia

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ABSTRACT

Pituitary adenylyl cyclase-activating polypeptide (PACAP) is a bioactive peptide with diverse effects in the nervous system. The present study investigated whether stimulation of PACAP receptors (PACAPRs) induces responses in neurons and satellite cells of the superior cervical ganglia (SCG), with special reference to intracellular Ca^{2+} ($[Ca^{2+}]_i$) changes. The expression of PACAPRs in SCG was detected by reverse transcription-PCR. PACAP type 1 receptor (PAC1R), vasoactive intestinal peptide receptor type (VPAC)1R, and VPAC2R transcripts were expressed in SCG, with PAC1R showing the highest levels. Confocal microscopy analysis revealed that PACAP38 and PACAP27 induced an increase in $[Ca^{2+}]_i$ in SCG, first in satellite cells and subsequently in neurons. Neither extracellular Ca^{2+} removal nor Ca^{2+} channel blockade affected the PACAP38-induced increase in $[Ca^{2+}]_i$ in satellite cells; however, this was partly inhibited in neurons. U73122 or xestospongin C treatment completely and partly abrogated $[Ca^{2+}]_i$ changes in satellite cells and in neurons, respectively, whereas VPAC1R and VPAC2R agonists increased $[Ca^{2+}]_i$ in satellite cells only. This is the first report demonstrating the expression of PACAPRs specifically, VPAC1 and VPAC2 in SCG and providing evidence for PACAP38-induced $[Ca^{2+}]_i$ changes in both satellite cells and neurons via Ca^{2+} mobilization.

Pituitary adenylyl cyclase-activating polypeptide (PACAP) belongs to the superfamily of metabolic, neuroendocrine, and neurotransmitter peptide hormones (52). The two biologically active forms (PACAP38 and PACAP27) share the same 27 amino acids at the N terminus and are members of the vasoactive intestinal peptide (VIP)/secretin/glucagon family (1, 26). The effects of PACAP in cells are mediated via class B G protein-coupled receptors (GPCRs) of the secretin receptor family. Three PACAP/VIP receptor genes have been identified: one encodes the preferred PACAP receptor (PACAPR) PAC1R, whereas the other two encode receptors that respond equally to PACAP and VIP, namely, VIP receptor type (VPAC)1R and VPAC2R. PAC1R not only activates a group II receptor signaling cascade via adenylyl cyclase (45), but is also coupled to the phospholipase (PL) C pathway (55). That is, PAC1R is coupled to Gαs and $G\alpha q_{/11}$ for activation of adenylyl cyclase and PLC, respectively (7, 11, 35, 41, 50), and binds to PACAP with a 1000-fold higher affinity than to VIP (15, 16).

PACAP and its receptors are mainly expressed in nervous tissues (51). Although it has various physiological functions, it primarily functions as a neurotransmitter, vasodilator, and immunomodulator (52). PACAPRs have been detected in the sympa-

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thetic superior cervical ganglia (SCG) (8, 28, 31, 32). PAC1R mRNA was found to be highly expressed in all principal neurons of SCG, but VPAC1R and VPAC2R mRNA were not detected by *in situ* hybridization (32). Another study showed that many nerve fibers and terminals innervating postganglionic sympathetic neurons were PACAP-immunoreactive, whereas VIP expression was rarely observed in intraganglionic neuronal processes (22). SCG neurons expressed PAC1R but not VIP/PACAP-nonselective VPAC1Rs, and low VPAC2R transcript levels were restricted to ganglionic non-neuronal cells (7). As mentioned above, some disagreement exists among the reported results to date.

There have been little studies analyzing the functions of SCG neurons and satellite cells. In case of nucleotide receptors, SCG possess at least two different types of receptors, P2X and P2Y, both of which are excitatory in nature and thus trigger noradrenaline release (4, 5). Peripheral nervous tissues mostly consist of neurons and satellite cells, and investigations have primarily focused on responses of neurons rather than those of satellite cells. A previous study described changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that are thought to be essential events in various cell functions (2) including neurons and satellite cells in SCG of rats. We recently reported that purinoceptors and protease-activated receptors (PAR)s are present in rat SCG, and that ATP and PAR2 agonists induced changes in $[Ca^{2+}]_i$ first in satellite cells and subsequently in neurons (23, 25). These results indicated that satellite cells are not just silent supporting cells but actively participate in nervous system function.

The present study investigated whether PACAP and VIP receptors (PAC1R, VPAC1R, and VPAC2R) are expressed in neurons and satellite cells of the rat sympathetic SCG by reverse transcription (RT)-PCR. We also examined the signaling mechanism of PACAP38- and PACAP27-induced $[Ca^{2+}]_i$ changes in SCG. We used intact SCG for these studies, since cellular signaling mechanisms may be altered in primary cell cultures. We are still far from an understanding of the mechanisms used by normal tissues *in vivo*, because in previous studies, the isolated/cultured cells lose their natural conformation and structure, and as a result, such cells may have an altered intracellular signaling. The signaling mechanism of PACAP and VIP receptors which induced $[Ca^{2+}]_i$ changes in SCG was also examined. We therefore assessed the IP_3- and cAMP-dependency of PACAP38-associated $[Ca^{2+}]_i$ changes in SCG cells. In addition, the functional relationship between neurons and satellite cells is discussed.

MATERIALS AND METHODS

Preparation of SCG. The study protocol including animal experiments was approved by and conducted under the authority of the Iwate Medical University Institutional Animal Care and Use Committee. Adult male Wistar rats (8–12 weeks old, weighing 250–350 g) were used. The animals were sacrificed using carbon dioxide gas and perfused via the left cardiac ventricle with Ringer's solution (147 mM NaCl, 4 mM KCl, and 2.25 mM $CaCl_2$) at room temperature (~23°C). SCG and associated connective tissues were removed in HEPES-buffered Ringer's solution (HR) composed of 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 5.5 mM glucose, 0.2% bovine serum albumin (Sigma, St. Louis, MO, USA), MEM amino acid solution (Gibco, Grand Island, NY, USA), 2.0 mM L-glutamine, and 10 mM HEPES (pH 7.4, adjusted with NaOH). Ca^{2+}-deficient solutions were prepared by replacing $CaCl_2$ with EGTA (1.0 mM; Sigma).

Ca^{2+}-sensitive dye loading and stimulation. Spatiotemporal changes in $[Ca^{2+}]_i$ in SCG were determined by ratiometry using the fluorescent dye Indo-1, whose emission spectrum shifts upon binding to Ca^{2+}. Dye loading was carried out using an acetoxymethyl ester of this dye (Indo-1/AM; Dojindo, Kumamoto, Japan). The specimens were transferred to HR containing 300 U/mL purified collagenase (Elastin Products, Owensville, MO, USA), 0.02% Cremophor-EL (Nacalai Tesque, Kyoto, Japan), and 10 μM Indo-1/AM, followed by incubation for 1 h at 37°C. The specimens were then transferred to coverslips coated with Cell-Tak (Collaborative Biomedical, Bedford, MA, USA) in Sykus-Moor chambers and continuously perfused with HR containing selected stimulants.

Confocal imaging of $[Ca^{2+}]_i$ dynamics. A real-time confocal microscope (RCM/Ab; Nikon, Tokyo, Japan) was used to evaluate cellular $[Ca^{2+}]_i$ changes. Indo-1-loaded cells were exposed to ultraviolet light (351 nm) and visualized with an inverted microscope equipped with an argon-ion laser (TE-300; Nikon), with the fluorescence emission passing through a water-immersion objective lens (Nikon C Apo 40×, N.A. 1.15) to a pinhole diaphragm. Eight frames were integrated to obtain images with maximal spatial resolution. Images were immediately stored on a high-speed hard drive and the ratio of fluorescence intensity < 440 nm to that > 440 nm (< 440 nm /

Table 1 *Primers used for PCR analysis of PAC1R, VPAC1R, and VPAC2R expression*

Receptor		Sequence 5'–3'	Position	Accession Code	Amplicon
PAC1R	F	AACGACCTGATGGGACTAAAC	458	NM_133511	413 bp
	R	CGGAAGCGGCACAAGATGACC	850		
VPAC1R	F	GAGAGGAAAGACAGCGTTGG	3331	NM_012685	183 bp
	R	CAGAAGGACCTGGGTGTTGT	3493		
VPAC2R	F	GCTTTCTGAGGCATGTAGGC	2522	NM_017238	250 bp
	R	CTGGAGGCCTTTCAAGAGTG	2751		
GAPDH	F	TTCAACGGCACAGTCAAGGC	1009	AF106860	812 bp
	R	TCCACCACCCTGTTGCTGTAGC	1820		

PAC1R primers are from Syed *et al.* (42). VPAC1R and VPAC2R primers were designed using the web-based tool Primer3Plus. Reaction conditions are described in the Materials and Methods. F, forward primer; R, reverse primer. All primers were assessed for sequence homology with other genes by BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

> 440 nm) was computed and used as a measure of relative $[Ca^{2+}]_i$. Ratio images were 512 × 480 pixels with a density resolution of approximately 0.3 μm, and were displayed with 256 colors (*i.e.*, 8 bits/pixel). In the pseudocolor table of ratio images, pink and blue/green colors represent high and low ratios (*i.e.*, $[Ca^{2+}]_i$), respectively.

Perfusion. SCG $[Ca^{2+}]_i$ dynamics were visualized in a Sykus-Moor perfusion chamber (1 mL capacity) immediately after dye loading. Specimens were perfused with HR at 1 mL/min at room temperature for a few minutes, and intact neurons and satellite cells were then examined under the microscope. To eliminate the variability of Indo-1/AM loading into neurons and satellite cells, only SCG that showed sufficient fluorescence signal intensity were selected for analysis. These specimens were perfused by replacing the HR as follows. After a brief wash with HR, the specimens were continuously perfused with HR containing the following agonists and/or antagonists: PACAP38 (1 μM) (Merck Millipore, Darmstadt, Germany); PACAP27 (1 μM) (Peptide Institute, Osaka, Japan); the PLC inhibitor U73122 (10 μM; a PLC), L-type Ca^{2+}-channel blocker diltiazem (50 μM), and adenylyl cyclase inhibitor SQ22536 (100 μM) (all from Sigma); the protein kinase (PK)A inhibitors PKI (14-22) (2 μM) and H89 (100 μM) and the PKC antagonist GF109203X (2 μM) (all from Enzo Life Sciences, Farmingdale, NY, USA); the transient receptor potential cation (TRPC) channel inhibitor and blocker of IP_3-dependent Ca^{2+} release 2-aminoethoxydiphenyl borate (2-APB; 100 μM) (Tocris, Bristol, UK); the VPAC1R and VPAC2R agonists [Ala 11, 22, 28] VIP (1 μM) and BAY 55-9837 (1 μM), respectively (R&D Systems, Minneapolis, MN, USA); the VPAC1R and VPAC2R agonist VIP (1 μM) and N-type Ca^{2+}-channel blocker ω-conotoxin GVIA (1 μM) (both from Nacalai Tesque, Kyoto, Japan); and the IP_3R blocker xestospongin C (2 μM) and nonspecific cation-channel blocker $GdCl_3$ (100 μM) (both from Wako, Osaka, Japan).

RNA extraction and RT-PCR. Total RNA was extracted from SCG using the RNeasy Micro kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration was determined by spectroscopy at 260 nm. Isolated RNA was used for RT-PCR on a PC-701 thermal cycler (ASTEC, Fukuoka, Japan) using the ReverTra Ace-α kit (Toyobo, Osaka, Japan). The primer sequences used are shown in Table 1 and were designed using the web-based tool Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The thermal cycling conditions were as follows: 94°C for 2 min; 35 cycles of 94°C for 45 s, 60°C for 30 s, and 72°C for 1 min, and 72°C for 10 min. PCR products were resolved by electrophoresis on 2% agarose gels stained with ethidium bromide. Images of the gels were captured using a Polaroid MP4 Land Camera (Polaroid, Minnetonka, MN, USA). Three independent experiments were performed.

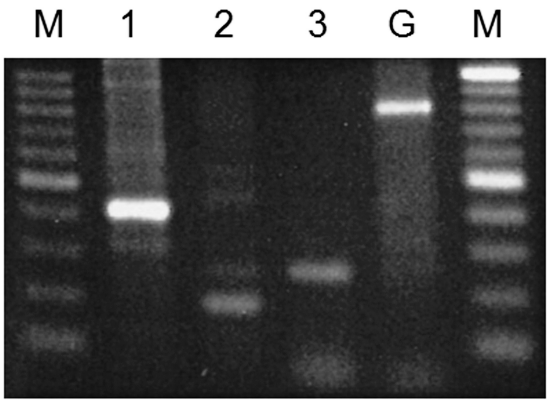
Ultrastructural analysis. To detect ultrastructural changes during the isolation procedure, we examined isolated SCG by electron microscopy. SCG were fixed in 1.25% glutaraldehyde (Nacalai Tesque) and 4% paraformaldehyde (Merck Millipore) in 0.1 M PBS for about 3.5 h at 20–25°C. Specimens were then post-fixed in 1% osmium tetroxide (Merck Millipore) in PBS for 1.5 h at 4°C, dehydrated in a graded series of ethanol, and embedded in Epon 812 (TAAB, Berks, Germany). Longitudinal and transverse sections were consecutively cut through the

nerve trunks using an ultramicrotome (2088 Ultratome; LKB, Bromma, Sweden). Semithin sections (about 1 μm thick) were stained with Toluidine Blue. Ultrathin sections (about 70 nm thick) were stained with uranyl acetate and lead citrate. Samples were examined by both light and electron microscopy (H-7100; Hitachi, Tokyo, Japan).

RESULTS

PAC1R, VPAC1R, and VPAC2R mRNA expression in SCG

PAC1R, VPAC1R, and VPAC2R transcript levels in SCG were detected by RT-PCR and graded as follows: + signified that the PCR product was detectable by ethidium bromide staining of an agarose gel; and ++ and +++ corresponded to bands of moderate and high intensity, respectively, on the gel (Fig. 1). All three receptors were expressed in SCG, with PAC1R showing the highest level.



Intensity of band	PAC1R	VPAC1R	VPAC2R
	+++	+	+

Fig. 1 RT-PCR analysis of PAC1R, VPAC1R, and VPAC2R mRNA expression. Amplicons were visualized by agarose gel electrophoresis and ethidium bromide staining. Expression levels were determined relative to the internal control glyceraldehyde 3-phophate dehydrogenase (GAPDH). Lane 1, PAC1R; lane 2, VPAC1R; lane 3, VPAC2R; G, GAPDH; M, 100 bp markers.

Effect of PACAP38 and PACAP27 on $[Ca^{2+}]_i$ dynamics

Large round neurons (~20–30 μm in diameter) and smaller, thinner satellite cells (~5–10 μm in diameter at the perinuclear region) surrounding them were visible in digital light micrographs (Fig. 2). Neuronal nuclei were often clearly observed due to the high fluorescence. SCG specimens were perfused with HR for 5–10 min before stimulation with selected reagents; spontaneous $[Ca^{2+}]_i$ changes were rarely observed. Damaged cells tended to have high $[Ca^{2+}]_i$; therefore, SCG cells showing high $[Ca^{2+}]_i$ under resting conditions were excluded from analyses (Fig. 2).

PACAP38 activates PAC1R, VPAC1R, and VPACR2 (*i.e.*, all PACAPRs) (14, 19, 49, 52). The patterns of $[Ca^{2+}]_i$ increase varied between neurons and satellite cells. Initially, PACAP38 (1 μM) elicited an increase in $[Ca^{2+}]_i$ in some satellite cells; an increase was subsequently detected in some neurons (Fig. 3), but with a relatively slow time course. Satellite cells sometimes exhibited oscillatory changes. Similar effects were observed in both cell types upon treatment with PACAP27 (data not shown); however, the frequency of $[Ca^{2+}]_i$ oscillations in satellite cells was lower than those induced by PACAP38.

Mechanism of PACAP-induced $[Ca^{2+}]_i$ dynamics

We investigated whether Ca^{2+} influx from the extracellular environment during stimulation was responsible for PACAP38-induced changes in the intracellular $[Ca^{2+}]_i$. We found that PACAP38 (1 μM)-induced $[Ca^{2+}]_i$ fluctuations in satellite cells persisted in the absence of extracellular Ca^{2+} ($[Ca^{2+}]_o$-free) (Fig. 4a). Similar effects were observed by treatment with $GdCl_3$ (100 μM), a nonspecific cation channel blocker (Fig. 4b). Neither diltiazem (50 μM) nor ω-conotoxin GVIA (1 μM) inhibited PACAP38-induced $[Ca^{2+}]_i$ changes in satellite cells (data not shown), and neuronal responses were only partly inhibited. These results imply that PACAP38-induced cellular responses involve a Ca^{2+} influx-dependent mechanism in neurons but not in satellite cells.

We next investigated the role of PLC and IP_3 in the PACAP38-induced $[Ca^{2+}]_i$ changes in SCG. Stimulation of G proteins activates PLC, which cleaves membrane-bound phosphatidyl inositol bisphosphate to generate IP_3 and diacylglycerol. The former promotes Ca^{2+} mobilization from internal stores (2). To determine whether PLC was activated following PACAPR stimulation, specimens were treated with the PLC inhibitor U73122 (10 μM), the sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin (2 μM), the IP_3R inhibitor xestospongin C (2 μM), and an inhibitor of TRPC channels as well as a blocker of IP_3-dependent Ca^{2+} release 2-APB (100 μM). All four agents completely inhibited PACAP38- or PACAP27-induced $[Ca^{2+}]_i$ changes in satellite cells (Fig. 5 and data not shown). In contrast, neuronal responses were only partly inhibited by U73122, thapsigargin, and xestospongin C, while PACAP-

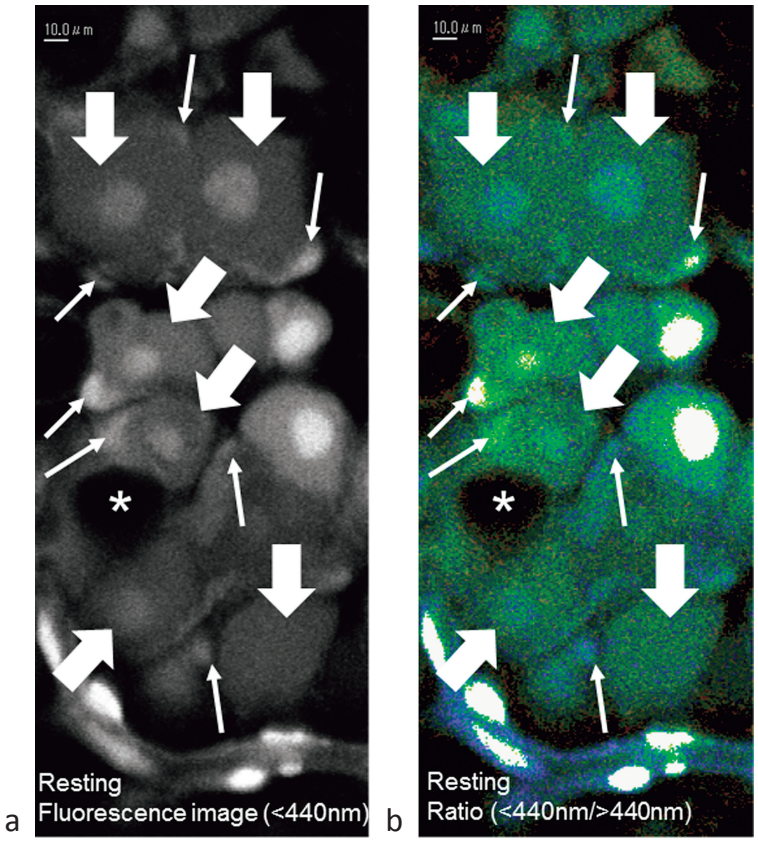


Fig. 2 Confocal micrographs of SCG loaded with Indo-1. **a**: Fluorescence signal detection at < 440 nm. **b**: Pseudocolor images showing the < 440 nm / > 440 nm ratio, reflecting $[Ca^{2+}]_i$ under resting conditions. Color scale bar: fluorescence ratio representing $[Ca^{2+}]_i$. Thick and thin arrows indicate neurons and satellite cells, respectively. Cells showing higher $[Ca^{2+}]_i$ under resting conditions (asterisk) were excluded from analyses.

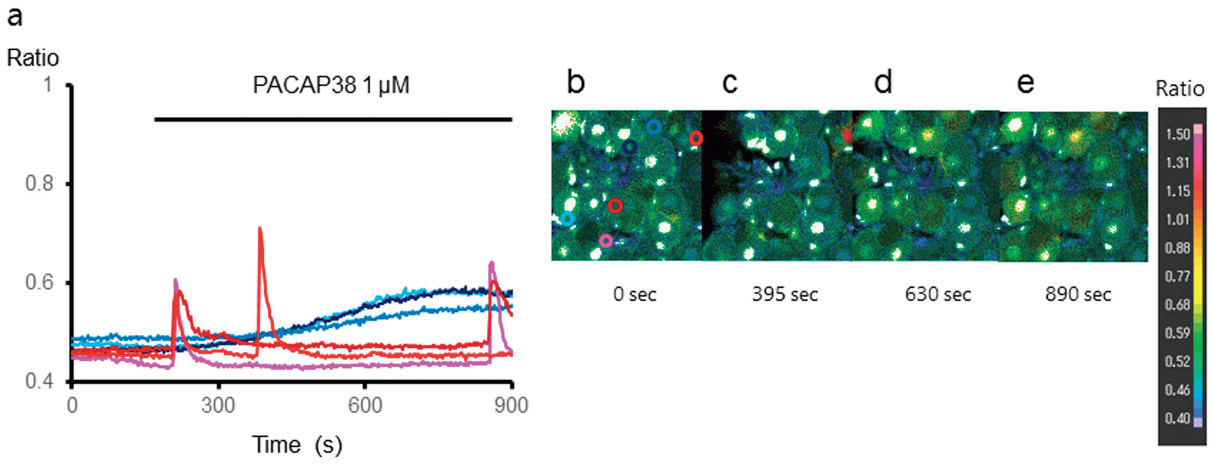


Fig. 3 Spatiotemporal changes in $[Ca^{2+}]_i$ in SCG neurons and satellite cells following PACAP38 stimulation. $[Ca^{2+}]_i$ was calculated based on the dye fluorescence intensity ratios at different wavelengths (colored scale bar). Time course of PACAP38-induced changes in $[Ca^{2+}]_i$ in specific areas (*i.e.*, regions of interest) of satellite cells (red lines) and neurons (blue lines) (~1 μm^2 in size). Changes in $[Ca^{2+}]_i$ are shown as pseudocolors ($n = 12$).

induced Ca^{2+} release and entry were completely blocked in the presence of 2-APB in neurons (Fig. 5d).

To determine whether protein kinases play a role in the PACAP-dependent $[Ca^{2+}]_i$ increase, we examined the effects of several kinase antagonists. H89 (100 μM) slightly inhibited the PACAP-induced increase in $[Ca^{2+}]_i$ in satellite cells (Fig. 6a). Similar effects were observed for PKI (14-22) (2 μM) (data not shown) and SQ22536 (100 μM) (Fig. 6b). In contrast, neuronal responses were not inhibited by these

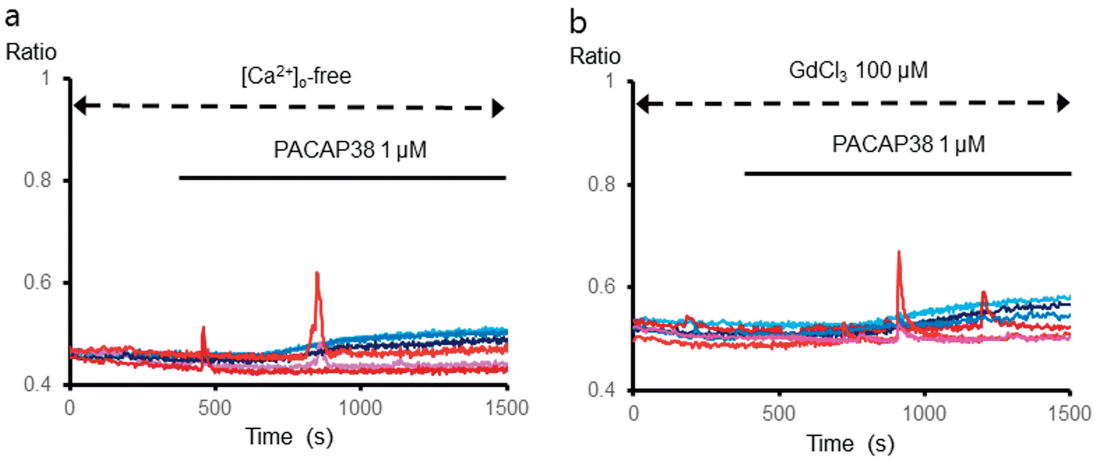


Fig. 4 Ion channels are only partly involved in PACAP38-induced $[Ca^{2+}]_i$ changes. The time course of $[Ca^{2+}]_i$ changes induced by PACAP38 in specific areas of SCG satellite cells and neurons (~1 μm^2 in size) was analyzed. $[Ca^{2+}]_o$-free (a) or 100 μM $GdCl_3$ (b) treatment slightly inhibited PACAP38-induced $[Ca^{2+}]_i$ increases in neurons. In satellite cells, both treatments failed to inhibit PACAP38-induced $[Ca^{2+}]_i$ increases ($n = 10$ and 9 for panels a and b, respectively).

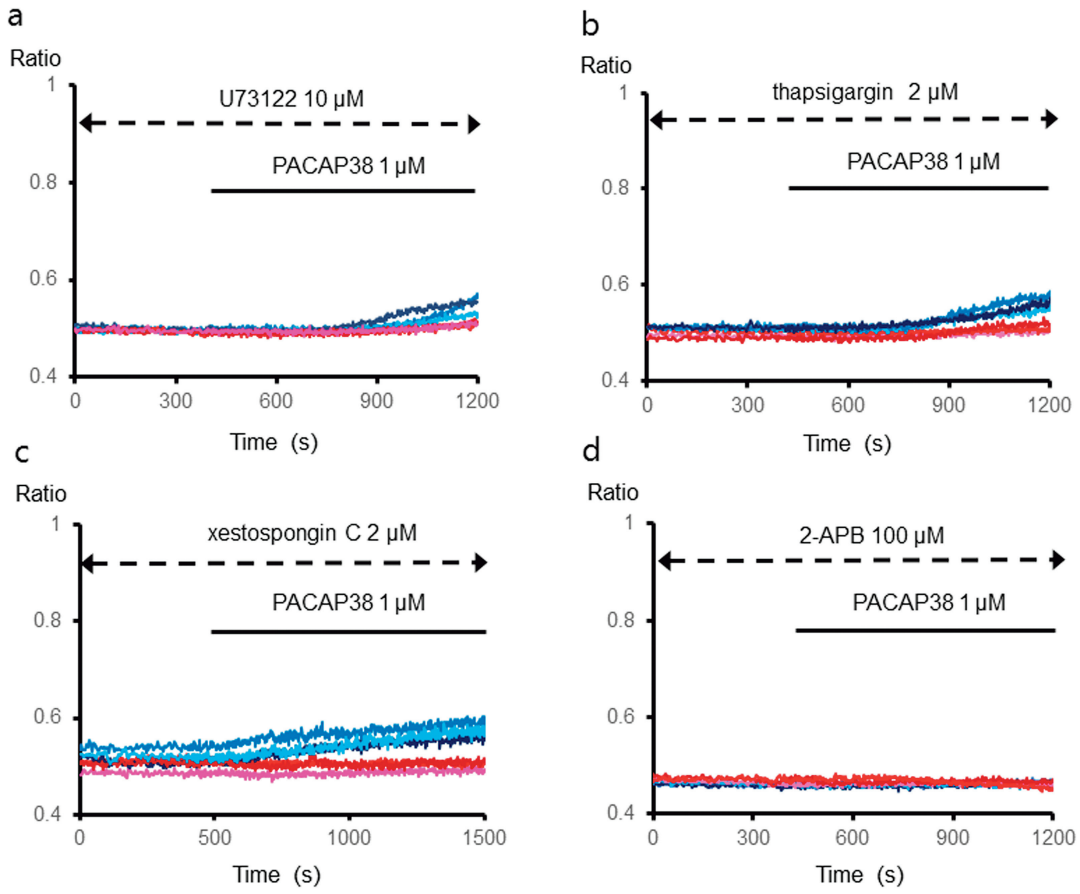


Fig. 5 Ca^{2+} mobilization from intracellular stores during PACAP38-induced $[Ca^{2+}]_i$ changes. **a**: PACAP38 (1 μM) had no effect on $[Ca^{2+}]_i$ in satellite cells after blocking PLC by treatment with U73122 (10 μM). **b**: Treatment with the sarcoendoplasmic reticulum Ca^{2+}-ATPase blocker thapsigargin (2 μM) and **c**: treatment with the IP_3 receptor antagonist xestospongin C (2 μM) also showed no effect on PACAP38-induced $[Ca^{2+}]_i$ increases in satellite cells. **a**–**c**: PACAP38 (1 μM) induced slight increases in $[Ca^{2+}]_i$ in neurons. **d**: Treatment with IP_3 receptor antagonist and the TRPC antagonist 2-APB (100 μM) completely inhibited PACAP38-induced $[Ca^{2+}]_i$ increases in both satellite cells and neurons ($n = 10$ each).

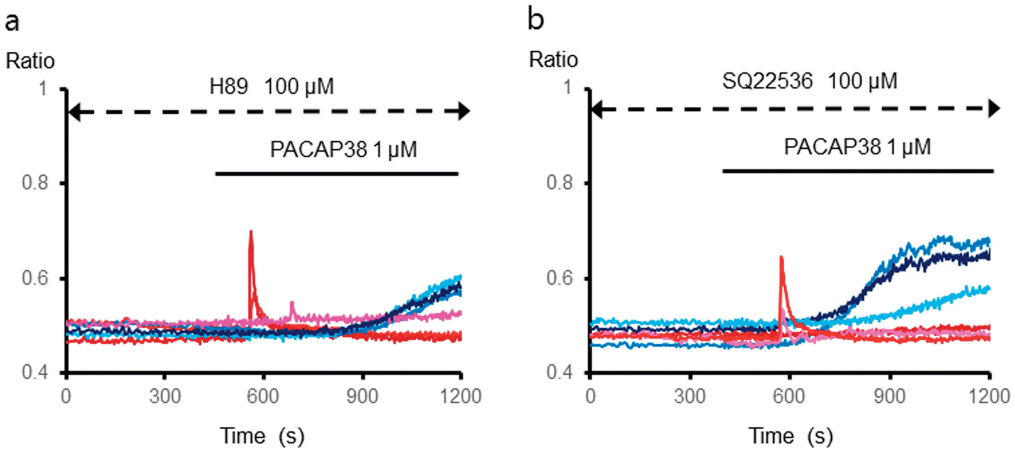


Fig. 6 Effect of PKA antagonists on PACAP38-induced $[Ca^{2+}]_i$ changes in SCG cells. Satellite cells and neurons were stimulated with 1 µM PACAP. **a**, **b**: The PKA antagonists H89 (100 µM) (a) and the adenylyl cyclase inhibitor SQ22536 (100 µM) (b) had no effect on $[Ca^{2+}]_i$ dynamics ($n = 9$, and 8 for panels a and b, respectively).

agents. These findings confirm the view that cAMP pathways (especially the PKA pathway) weakly contribute to PACAP38-mediated $[Ca^{2+}]_i$ increase in satellite cells in SCG.

To determine whether SCG satellite cells and neurons express PAC1R, VPAC1R, and VPAC2R, samples were treated with VIP (1 µM), [Ala 11, 22, 28] VIP (1 µM) which activates PAC1R, VPAC1R, and VPAC2R (15, 16, 52) and BAY 55-9837, the VPAC1R/VPAC2R agonist (1 µM). All three agents induced increases in $[Ca^{2+}]_i$ in satellite cells (Fig. 7), with BAY 55-9837 showing more potent effects (Fig. 7b). However, these agents had little effect on neurons (Fig. 7).

Ultrastructure of isolated SCG

Many intact neurons and satellite cells with normal ultrastructure were observed in SCG. Neuronal somata were wrapped by a satellite cell sheath (Fig. 8a), and neurons and satellite cells were bound by separate plasma membranes; the two cell types were separated by an intercellular space of variable size (Fig. 8). Some portions of the neuronal surface were not covered by satellite cells (Fig. 8b). There were no ultrastructural differences between treated specimens and controls with respect to Indo-1/AM loading (data not shown). Pretreatment of SCG with the various reagents did not cause any ultrastructural changes.

DISCUSSION

The main finding of the present study was that PACAP caused increases in $[Ca^{2+}]_i$ in SCG, first in satellite cells and then in neurons. Satellite cells expressed PAC1R, VPAC1R, and VPAC2R, whereas neurons primarily expressed PAC1R. PACAP and VIP peptides have various effects in different tissues (52). In the nervous system, PACAP has been shown to exert neurotrophic and neuroprotective effects in cerebral ischemia (30, 33, 36–39, 43, 48). However, there are no reports to date regarding the role of PACAPs in sympathetic nervous tissue specifically, in SCG. We demonstrate for the first time that PACAPR is expressed in SCG and that PACAPs induce $[Ca^{2+}]_i$ changes in both the neurons and satellite cells, highlighting a role for peptides in sympathetic nerve activation.

We previously observed that PAR2 activation induced an increase in $[Ca^{2+}]_i$ in SCG satellite cells and neurons, indicating that sympathetic nerves can be activated by proteases that are upregulated in response to inflammation and other pathological conditions (25). We also reported the expression of P2Y and P2X receptors in rat SCG neurons and satellite cells, respectively (23), which suggested that these receptors may be important for neuron-satellite cell signaling. However, their precise functions have yet to be elucidated. In both our experiments, reagents induced an increase in $[Ca^{2+}]_i$ in many satellite cells followed by neurons (23, 25). This phenomenon was also observed in the present study. It is unclear whether a subset of neurons express only PAC1R; since neurons are enveloped by satellite cells, some may not have been exposed to HR containing the stimulants, especially VPAC1 and VPAC2. PAC1R was also found to bind to PACAP with a 1000-fold higher affinity than to VIP (15, 16). Thus, neurons

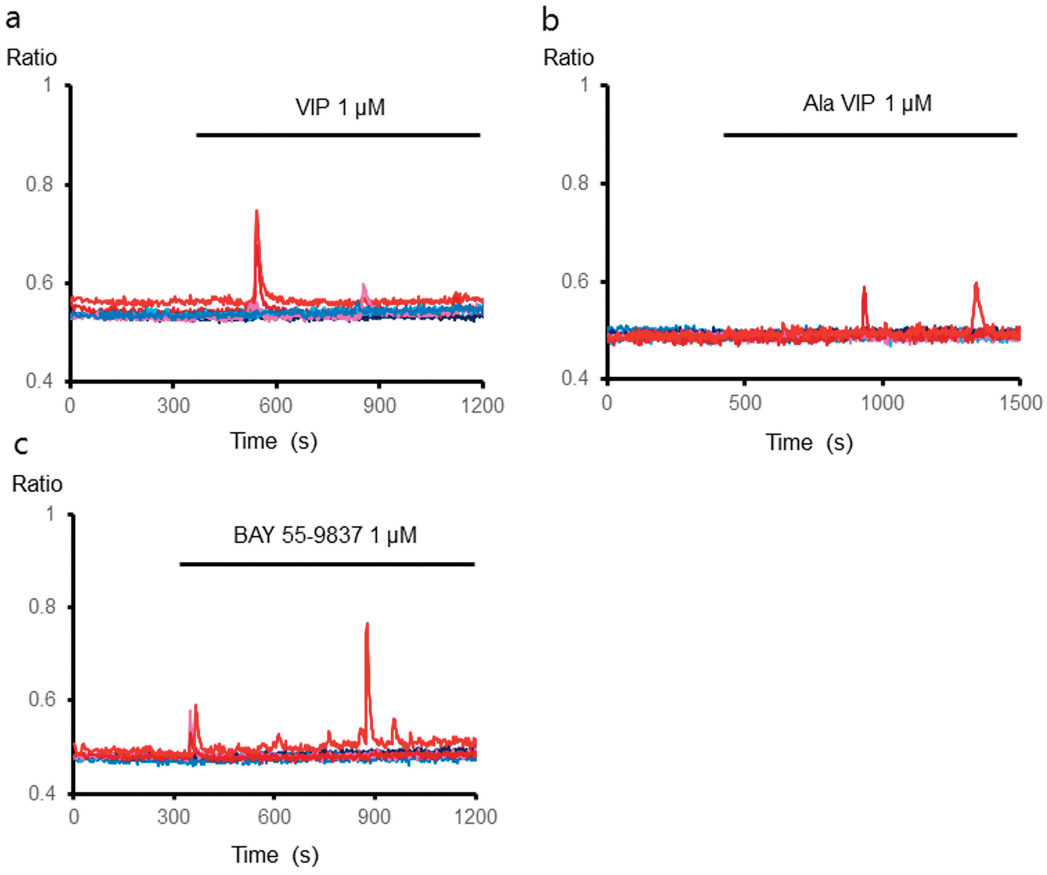


Fig. 7 Effect of VIP receptor agonists on $[Ca^{2+}]_i$ dynamics. **a**: VIP (1 μM); **b**: [Ala 11, 22, 28] VIP (VPAC1R agonist; 1 μM); **c:** BAY 55-9837 (VPAC2 receptor agonist; 1 μM). All three agonists induced $[Ca^{2+}]_i$ changes in satellite cells only ($n = 9$, 9, and 8 for panels a, b, and c, respectively).

may have VIP receptors that are not detectable by the methods used in this study. Results from our imaging and RT-PCR experiments indicate that satellite cells in SCG express PAC1R, VPAC1R, and VPAC2R.

Changes in $[Ca^{2+}]_i$ upon activation of GPCRs such as PACAPRs have been attributed to IP_3-mediated mobilization of Ca^{2+} from internal stores such as the sarcoendoplasmic reticulum (2). The inhibition of PACAP-induced $[Ca^{2+}]_i$ responses by U73122, thapsigargin, xestospongin C, and 2-APB in neurons indicated that these are primarily IP_3-dependent. Especially, 2-APB was originally developed as a membrane-permeable inhibitor of IP_3R (27) and has since been widely used to examine the functions of these receptors and other Ca^{2+} signaling mechanisms such as store-operated Ca^{2+} entry (6). In our experiments, 2-APB completely blocked PACAP-induced Ca^{2+} release and entry, consistent with previous studies including our own (34). In the absence of extracellular Ca^{2+} and the presence of some Ca^{2+} channel blockers, Ca^{2+} influx is independent of IP_3 and dependent on cyclic (c)AMP. In dendrites of hippocampal pyramidal neurons, PKA activation by cAMP enhanced L-type channel currents (17, 20). In our study, U73122 and other inhibitors of Ca^{2+} release did not completely inhibit $[Ca^{2+}]_i$ increase in neurons, suggesting that they possess both IP_3-independent and -dependent Ca^{2+} mobilization systems. In addition, VIP, VPAC1, and VPAC2 agonists failed to induce $[Ca^{2+}]_i$ responses in neurons. Some studies have shown that the β-adrenergic receptor agonist isoproterenol failed to induce $[Ca^{2+}]_i$ responses in rat parotid and lacrimal gland acinar cells (18, 40, 47). However, isoproterenol as well as forskolin promoted Ca^{2+} release from intracellular stores by stimulating muscarinic and α-adrenergic receptors in rodent parotid acinar cells, which involved PKA-dependent phosphorylation of IP_3R (3, 9, 44). It is possible that cAMP caused no changes in $[Ca^{2+}]_i$ in SCG neurons; we suggest that they have both IP_3-dependent $[Ca^{2+}]_i$ release and cAMP-dependent Ca^{2+} influx mecha-

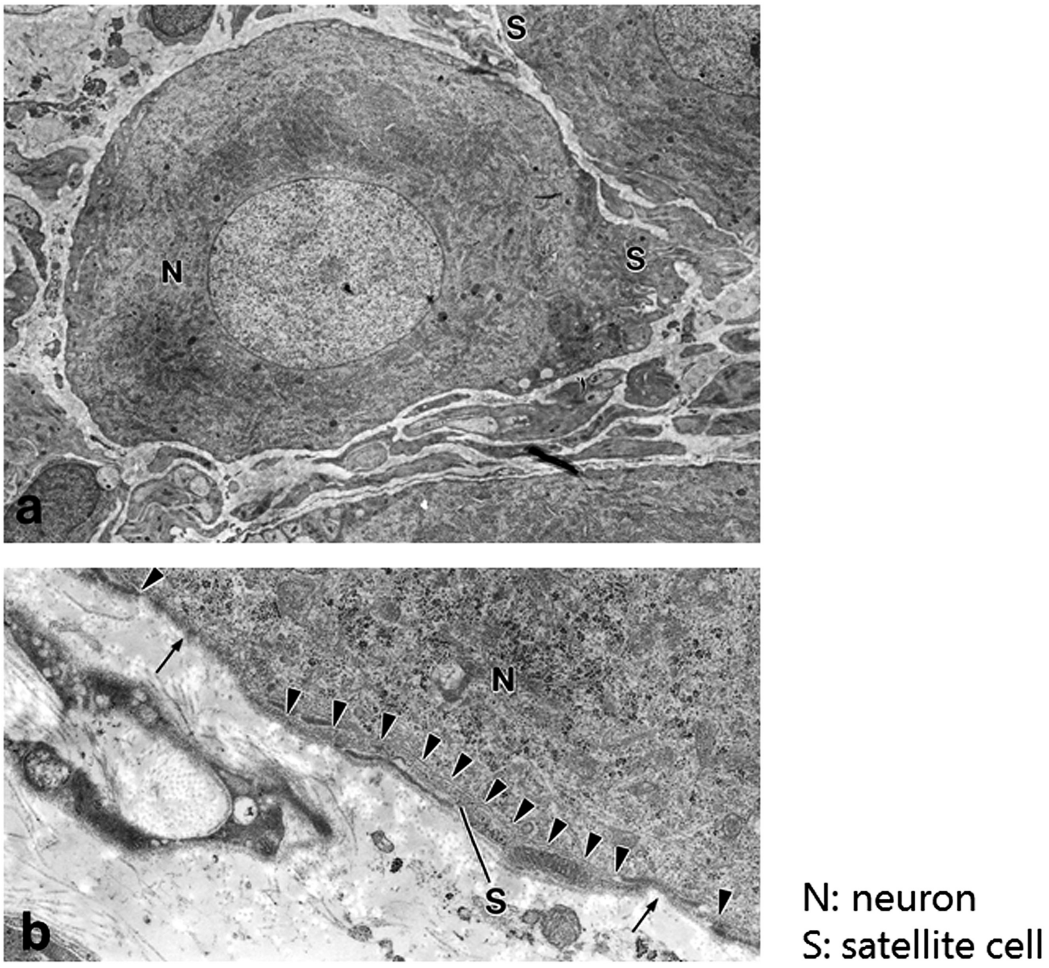


Fig. 8 **a**: Electron micrographs showing neurons (N) covered by satellite cells (S). **b**: Arrows indicate areas where the neuron is not covered with satellite cells; arrowheads indicate the basal lamina of both neurons and satellite cells. Magnification: 3,200× (a) and 15,000× (b).

nisms.

PACAP-mediated $[Ca^{2+}]_i$ mobilization of satellite cells was mainly IP_3-dependent. When Ca^{2+} mobilization was inhibited, PACAP failed to induce $[Ca^{2+}]_i$ changes in satellite cells. However, VPAC1R and VPAC2R agonists stimulated cAMP-dependent Ca^{2+} influx; cAMP has been shown to modulate Ca^{2+} signaling and IP_3-induced Ca^{2+} release from the endoplasmic reticulum (ER) (21, 24, 54). PKA, which is activated by cAMP, can phosphorylate all IP_3R subtypes to modulate IP_3-induced Ca^{2+} release from the ER (9, 10, 12, 13, 29, 46, 53). In our experiments, it is possible that PKA phosphorylated or interacted with IP_3Rs in satellite cells, in which IP_3-dependent Ca^{2+} mobilization is more dominant than in neurons. This mechanism may depend on cAMP signaling. PKA activation was shown to enhance IP_3-induced Ca^{2+} release in mouse parotid acinar cells and induce IP_3R2 phosphorylation (10). In addition to VIP, VPAC1, and VPAC2, PACAP-induced $[Ca^{2+}]_i$ was also altered by treatment with H89, PKI, and SQ22536. This suggests that IP_3Rs interact with PKA in satellite cells of SCG. Thus, PACAP-mediated responses in various cell types are likely IP_3-dependent, although the precise mechanism by which Ca^{2+} is mobilized remains to be elucidated.

In conclusion, $[Ca^{2+}]_i$ changes in satellite cells stimulated by PACAP and VIP analogs are exclusively caused by Ca^{2+} mobilization from internal stores and is IP_3-dependent, whereas IP_3-dependent Ca^{2+} mobilization and Ca^{2+} influx play an important role in neuronal responses to PACAPs. Satellite cell activation by PACAPs and VIPs may affect neuronal activity, although our results indicate that neuronal and satellite cell responses were independent. However, the mechanism underlying PACAP-mediated

intracellular signaling in sympathetic neurons await clarification in future studies.

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CONFLICTS OF INTEREST

The authors declare no competing financial interests.

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