Intracellular Ca\(^{2+}\) mobilization pathway via bradykinin B\(_1\) receptor activation in rat trigeminal ganglion neurons

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Abstract
Bradykinin (BK) and its receptors, B\(_1\) and B\(_2\), in trigeminal ganglion (TG) neurons are involved in the regulation of pain. Recent studies have revealed that B\(_1\) receptors are expressed in neonatal rat TG neurons; however, the intracellular signaling pathway following B\(_1\) receptor activation remains to be elucidated. To investigate the mechanism by which B\(_1\) receptor activation leads to intracellular Ca\(^{2+}\) mobilization, we measured the intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) in primary-cultured TG neurons. The application of Lys-[Des-Arg\(^9\)]BK (B\(_1\) receptor agonist) increased the [Ca\(^{2+}\)\(_i\)] in these TG neurons even in the absence of extracellular Ca\(^{2+}\). Pretreatment with inhibitors of ryanodine receptors or sarcoplasmic reticulum Ca\(^{2+}\)-ATPase suppressed the increase in Lys-[Des-Arg\(^9\)]BK-induced [Ca\(^{2+}\)\(_i\)]. The Lys-[Des-Arg\(^9\)]BK-induced [Ca\(^{2+}\)\(_i\)] increase was unaffected by phospholipase-C inhibitor. B\(_1\) receptor activation-induced [Ca\(^{2+}\)\(_i\)] increase was suppressed by phosphodiesterase inhibitor and enhanced by adenylyl cyclase inhibitor. These results suggest that B\(_1\) receptor activation suppresses intracellular cAMP production via adenylyl cyclase inhibition and mobilizes intracellular Ca\(^{2+}\) via ryanodine receptors that access intracellular Ca\(^{2+}\) stores.

Keywords Adenylyl cyclase · Ca\(^{2+}\) mobilization · Cyclic AMP · G protein · Ryanodine receptors · Trigeminal ganglion neuron

Introduction
Bradykinin (BK) and related peptides play important roles in the modulation of physiological and pathological processes, including pain and inflammation [1]. The kallikrein–kinin system comprises kininogens, proteolytic kallikrein enzymes, BK and Lys-BK (kallidin; produced through the cleavage of kininogens by kallikreins), [Des-Arg\(^9\)]BK and Lys[Des-Arg\(^9\)]BK (produced through the cleavage of BK and kallidin, respectively) and BK receptors [2]. Both BK and Lys-BK are vasoactive peptides synthesized by the kallikrein–kinin system. Their metabolites without the C-terminal arginine residue act as ligands of BK receptors [1]. The BK receptors localized to the plasma membrane belong to the G protein-coupled receptor (GPCR) family and are classified into two subtypes, B\(_1\) and B\(_2\). It has been shown that BK itself activates B\(_2\) receptors [1] and that BK has a 100- to 20,000-fold higher affinity for B\(_2\) receptors than for B\(_1\) receptors [1]. Both Lys-BK and Lys[Des-Arg\(^9\)]BK have higher affinities for B\(_1\) receptors than do BK and [Des-Arg\(^9\)]BK, respectively. The only natural kinin sequence with a subnanomolar affinity for B\(_1\) receptors is Lys[Des-Arg\(^9\)]BK [1]. For mammalian BK receptors, the order of agonist affinity is: Lys[Des-Arg\(^9\)]BK > Lys-BK ≈ [Des-Arg\(^9\)]BK > BK for B\(_2\) receptors; BK ≈ Lys-BK » [Des-Arg\(^9\)]BK > Lys[Des-Arg\(^9\)]BK for B\(_1\) receptors [1]. Thus, the agonist showing the highest affinity for B\(_1\) receptors is Lys[Des-Arg\(^9\)]BK, with much higher affinities than BK itself.

Neuropathic pain, which is mediated by both B\(_1\) and B\(_2\) receptor activation in the orofacial area, is often induced by injuries to trigeminal ganglion (TG) neurons or glial cells [2–4]. B\(_1\) receptors have been suggested as an attractive target for the control of neuropathic pain [2]. In a previous study [3], we demonstrated functional expression of B\(_1\) and B\(_2\) receptors in TG neurons, observing that BK elicited...
increases in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) that were inhibited by B\(_2\) receptor antagonists, but not by B\(_1\) receptor antagonists, whereas application of Lys-[Des-Arg\(^9\)]BK induced increases in [Ca\(^{2+}\)]\(_i\) that were sensitive to a B\(_1\) receptor antagonist. We therefore concluded that B\(_1\) receptors in TG neurons, similar to those elsewhere in the brain, show high selectivity for Lys-[Des-Arg\(^9\)]BK [3]. In addition, the activation of B\(_2\) receptors induced both the influx of Ca\(^{2+}\) from the extracellular medium and the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores [3]. However, the intracellular signaling pathway by which Lys-[Des-Arg\(^9\)]BK induces Ca\(^{2+}\) mobilization in response to the activation of B\(_1\) receptors had not yet been fully elucidated.

Intracellular Ca\(^{2+}\) is mobilized by two closely coupled components: Ca\(^{2+}\) entry from the extracellular space and the release of Ca\(^{2+}\) from intracellular stores. Ca\(^{2+}\) release from intracellular stores is mediated by inositol 1,4,5-trisphosphate (IP\(_3\)) receptors or ryanodine receptors. Ligand binding to the GPCR leads to phospholipase C (PLC) activation that in turn induces the production of IP\(_3\) and subsequent IP\(_3\)-induced Ca\(^{2+}\) release. Ryanodine receptors are known to elicit Ca\(^{2+}\)-induced Ca\(^{2+}\) release following depolarization-induced Ca\(^{2+}\) entry and/or Ca\(^{2+}\) release via IP\(_3\) receptors.

In the study reported here, we measured [Ca\(^{2+}\)]\(_i\) in primary-cultured rat TG neurons and used various agonists and antagonists to investigate the intracellular signaling pathway that is activated by the administration of Lys-[Des-Arg\(^9\)]BK.

### Materials and methods

#### Ethical approval

All animals were treated in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science approved by the Council of the Physiological Society of Japan, and the American Physiological Society. This study also followed the guidelines established by the U.S. National Institutes of Health (Bethesda, MD, USA) on the care and use of animals for experimental procedures. The study was approved by the Ethics Committee of Tokyo Dental College (Approval no. 292503).

#### Isolation of trigeminal ganglion cells

Trigeminal ganglion cells were isolated from neonatal Wistar rats (7–8 days old) under pentobarbital sodium anesthesia (50 mg/kg), following the administration of isoflurane (3.0% vol). TG cells were dissociated by enzymatic treatment with Hanks’ balanced salt solution (137 mM NaCl, 5.0 mM KCl, 2.0 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.44 mM KH\(_2\)PO\(_4\), 0.34 mM Na\(_2\)HPO\(_4\), 4.17 mM NaHCO\(_3\), 5.55 mM glucose) containing 20 U/ml papain (Worthington, Lakewood, NJ, USA) for 20 min at 37 °C, followed by dissociation by trituration. After dissociation, the TG cells were plated on 35-mm diameter dishes (ibidi GmbH, Planegg, Germany). The primary culture of the TG cells was performed in Leibovitz’s L-15 medium (Life Technologies, Carlsbad, CA, USA), containing 10% fetal bovine serum, 1% amphotericin B, 1% fungizone (Life Technologies), 26 mM NaHCO\(_3\) and 30 mM glucose (pH 7.4). The cells were maintained in culture for 48 h at 37 °C in a humidified atmosphere containing 95% oxygen and 5% CO\(_2\) to allow cell attachment to the bottom of dishes. For measurement of [Ca\(^{2+}\)]\(_i\), the temperature of the extracellular medium was maintained at 32 °C (Warner Instruments, Hamden, CT, USA) to avoid thermal stimulation of cells.

#### Solutions and reagents

Hanks’ balanced salt solution was used as the standard extracellular solution. A solution containing a high concentration of extracellular K\(^+\) (91 mM NaCl, 50 mM KCl, 2.0 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 0.44 mM KH\(_2\)PO\(_4\), 0.34 mM Na\(_2\)HPO\(_4\), 4.17 mM NaHCO\(_3\), 5.55 mM glucose; pH 7.4) was used to distinguish TG neurons from glial cells through activation of depolarization-induced increases in [Ca\(^{2+}\)]\(_i\) in the neurons. The endogenous potent and highly selective bradykinin B\(_1\) receptor agonist Lys-[Des-Arg\(^9\)]BK [3], the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA, 100 nM [5]), the ryanodine receptor inhibitor dantrolene (sodium salt, 1 μM [5, 6]), the phosphodiesterase (PDE) inhibitor isobutylmethylxanthine (IBMX, 50 μM [5]), the phospholipase C inhibitor U73122 (100 nM [7]) and the adenylyl cyclase inhibitor SQ22536 (1 μM [5, 8]) were obtained from Tocris Bioscience (Bristol, UK). Xestospongin C [9], which antagonizes the calcium-releasing action of IP\(_3\) at the receptor level without interacting with the IP\(_3\)-binding site, was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), except where indicated.

#### Measurement of the [Ca\(^{2+}\)]\(_i\) concentration

Primary-cultured TG cells were loaded with 10 μM fura-2 acetoxyethyl ester (DOJINDO, Kumamoto, Japan) and 0.1% (w/v) pluronic F-127 acid (Life Technologies) in Hanks’ solution (90 min at 37 °C, 5% CO\(_2\)). The cultured TG cells were then rinsed with fresh Hanks’ solution. A dish containing fura-2-loaded TG cells was mounted onto the stage of a microscope (model IX73; Olympus Corp., Tokyo, Japan) equipped with HCImage software, an excitation wavelength selector and an intensified charge-coupled device camera system (Hamamatsu Photonics, Hamamatsu, Japan). Fura-2 fluorescence emissions were recorded at...
510 nm under alternating excitation wavelengths of 380 nm (F380) and 340 nm (F340). The [Ca$^{2+}$]$_i$ was measured as the fluorescence ratio of F340 and F380 (F$_{380}$/F$_{340}$), expressed in F/F$_{0}$ units; that is, the F$_{380}$/F$_{340}$ value (F) was normalized to the resting value (F$_{0}$). The F/F$_{0}$ baseline was set at 1.0. We evaluated [Ca$^{2+}$]$_i$ responses as changes in the F/F$_{0}$ values using the formula:

\[
\text{Change in fluorescence (\Delta F)} = \frac{F}{F_{0peak}} - \frac{F}{F_{0base}}
\]

where F/F$_{0peak}$ was the value obtained at peak [Ca$^{2+}$]$_i$ response; F/F$_{0base}$ indicates the value just before the application of certain pharmacological agents.

### Statistical and offline analysis

The data are expressed as the mean ± standard error of the mean of N observations, where N represents the number of independent experiments or cells. The Wilcoxon rank-sum test, Kruskal–Wallis test or Mann–Whitney U test were used to determine nonparametric statistical significance. A p value of < 0.05 was considered to be significant. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

### Results

**Lys-[Des-Arg$^9$]BK, a B$_1$ receptor agonist, induced [Ca$^{2+}$]$_i$ increases in TG neurons**

Primary-cultured TG neurons exhibited a round-shaped cell body that ranged in diameter from 7.2 to 53.6 μm (Fig. 1a). We first examined the response of these neurons to Lys-[Des-Arg$^9$]BK-induced [Ca$^{2+}$]$_i$, in both the presence and absence of external Ca$^{2+}$. In the presence of extracellular Ca$^{2+}$ (2.0 mM), the first application of Lys-[Des-Arg$^9$]BK (10 nM) evoked transient increases in [Ca$^{2+}$]$_i$ to peak values of 0.61 ± 0.07 ΔF units, and the second application of this molecule evoked transient increases in [Ca$^{2+}$]$_i$, to peak values of 0.54 ± 0.07 ΔF units (Fig. 1b, c). Following the removal of Ca$^{2+}$ from the extracellular solution, repeated addition of Lys-[Des-Arg$^9$]BK (10 nM) again produced rapid and transient increases in [Ca$^{2+}$]$_i$, reaching peak values of 0.18 ± 0.05 ΔF units for the first application, and 0.11 ± 0.02 ΔF units for the second application (Fig. 1b, c). There was no significant difference in the peak values between the first and second applications of Lys-[Des-Arg$^9$]BK in either the presence or absence of extracellular Ca$^{2+}$ (Fig. 1c). However, there were significant differences in the amplitudes of the Lys-[Des-Arg$^9$] BK-induced [Ca$^{2+}$]$_i$, increases as a function of the presence or absence of extracellular Ca$^{2+}$ (Fig. 1c). After extracellular Ca$^{2+}$ was restored to the extracellular solution, the baseline level of [Ca$^{2+}$]$_i$ increased, and Lys-[Des-Arg$^9$] BK-induced [Ca$^{2+}$]$_i$ increases could also be observed (Fig. 1b, c). Irrespective of cell body diameter (Fig. 1a), 119 of 127 neurons (from 16 experiments) responded to Lys-[Des-Arg$^9$]BK.

### Effects of inhibition of ryanodine receptors and SERCAs

Following repeated application of Lys-[Des-Arg$^9$]BK, the addition of the SERCA inhibitor CPA (100 nM) gradually increased [Ca$^{2+}$]$_i$ in both the presence (Fig. 2a) and absence (Fig. 2c) of extracellular Ca$^{2+}$. After the CPA-induced [Ca$^{2+}$]$_i$ increase reached a plateau, subsequent application of Lys-[Des-Arg$^9$]BK resulted in a further increase in [Ca$^{2+}$]$_i$ (Fig. 2a) in the presence of extracellular Ca$^{2+}$, but only quite small [Ca$^{2+}$]$_i$ increases occurred in the absence of extracellular Ca$^{2+}$ (Fig. 2c). In both the presence (Fig. 2b) and absence (Fig. 2d) of extracellular Ca$^{2+}$, the ΔF values of the [Ca$^{2+}$]$_i$ increases induced by Lys-[Des-Arg$^9$]BK in the presence of 100 nM CPA were significantly smaller than those induced in the absence of CPA.

The ryanodine receptor inhibitor dantrolene (1.0 μM dantrolene sodium salt) significantly abolished Lys-[Des-Arg$^9$] BK-induced [Ca$^{2+}$]$_i$ increases, reducing them to ΔF values of 0.10 ± 0.02 and 0.20 ± 0.03 in the absence (Fig. 3a, b) and presence (Fig. 3c, d), respectively, of external Ca$^{2+}$. Notably, the Lys-[Des-Arg$^9$]BK-induced [Ca$^{2+}$]$_i$ increases were observed after Ca$^{2+}$ was restored to the extracellular medium following Ca$^{2+}$-free conditions (Fig. 3a).

### Effects of PLC inhibition

To examine the effects of PLC or IP$_3$ receptor inhibition, we first measured the increase in Lys-[Des-Arg$^9$]BK-elicted [Ca$^{2+}$]$_i$ in the absence of extracellular Ca$^{2+}$ (Fig. 4a). We then restored extracellular Ca$^{2+}$ and allowed the [Ca$^{2+}$]$_i$, to reach a steady state, following which we once again observed increases in Lys-[Des-Arg$^9$]BK-induced [Ca$^{2+}$]$_i$. When we applied the PLC inhibitor U73122 (100 nM) in the absence of extracellular Ca$^{2+}$, the baseline value of [Ca$^{2+}$]$_i$, further gradually increased. In the presence of U73122 but absence of extracellular Ca$^{2+}$, Lys-[Des-Arg$^9$]BK again increased the [Ca$^{2+}$]$_i$. We did not observe any significant effect of the presence or absence of U73122 on the ΔF values resulting from the application of Lys-[Des-Arg$^9$]BK in the absence of extracellular Ca$^{2+}$ (Fig. 4a, b).

In addition, application of xestospongin C (1 μM [9]) did not show any effects on the Lys-[Des-Arg$^9$]BK-induced [Ca$^{2+}$]$_i$ increases in the presence of extracellular Ca$^{2+}$ (Fig. 4c, d).
Effects of intracellular cAMP increases and adenylyl cyclase inhibition

In the presence of extracellular Ca\(^{2+}\), repeated application of Lys-[Des-Arg\(^9\)]BK elicited \([Ca^{2+}]_i\) increases (Figs. 5a, 6a). IBMX (50 µM), a selective PDE inhibitor that raises intracellular cAMP levels, significantly and reversibly inhibited the increases in \([Ca^{2+}]_i\) induced by Lys-[Des-Arg\(^9\)]BK (Fig. 5a, b). Conversely, SQ22536 (0.1 µM), an adenylyl cyclase inhibitor that decreases intracellular cAMP levels, significantly enhanced the increases in \([Ca^{2+}]_i\) induced by Lys-[Des-Arg\(^9\)]BK in the presence of extracellular Ca\(^{2+}\) (Fig. 6a, b).
Discussion

The results of our study show that B1 receptor activation by Lys-[Des-Arg9]BK in TG neurons induced increases in the [Ca2+]i, in both the presence and absence of extracellular Ca2+, thereby indicating that B1 receptors are capable of mobilizing Ca2+ by triggering Ca2+ release from intracellular stores. Notably, almost all of the primary-cultured TG neurons (93.7%) responded to Lys-[Des-Arg9]BK. The Ca2+ was removed and 100 nM CPA was applied (black bar at top of graph), which gradually elicited an increase in [Ca2+]i, with the subsequent application of Lys-[Des-Arg9]BK (10 nM) inducing a considerably small transient [Ca2+]i increase. Gray boxes on the upper-right side of graphs in a and c indicates the timing for application of the high extracellular-K+ (50 mM) solution. d Summary bar graph showing [Ca2+]i increases following the first (upper bar) and second (middle bar) application of 10 nM Lys-[Des-Arg9]BK in the presence of external Ca2+ (2.0 mM) and following the application of 10 nM Lys-[Des-Arg9]BK with 100 nM CPA (lowermost bar) in the presence of extracellular Ca2+ (gray box on the upper-right side of graph). Each bar denotes the mean ± SE of four experiments. Statistical significance between the bars in b and d (shown by solid lines) is indicated by asterisks: *p < 0.05.

Fig. 2 Effects of sarcoplasmic reticulum Ca2+-ATPase inhibitors on [Ca2+]i. a Representative [Ca2+]i trace upon additions of Lys-[Des-Arg9]BK (upper white boxes) is shown. Application of 100 nM of cyclopiazonic acid (CPA; black bar at top of graph) gradually elicited an increase in [Ca2+]i, and the subsequent application of Lys-[Des-Arg9]BK (10 nM) induced a further increase in transient [Ca2+]i. b Summary bar graph showing [Ca2+]i increases following the first (upper bar) and second (middle bar) application of 10 nM Lys-[Des-Arg9]BK in the presence of external Ca2+ (2.0 mM) and following the application of 10 nM Lys-[Des-Arg9]BK with 100 nM CPA (lowermost bar) in the presence of extracellular Ca2+ (gray box on the upper-right side of graph). Each bar denotes the mean ± SE of seven experiments. c Following the repetitive [Ca2+]i increases triggered by Lys-[Des-Arg9]BK (white boxes at top of graph), extracellular distribution of the cell body diameter of TG neurons in the present study is consistent with that reported in our previous study [10]. The amplitudes of the Lys-[Des-Arg9]BK-induced [Ca2+]i increases in the absence of extracellular Ca2+ were significantly smaller than those in the presence of extracellular Ca2+ (Fig. 1), indicating that Lys-[Des-Arg9]BK mobilizes Ca2+ not only by releasing it from intracellular stores, but also by inducing Ca2+ influx from the extracellular medium. These results are in agreement with those from
our previous study showing that B2 receptor activation in TG neurons also induced both Ca\^{2+} release and Ca\^{2+} influx [3]. In vascular smooth muscle cells, Mathis et al. observed that B1 receptor activation not only elevated [Ca\^{2+}]_i by inducing the release of Ca\^{2+} from intracellular Ca\^{2+} stores, but also produced [Ca\^{2+}]_i oscillations that were dependent on Ca\^{2+} influx from the extracellular medium [11]. In embryonic chick heart cells, El-Bizri et al. observed BK-activated T-type and L-type voltage-dependent Ca\^{2+} currents that were partially inhibited by a B1 receptor antagonist [12]. However, Kitakoga and Kuba reported that in their study BK did not elicit Ca\^{2+} currents in TG neurons [13]. Recently, Ifuku et al. demonstrated that microglial migration mediated by the activation of B1 receptors depends on the Ca\^{2+} entry mode (or “reverse mode” producing Ca\^{2+} influx) of Na\(^+\)/Ca\^{2+} exchanger (NCX) activity (NCX-induced Ca\^{2+} influx;
We previously reported the expression of NCX isoforms (NCX1, NCX2, and NCX3) in primary-cultured rat TG neurons and observed reverse mode of NCX activity that was functionally coupled to voltage-dependent Na⁺ channels [15]. Although further study will be needed to clarify the extracellular Ca²⁺ influx pathway induced by B₁ receptor activation in TG neurons, the results from our present study show that B₁ receptors mobilized intracellular Ca²⁺ via tryanodine receptors that access intracellular Ca²⁺ stores (see below).
We found that a SERCA pump inhibitor, CPA, reduced the ΔF amplitude of B₁ receptor activation-induced [Ca²⁺]ᵢ increases, while increasing the baseline values of the [Ca²⁺]ᵢ in both the absence and presence of external Ca²⁺. This process resulted from both the leakage of Ca²⁺ from the intracellular Ca²⁺ store and the accumulation of [Ca²⁺]ᵢ by suppression of movement of Ca²⁺ into that store. In the absence of extracellular Ca²⁺, CPA almost completely abolished the Lys-[Des-Arg⁹]BK-induced [Ca²⁺]ᵢ increases, compared with those in the absence of CPA, suggesting...
The B₁ receptor is directly coupled to G proteins of the G₁₂ and G₃ families [1]. The B₁ agonist generated by the degradation of BK activates B₁ receptors coupled to the G₁₂ family [1, 14]. Activation of the G₁₂ family mediates the phosphoinositide turnover signaling pathway, resulting in an [Ca²⁺]ᵢ increase through the generation of IP₃ via activation of PLC. Interestingly, however, administration of not only the PLC inhibitor U73122, but also the membrane-permeable IP₃ receptor blocker xestospongin C did not affect the Lys-[Des-Arg⁹]BK-induced Ca²⁺ release increases. Thus, these results suggest that the PLC-IP₃ signaling pathway might not contribute to the Lys-[Des-Arg⁹]BK-induced Ca²⁺ release from the intracellular stores via ryanodine receptors in the TG neurons. In addition, the subsequent Ca²⁺ release from ryanodine receptors elicited by IP₃-mediated Ca²⁺ release might also be unlikely. In the present study, although dantrolene almost completely suppressed the Lys-[Des-Arg⁹]BK-induced Ca²⁺ release in TG neurons, we observed a residual component of the [Ca²⁺]ᵢ increase during application of dantrolene in both the presence and absence of extracellular Ca²⁺. Therefore, we cannot exclude the contribution of the PLC-IP₃ signaling cascade to Lys-[Des-Arg⁹]BK-induced Ca²⁺ mobilization in TG neurons. However, the Lys-[Des-Arg⁹]BK-induced Ca²⁺ mobilization may be mediated by another signaling pathway, such as the cAMP-dependent pathway, rather than a PLC-coupling Gq pathway (see below).

Since both U73122 [16] and xestospongin C [17] exert inhibitory effects on not only the IP₃-mediated Ca²⁺ release but also the SERCA pumps, in our study they increased the baseline F/I₀ value. Therefore, the U73122- and xestospongin C-induced increases in the baseline F/I₀ value resulted from SERCA inhibition. The SERCA inhibitor CPA also increased the baseline F/I₀ value. The presence of CPA almost completely suppressed the Lys-[Des-Arg⁹]BK-induced Ca²⁺ increases, while exposure to U73122 and xestospongin C did not affect the increase. These results suggest that U73122 and xestospongin C more efficiently inhibit the PLC-IP₃ signaling pathway than do the SERCA pumps in TG neurons.

In contrast, activation of the G₁₂ family suppresses the production of cAMP from ATP. In the present study, increasing the intracellular cAMP level by applying the selective PDE inhibitor IBMX reduced the amplitude of Lys-[Des-Arg⁹]BK-induced [Ca²⁺]ᵢ increases (PDE hydrolyzes cAMP into inactive 5'-AMP). Decreases in intracellular cAMP levels induced by the inhibition of adenylyl cyclase (by SQ22536) had the opposite effect. These results indicate that B₁ receptor activation increases the [Ca²⁺]ᵢ, by suppressing adenylyl cyclase activity and thereby decreasing intracellular cAMP level.

In our recent study, activation of the P2Y₁₂ receptor, a G protein-coupled nucleotide receptor expressed in TG neurons, also increased the [Ca²⁺]ᵢ; the P2Y₁₂ receptor-induced [Ca²⁺]ᵢ increase is also sensitive to a ryanodine receptor inhibitor [5]. In addition, in the same study, application of SQ22536 to the primary-cultured TG neurons resulted in a concentration-dependent increase in [Ca²⁺]ᵢ, while the application of IBMX inhibited the P2Y₁₂ receptor activation-mediated [Ca²⁺]ᵢ increase. These results are in agreement with our present results, showing that, in the TG neurons, a decrease in intracellular cAMP levels due to the suppression of adenylyl cyclase following the activation of B₁ receptor increases the [Ca²⁺]ᵢ, thereby suggesting that intracellular Ca²⁺ mobilization by Lys-[Des-Arg⁹]BK may possibly be regulated by a cAMP-dependent Gq pathway.

It has been demonstrated that activation of cAMP-dependent protein kinase (protein kinase A) results in inhibition of PLC activity, IP₃ production and subsequent IP₃-induced Ca²⁺ mobilization during smooth muscle relaxation [18, 19], implying that a reduction of cAMP production is capable of enhancing IP₃-induced Ca²⁺ release. The results of our present study show that the PLC inhibitor and IP₃ receptor blocker did not have any effect on the Lys-[Des-Arg⁹]BK-induced Ca²⁺ increases in TG neurons, while the reduction of cAMP levels by B₁ receptor activation triggered an increase in the [Ca²⁺]ᵢ via ryanodine receptors. Taken together, our results suggest that intracellular Ca²⁺ mobilization may possibly be regulated by a cAMP-dependent Gq pathway, but not by PLC activity or IP₃-induced Ca²⁺ release. Further studies are needed to clarify the detailed mechanism of cAMP-dependent Ca²⁺ release via ryanodine receptors, following activation of not only B₁ receptors but also P2Y₁₂ receptors.

The expression of B₁ receptors is induced rapidly in response to tissue damage or inflammation [20]. This expression pattern of B₁ receptors suggests that they may play a role in chronic inflammation [1]. The morphological and functional expression of B₁ receptors in TG and dorsal root ganglion (DRG) neurons, however, is controversial. In DRG neurons, constitutive B₁ receptor expression, assessed immunohistochemically, has been reported [21, 22], while B₁ receptor activation-induced [Ca²⁺]ᵢ responses have not been observed in DRG neurons [23]. Although constitutive expression of the B₁ receptor has been described in TG neurons [22], in our previous study, we were unable to observe a clear localization of B₁ receptors in cryosections prepared from intact TG tissue, although we did observe weak immunoreactivity for these receptors in primary-cultured TG neurons [3]. Interestingly, the B₁ receptor mRNA expression was barely detectable in the intact tissue; however, in primary-cultured TG neurons,
the B$_1$ receptor mRNA expression has been reported to depend on the length of the culture period [24]. In addition, both the results from our previous [3] and present studies clearly show that Lys-[Des-Arg$^9$]BK, a metabolite of endogenous BK in peripheral tissues [25], is capable of triggering $[\text{Ca}^{2+}]_i$ changes in TG neurons, in contrast to DRG neurons. These morphological and mRNA expression patterns of B$_1$ receptors in primary-cultured TG neurons or intact TG tissue suggest that B$_1$ receptor expression is induced in TG neurons as a result of tissue damage and/or inflammation. They also suggest a role for B$_1$ receptors in modulating nociceptive functions. Although further studies are required to determine the expression pattern of B$_1$ receptors in TG neurons, these neurons clearly express B$_1$ receptors that mobilize intracellular Ca$^{2+}$ [1–4].

In conclusion, we have demonstrated the expression of B$_1$ receptors in primary-cultured TG neurons and clarified the intracellular signaling pathway that follows B$_1$ receptor activation. An agonist for B$_1$ receptors, Lys-[Des-Arg$^9$]BK, mobilizes Ca$^{2+}$, via activation of the intracellular Ca$^{2+}$ releasing pathway that is mediated by ryanodine receptors. The intracellular signaling pathways that increase the $[\text{Ca}^{2+}]_i$ are activated by suppression of intracellular cAMP production. Thus, the effects of B$_1$ receptors in TG neurons may possibly be mediated by a G$_i$ pathway.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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