The Central Nervous System as Target for Antihypertensive Actions of a Proline-Rich Peptide from Bothrops jararaca Venom

Claudiana Lameu,5 Mirian A. F. Hayashi,2,3 Juliano R. Guerreiro,4 Eduardo F. Oliveira,2 Ivo Lebrun,5 Vera Pontieri,2 Kátia L. P. Morais,2 Antonio C. M. Camargo,2,6 Henning Ulrich1*

Abstract
Pyroglutamyl proline-rich oligopeptides, present in the venom of the pit viper Bothrops jararaca (Bj-PROs), are the first described naturally occurring inhibitors of the angiotensin I-converting enzyme (ACE). The inhibition of ACE by the decapeptide Bj-PRO-10c (<ENWPHPQIPP) and other Bj-PROs was classically used to explain the pharmacological effects of these venom peptides in mammals resulting in a decrease of blood pressure. Recent studies, however, suggest that ACE inhibition alone is not sufficient for explaining the antihypertensive actions exerted by these peptides. In this study, we show that intracerebroventricular injection of Bj-PRO-10c induced a significant reduction of mean arterial pressure (MAP) together with a decrease of heart rate (HR) in spontaneously hypertensive rats, indicating that Bj-PRO-10c may act on the central nervous system. In agreement with its supposed neuronal action, this peptide dose-dependently evoked elevations of intracellular calcium concentration ([Ca^{2+}]) in primary culture from postnatal rat brain. The N-terminal sequence of the peptide was not essential for induction of calcium fluxes, while any changes of C-terminal Pro or Ile residues affected Bj-PRO-10c’s activity. Using calcium imaging by confocal microscopy and fluorescence imaging plate reader analysis, we have characterized Bj-PRO-10c-induced [Ca^{2+}], transients in rat brain cells as being independent from bradykinin-mediated effects and ACE inhibition. Bj-PRO-10c induced pertussis toxin-sensitive G_i/o-protein activity mediated through a yet unknown receptor, influx and liberation of calcium from intracellular stores, as well as reduction of intracellular AMP levels. Bj-PRO-10c promoted glutamate and GABA release that may be responsible for its antihypertensive activity and its effect on HR. © 2010 International Society for Advancement of Cytometry

Key terms
pyroglutamyl proline-rich oligopeptides; calcium signaling; G-protein coupled receptor; neurotransmitter release; cardiovascular homeostasis; neuronal cells; snake peptide; calcium imaging by confocal microscopy; fluorescence imaging plate reader analysis

Animal toxins have largely contributed not only to the discovery and development of pharmaceutical compounds but also for the identification of protein targets for therapeutic interventions (1). In early 80s, a new class of therapeutic agents for the treatment of hypertension, namely the active site directed inhibitors of the angiotensin I-converting enzyme (ACE), was introduced (2). The pharmacological and biochemical properties of the bradykinin-potentiating peptides (BPPs), which are mainly proline-rich oligopeptides found in the venom of the Brazilian snake Bothrops jararaca (Bj-PROs) (3,4), were crucial for the development of the antihypertensive drug named Captopril (2,5). This compound represents one of the best examples of a target-driven drug discovery (reviewed in Ref. 6).

Bj-PROs belong to a class of oligopeptides consisting of 5–14 amino acids with both a canonical N-terminal pyroglutamyl moiety and a C-terminal prolyl residue...
The bradykinin (BK) potentiation of the isolated smooth muscle contracting activity of Bj-PROs and its antihypertensive effects were associated with ACE inhibition (5). However, the dissociation between the potentiation of BK-mediated effects on smooth muscle contraction and ACE inhibition was already demonstrated long time ago (3,7). Moreover, neither all antihypertensive acting Bj-PROs are inhibitors of ACE nor their cardiovascular activity in spontaneous hypertensive rats (SHRs) are related to the inhibition of plasma ACE activity (8).

It is also worth mentioning that, at least in snakes, the Bj-PRO-10c is present in the same precursor protein of a C-type natriuretic peptide (CNP) (9). In situ hybridization studies also showed the expression of these peptides in brain regions correlated with neuroendocrine functions, which include aldosterone secretion, excretion of sodium, and diuresis (10). Intriguingly, biodistribution studies of Bj-PRO-10c revealed the accumulation of this peptide in various mouse tissues, including the brain (11). Moreover, Bj-PRO-10c evoked changes in arterial blood pressure that were followed by a significant reduction of heart rate (HR) (8). However, this effect seems to be contradictory because it is expected that the reflex discharge of baroreceptors triggered by the reduction of blood pressure should be accompanied by an increase and not a decrease of the HR (12). As possible explanation, Bj-PRO-10c could act on the central nervous system (CNS), thus influencing the sympathetic and parasympathetic autonomic activities involved in the baroreflex control of the HR (13–15). Taken together, these results strongly suggested a potential CNS action of Bj-PRO-10c. Accordingly, we describe here that intracerebroventricular (ICV) injection of this peptide in SHRs decreases the arterial blood pressure together with a reduction of the HR.

This study provides evidence that Bj-PRO-10c induces transient increases of free intracellular calcium concentration ([Ca$^{2+}$]$_i$) in neuronal cells through a mechanism by activation of a yet unknown Ca$_{i}$-protein coupled receptor and promotes release of neurotransmitters in neuronal cells, namely GABA and glutamate, which may contribute to central cardiovascular effects exerted by Bj-PRO-10c.

**Materials and Methods**

**Synthesis and Purification of Peptides**

Peptides were synthesized essentially as previously described by Gomes et al. (16). Briefly, the synthesis of Bj-PRO-10c (<ENWPHPQIPP>) and analogs were performed on an automated PSSM-8 peptide synthesizer (Shimadzu Corp., Kyoto, Japan) by a stepwise solid-phase method using N-9-fluorenlymethoxycarbonyl (Fmoc) chemistry (Novabiochem-EMD Chemicals, San Diego, CA). Cleavage of the peptide from the resin was achieved by treatment with a mixture of trifluoroacetic acid (TFA)/1,2-ethanediol/ethyl methyl sulfide for 2 h at room temperature. After removal of the resin by filtration and washing twice with TFA, the crude synthetic peptide was purified by preparative reversed-phase high-performance liquid chromatography (HPLC) (Shimadzu Corp.) on a YMC-Pack ODS column (20 mm × 150 mm (YMC, Kyoto, Japan), using a linear gradient from 3 to 20% CH$_3$CN in 0.1% TFA, at a flow rate of 7 ml/min. Both, the purity and primary structure of each synthetic peptide were confirmed by analytical HPLC and MALDI-TOF mass spectrometry (Amer sham Biosciences, Uppsala, Sweden). Samples were frozen in liquid nitrogen and then freeze dried (Edwards Freeze Dryer Super Modulyst Pirani 1001, Thermo Fisher Scientific, Wal tham, MA) for 48 h at –50 °C under vacuum. Following freeze drying (17), the counter ion TFA was completely exchanged as determined mass spectrometry.

**Microinjection of Bj-PRO-10c in the Lateral Ventricle of SHRs**

Male spontaneously hypertensive rats (SHRs) (270–350 g) from the animal house of the Instituto de Ciencias Biológicas (University of São Paulo, Brazil) were used. These animals were transported to a room adjacent to the test laboratory for 48 h after arrival, to become acclimatized to laboratory conditions. They were housed in groups of six per cage under a 12:12 dark/light cycle (lights on at 07:00 h) at 23 °C ± 1 °C, and free access to food and water was given. All procedures involving animals were approved by the local Ethics’ Committee of the Institute of Chemistry at the University of São Paulo. Animals were anesthetized with ketamine (185 mg/kg, i.p.) and xylazine (9.2 mg/kg, i.p.). The rats were fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and a guide cannula was stereotaxically implanted in the lateral ventricle. The following coordinates were used relative to bregma: A-P: 0.8 mm, L ± 1.5 mm, V: 4.6 mm (18). A set of two anchoring screws was fixed to the skull and dental acrylic was layered over them and the cannula. After surgery, the animals were then housed in groups of two for a postoperative recovery period of one week. Twenty hours before the experiment, under anesthesia with ketamine (185 mg/kg, i.p.) and xylazine (9.2 mg/kg, i.p.), a polyethylene catheter (PE-10 connected to PE-50) was introduced into the abdominal aorta through a femoral artery for measurements of mean arterial pressure (MAP). After recovery from anesthesia, the rats were kept in individual cages with free access to water and chow until the end of the experiments. Before drug administration, the MAP was monitored for 1 h (baseline period). Then, Bj-PRO-10c (0.7 nmol/kg) was microinjected into the lateral ventricle using a thin dental needle (o.d. 0.3 mm) linked to a 5-μl Hamilton syringe by means of a polyethylene tube. The injection needle was introduced through the guide cannula until its lower end was 2 mm below the guide cannula. Microinjections of the same volume of physiological saline served as vehicle control. MAP values were registered at 2 min intervals for the entire recording period of 6 h.

**Preparation of Rat Brain Cell Culture**

One-day-old male Wistar rats were killed by decapitation in agreement with the guidelines of the Ethics Committee of the Institute of Chemistry, University of São Paulo, Brazil. Whole-brain primary neuronal culture (Neu-W) was prepared from dissected brain. Pia mater and blood vessels were carefully removed, and brain cells were dissociated in the presence of trypsin. The cell suspension was plated on a poly-L-lysine-
precoated tissue culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% horse serum (Gibco BRL, Grand Island, NY) as previously described (19). Following 3 days of culture, 10 µM cytosine-arabinoside was added to eliminate cells of nonneuronal origin. Cells were cultivated at 37°C in a water-saturated atmosphere containing 5% CO2 for 10–15 days before using them for calcium measurements. Immunohistochemical analysis indicated that these cultures of neural cells contained 85–90% neurons and around 10–15% astroglial cells (19).

Culture of Neuronal SK-N-AS and Glial C6 Cells
SK-N-AS human neuroblastoma cells (ATCC No. CRL-2137) and C6 rat glioma cells (ATCC No. CRL-107) were cultured in DMEM containing 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). For SK-N-AS cell culture, the medium was further supplemented with 1 mM nonessential amino acids. All cells were maintained at 37°C in 5% CO2 atmosphere.

Calcium Measurements by Microfluorimetry
Changes in [Ca2+]i were determined by microfluorimetry using the FlexStation III (Molecular Devices Corp., Sunny Valley, CA), following the instructions of the manufacturer (20). Briefly, the cells were seeded a night before starting the experiment, using the FlexStation III (Molecular Devices Corp., Sunny Valley, CA), following the instructions of the manufacturer (20). For SK-N-AS cell culture, the medium was further supplemented with 1 mM nonessential amino acids. All cells were maintained at 37°C in 5% CO2 atmosphere.

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Calcium Imaging in Single Cells
Measurements of transient changes in [Ca2+]i in SK-N-AS and Neu-W cells were done by calcium imaging by using a LSM 510 Meta confocal microscope (Zeiss, Jena, Germany). Forty-eight hours before calcium measurements, 2.5 × 10^5 cells were seeded and allowed to attach on cover slides in p60-mm culture dishes (Nalge Nunc International, Rochester, NY). Cells were loaded for 30 min at 37°C with 4 µM Fluo-3-AM (Sigma Chemical, St. Louis, MO) in the presence of 0.5% Me2SO and 0.1% of the nonionic surfactant pluronic acid F-127, followed by three washes with DMEM containing 10% FBS. Fluo-3-AM fluorescence was excited with the Argon ion laser line at 488 nm, and fluorescence emission was collected at 510–530 nm using a band pass filter. First [Ca2+]i levels of nonstimulated cells and then the changes in [Ca2+]i, after addition of agonists (namely, Bj-PRO-10c, control peptides or BK) were recorded at 1.5 s intervals for a further 2 min. During the intervals between agonist applications, cells were washed with incubation medium. The ionophore 4-Br-A23187 (5 µM) and the chelating compound EGTA (10 mM) were used to determine the maximal (Fmax) and minimal (Fmin) fluorescence values, respectively. Intracellular free calcium concentrations were calculated from relative fluorescence values using the equation [Ca2+]i = Kd (F – Fmin)/(Fmax – F), assuming a 450 nM Kd for fluo-3 calcium binding (21). Calculated concentrations are mean values of data from at least 10 individually analyzed cells.

Measurement of Intracellular Cyclic AMP Concentration
Neu-W or C6 cells were grown in 96-well plates. Cells were washed with medium and then incubated for 10 min in the absence or presence of 1 µM Bj-PRO-10c in DMEM without FBS. Then, 50 µM of forskolin was added and incubated with the cells for further 60 min. Following cell lysis, cAMP contents were determined using the Enzyme Immunoassay System (Amersham Biosciences, Uppsala, Sweden) according to manufacturer’s instructions.

Radioligand Receptor Binding Assays with Bj-PRO-10c
Bj-PRO-10c receptor binding was profiled in a commercially available panel of 105 radioligand binding assays (MDS Pharma Services, Taiwan, China). These assays characterize potential interactions of radioligands with agonist binding sites of a wide range of G-protein coupled receptors (GPCRs). The experiments are based on a competition assay, where Bj-PRO-10c (10 µM) competes with radiolabeled known GPCR agonists or antagonists for the receptor binding site. Each radioligand binding assay has been fully characterized and validated for ligand binding to the respective receptor. The specific list of the assays performed in the presence of 10 µM Bj-PRO-10c is documented as Supplementary Material and further details of the methodologies for each assay can be found at URL: http://www.mdsps.com/. Experiments were carried out in duplicate.

Measurement of Neurotransmitter Release in Neuronal Cells
Amounts of GABA, glutamate, aspartate, and glycine released by the neuronal cell primary culture were determined using a HPLC system (Shimadzu, Kyoto, Japan). Approximately 3 × 10⁶ Neu-W cells were kept for 2–5 h in a serum-free culture medium. Then, the cells were washed three times with HEPES-buffered Krebs-Ringer solution (HKR buffer) containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 5.5 mM glucose, and 50 mM HEPES (pH 7.4). Initially, HKR buffer was collected without previous stimulation of cells for the determination of basal GABA, glutamate, aspartate, and glycine release. Then, neurotransmitter release was quantified following exposure of cells to 1 µM Bj-PRO-10c for 3 min. Collected HKR buffer fractions were transferred...
into tubes on ice and filtered through membranes of 0.22 μm (Millipore Corp., MA) for removal of cell debris. Derivatization of secreted amino acids was done with a phenylisothiocyanate reagent (75% ethanol/water (7/1), 12.5% triethylamine, 12.5% phenylisothiocyanate), and samples were analyzed by HPLC (Shimadzu Co.) using a C18 analytical column (250 mm, 4.6 mm, 5 μm, Merck, Whitehouse Station, NJ). Elution times and integrated peak areas were compared with standard pure compounds (GABA, glutamate, aspartate, or glycine) for quantification of concentrations of these amino acids in the sample.

**Statistical Analysis**

Statistical analysis was conducted by using one-way ANOVA, employing the GraphPad3 software. Differences were considered significant when $P < 0.05$. The student t-test was employed for verification of statistical significance of neurotransmitter release in the presence or absence of Bj-PRO-10c and peak values for cardiovascular parameters of Bj-PRO-10c-or vehicle-treated SHRs.

**RESULTS**

**Effect of Bj-PRO-10c on the Central Nervous System of Spontaneously Hypertensive Rats**

The cardiovascular parameters, namely MAP and HR, of conscious SHRs were recorded after ICV injection of 0.7 nmol/kg Bj-PRO-10c. This peptide induced long-lasting antihypertensive activity, which was visible for up to 6 h. A significant reduction of MAP was observed (Fig. 1A) together with a decrease of HR (Fig. 1B). The peak change in MAP produced by Bj-PRO-10c in SHRs was $-28 \pm 4$ mm Hg. The changes in MAP were associated with significant reductions in HR [peak change, $-122 \pm 33$ beats per minute (bpm) (Fig. 1B)]. The vehicle control also induced small, statistically non-relevant alterations in HR due to bolus injection, as already observed in published studies (22).
Characterization of Bj-PRO-10c-Induced [Ca$^{2+}$],
Transients in Neuronal Cells

Bj-PRO-10c-induced transients of [Ca$^{2+}$], after stimulation with 0.1–10 μM of the peptide were monitored by microfluorimetry. Fluorescence imaging plate reader analysis was already shown to be a sensitive and reproducible assay for dose- dependent induction of GABA$_B$ receptor-mediated [Ca$^{2+}$], elevations (23).

Dose–response curves for Bj-PRO-10c-induced elevations of [Ca$^{2+}$], in Neu-W, SK-N-AS, and C6 cells were individually determined, showing potencies (pD$_2$ values) of 7.23 ± 0.09 and 7.19 ± 0.06, for Neu-W and SK-N-AS cells, respectively, while no significant [Ca$^{2+}$], response could be observed in C6 rat glioma cells (Figs. 2A and 2B). Bj-PRO-10c-provoked increases in [Ca$^{2+}$], were transient, obtaining peak values within 20 s and returning to basal values 120 s after stimulation onset (Figs. 2C and 2D).

Although being a strong inhibitor of ACE (10), this specific property of Bj-PRO-10c was not involved in [Ca$^{2+}$], transients elicited by this peptide in neuroblastoma cells, as 1 μM of the ACE inhibitor captopril, an excess concentration in view of its dissociation constants of 0.5 and 8.3 nM for binding to N- and C-terminal sites of ACE, respectively (reviewed by Ref. 6), did not provoke any changes of [Ca$^{2+}$], in these cells (Figs. 3A and 3B). In agreement with these results, ruling out any participation of the kinin-kallikrein system and BK-B2 receptors, pretreatment of Neu-W cells for 1 or 5 min with 1 μM of Bj-PRO-10c did not potentiate BK-induced [Ca$^{2+}$], transients (data not shown).

Repeated applications of Bj-PRO-10c in 2 min intervals to the same cell preparation resulted in desensitization of putative receptors for this peptide, which was not reversed by removal of the peptide from the cells by washing (Fig. 3C). On the other hand, the response to BK triggered [Ca$^{2+}$], transients, known to be mediated by the BK-B2 receptor, was not affected by the previous stimulation with Bj-PRO-10c (Figs. 3B, 3C, 4A, and 4B), suggesting independent pathways for Bj-PRO-10c and BK, since a long-lasting BK-B2 receptor desensitization is usually expected after agonist binding (24).

Bj-PRO-10c analogs were synthesized to verify the specificity of the observed [Ca$^{2+}$], mobilization response triggered by this snake peptide. A partially scrambled sequence of Bj-PRO-10c, with five N-terminal amino acid sequences in random order (N5-scrambled) (PHNWQPQIPP), suggested the importance of the C-terminal motif for this biological activity, since no difference in the [Ca$^{2+}$], transients could be observed when this peptide was employed for cell stimulation compared to the effects elicited by the unmodified Bj-PRO-10c (c<ENWHPQPQIPP) (Fig. 5). In the same way, a truncated form of Bj-PRO-10c, lacking the first three N-terminal amino acid residues (without c<ENW) (PHQPQIPP), also did not reveal any difference in its potency of inducing [Ca$^{2+}$], transients compared to the unmodified Bj-PRO-10c. However, the inverted Bj-PRO-10c peptide (PPIQPHPWNE) with the same amino acid composition as Bj-PRO-10c and oppositely ordered from C- to N-terminal, did not show any activity.

Characterization of the Bj-PRO-10c-Induced Signal Transduction

Bj-PRO-10c-induced [Ca$^{2+}$], transients require the influx of extracellular calcium as well as involve the calcium release from the intracellular stores (Fig. 6). The presence of calcium chelator EGTA or thapsigargin, an inhibitor of endoplasmic reticulum Ca$^{2+}$-ATPase (25) resulting in depletion of intracellular calcium stores, led to an almost complete loss of the Bj-PRO-10c-induced [Ca$^{2+}$], response.

BK produced by developing and mature brain neurons, activating the BK-B2 receptor with well-characterized signal transduction (26), was used as control, either in the absence or presence of inhibitors. The induced signal transduction includes activation of phospholipase C-β (PLC-β) and liberation of calcium from intracellular inositol-triphosphate (IP$_3$)-sensitive stores (27). As expected, preincubation with U-73122, a specific inhibitor of PLC-β activity (28), inhibited the BK-induced [Ca$^{2+}$], increase. In agreement, depletion of extracellular calcium by EGTA did not affect the mobilization of intracellular calcium by BK.

Moreover, pretreatment of Neu-W cells for 18 hours with 100 ng/ml of pertussis toxin (PTX), an inhibitor of G$_i$, 202
Protein coupled metabotropic receptor activation, largely diminished Bj-PRO-10c-induced \([\text{Ca}^{2+}]_i\) transients, while 50 nM ryanodine for inhibiting calcium-induced calcium release (CICR) mechanism (29) also resulted in an almost complete blockade of Bj-PRO-10c-induced responses. Pretreatment of Neu-W cells with 1 μM HOE-140, a BK-B2 receptor antagonist, did not interfere with Bj-PRO-10c-induced responses. In control experiments, Gq-protein coupled BK-B2 receptor activity was not affected by preincubation with PTX but was completely blocked by HOE-140 (Fig. 6).

Inhibition of Bj-PRO-10c-induced \([\text{Ca}^{2+}]_i\) responses by PTX (Fig. 6) already suggested the involvement of Gi/o-protein action. As further evidence for the participation of a Gi/o-protein, addition of 1 μM Bj-PRO-10c resulted in an almost complete inhibition of forskolin-induced cAMP accumulation in Neu-W cells, while in C6 glioma cells such inhibition could not be observed (Fig. 7). It is worthwhile to note that inhibition of cAMP accumulation by Bj-PRO-10c was not observed in the absence of forskolin.

Putative Bj-PRO-10c Receptor Search

Aiming to identify the putative receptor for Bj-PRO-10c, this peptide was used as ligand for an affinity screening against 105 known G-protein-coupled seven-transmembrane receptors, including the BK-B2 receptor (Table 1 in Supp. Info.). However, no specific binding to any of the evaluated receptors could be observed for Bj-PRO-10c.

Effects of Bj-PRO-10c on Neurotransmitter Release

Release of excitatory (aspartate and glutamate) and inhibitory (glycine and GABA) neurotransmitters by Neu-W cells was quantified following stimulation with Bj-PRO-10c. In the presence of this peptide, GABA release was significantly increased (from 24.5 ± 1.2 to 65.0 ± 3.3 nmol/sample) (Fig. 8A). Moreover, application of 1 μM Bj-PRO-10c resulted in a 1.5-fold increase of glutamate release (from 1.8 ± 0.1 to 2.7 ± 0.7 nmol/sample) (Fig. 8B). On the other hand, release of glycine was not significantly affected by the presence of 1 μM of Bj-PRO-10c (from 117.7 ± 5.9 to 122.3 ± 6.1 nmol/sample) (Fig. 8A), while release of aspartate could not be detected nei-
Bj-PRO-10c-induced Effects in CNS

**DISCUSSION**

A number of snake toxins possess endogenous counterparts in vertebrates. This may be also true for proline-rich oligopeptides (PROs) because PRO sequences are present in CNP precursors, typically found in mammalian CNS (9). Moreover, expression of Bj-PRO-precursors is not restricted to the venom gland, as it has also been identified by in situ hybridization in snake brain correlated with neuroendocrine functions (10).

The results of this study suggest that antihypertensive actions exerted by Bj-PRO-10c may involve actions on the CNS. It is known that prototypes of antihypertensive drugs, such as clonidine and \( \alpha \)-methyldopa, change efferent autonomic activity in the cardiovascular system leading to a decline of MAP and HR (13). Aiming to study the effects of this snake peptide on the mammal CNS, 0.7 nmol/kg of Bj-PRO-10c were microinjected into the lateral ventricle of conscious SHRs. Bj-PRO-10c evoked antihypertensive and bradycardic responses. Bj-PRO-10c injection into the brain induced similar effects to those observed with intravenous peptide administration (8). As a previous biodistribution study revealed that 1% of radiolabeled peptide was detected in the brain (11), we have microinjected into the lateral ventricle of SHRs, 1% of the dose, which had provoked the highest antihypertensive response in the work of Lanzer et al. (8).

Cellular mechanisms were investigated in this study to explain Bj-PRO-10c actions in the brain. Primary cultures of postnatal rat brain (Neu-W cells) were used to evaluate Bj-PRO-10c-evoked effects on calcium signaling and neurotransmitter release. Calcium is an important second messenger in the CNS, controlling almost every cellular process, including gene expression, signal transmission, and neurotransmitter liberation (reviewed in Ref. 30). The data presented herein show that Bj-PRO-10c induced \([Ca^{2+}]_i\), transients in neuronal Neu-W and SK-N-AS cells, but not in glial C6 cells. Bj-PRO-10c-induced \([Ca^{2+}]_i\), responses increased with rising peptide concentrations in both Neu-W and SK-N-AS cells. Similarly to BK-induced responses (data not shown), maximal peak

![Figure 4. Confocal imaging of \([Ca^{2+}]_i\), transients in Neu-W cells after stimulation with Bj-PRO-10c or BK. Experimental procedures were the same as described in the legend of Figure 3. (A) 1, non-stimulated Neu-W cells; 2, cells following application of 1 \( \mu \)M of Bj-PRO-10c; 3, \([Ca^{2+}]_i\), levels after cell recovery; 4, cells after addition of 1 \( \mu \)M of BK; 5, \([Ca^{2+}]_i\), levels after cell recovery; and 6, 5 \( \mu \)M of calcium ionophore Br-A23187. Time points of image capture after application of each compound are indicated in the figure. (B) Representative traces of \([Ca^{2+}]_i\), transients after application of Bj-PRO-10c and BK, respectively. Arrows indicate the time points of ligand application following the washing step.](image)

![Figure 5. Essential structure conservation for Bj-PRO-10c activity. A partially scrambled peptide with the first five N-terminal amino acids in a random sequence (N5-scrambled) and a truncated Bj-PRO-10c without the first three N-terminal amino acids (without \( \ \square \)) were synthesized and used to evaluate whether the Bj-PRO-10c N-terminus was important for the activity of the peptide. \( \angle \)N-terminal pyroglutamate residue. As negative control, a peptide consisting of an inverted sequence of Bj-PRO-10c was used. Peak values of \([Ca^{2+}]_i\), elevations induced by 1 \( \mu \)M of each peptide were determined by microfluorimetry using FlexStation III. \([Ca^{2+}]_i\), peak values observed after treatment with 1 \( \mu \)M Bj-PRO-10c were considered as 100% activation. The shown data are mean values ± S.E. of five independent experiments. *P < 0.05 compared to the control measurements in the presence of Bj-PRO-10c.](image)
In their biological activity when compared to native amino acids in a random order, did not reveal any reduction and the N5-scrambled peptide, with the five N-terminal lacking the three N-terminal amino acids (without line by alanines (data not shown). Both, the truncated peptide was observed following substitutions of any isoleucine or proline. The truncation of Bj-PRO-10c down to a high content of prolines at the C-terminus is not sufficient to determine a BK-potentiating activity (16). We have now shown that all amino acid residues forming this Ile-Pro-Pro motif are relevant for the efficiency of Bj-PRO-10c in inducing \([Ca^{2+}]_i\) transients, as a partial loss of this biological activity was observed following substitutions of any isoleucine or proline by alanines (data not shown). Both, the truncated peptide lacking the three N-terminal amino acids (without \(<\)ENW) and the N5-scrambled peptide, with the five N-terminal amino acids in a random order, did not reveal any reduction in their biological activity when compared to native Bj-PRO-10c, suggesting that the property of Bj-PRO-10c in inducing \([Ca^{2+}]_i\) responses does not depend on its N-terminal sequence. The specificity of Bj-PRO-10c-induced responses was also corroborated by the lack of activity of the inverted peptide sequence.

Bj-PRO-10c-evoked \([Ca^{2+}]_i\) responses did not depend on ACE inhibition and BK-B2 receptor activation, as Bj-PRO-10c did not affect the BK-induced response. BK-B2 receptor participation was further ruled out, as HOE-140 did not block Bj-PRO-10c-promoted calcium fluxes. Moreover, prior exposure of neuronal cells to 1 \(\mu M\) Bj-PRO-10c to desensitize the putative receptor did not interfere with BK-induced calcium responses. In accordance with these results, previous stimulation of HEK 293 cells with BK did not affect Bj-PRO-10c-mediated responses (data not shown). Although Bj-PRO-10c is a BK-potentiating peptide, this peptide does not potentiate the BK-induced calcium mobilization in neuronal cells. Our results point to a novel, so far not described mechanism, leading to calcium mobilization and possibly to antihypertensive activity.

Both, influxes of extracellular calcium and subsequent liberation of calcium from ryanodine-sensitive intracellular stores, a mechanism known as the CICR, were involved in Bj-PRO-10c-evoked \([Ca^{2+}]_i\) responses. In agreement with this proposed mechanism, inhibition of PLC-\(\beta\) did not interfere with the increase of \([Ca^{2+}]_i\), induced by Bj-PRO-10c, indicating that the formation of IP3 does not participate in the signaling pathway of this peptide. A similar mechanism was responsible for \([Ca^{2+}]_i\) transients mediated by nicotinic acetylcholine receptors in neuronal-differentiated P19 cells (31).

Several mechanisms involving heterotrimeric G proteins in the modulation of calcium fluxes have been described (32–

**Figure 6.** Evaluation of Bj-PRO-10c- and BK-induced \([Ca^{2+}]_i\) transients in Neu-W cells in the presence of inhibitors. Peak values of agonist (1 \(\mu M\) Bj-PRO-10c or BK)-induced \([Ca^{2+}]_i\), levels were obtained in the absence (control) or after preincubation with several inhibitors of calcium-signal transduction pathways or a BK-B2 receptor antagonist. Calcium influx and calcium release from intracellular stores were inhibited by pre-incubation of cells with 10 mM EGTA or 200 ng/ml thapsigargin for 5 or 30 min, respectively. The participation of IP3- and ryanodine-sensitive calcium stores in Bj-PRO-10c-induced \([Ca^{2+}]_i\) responses was studied following 30 min preincubation with 20 \(\mu M\) of the PLC-\(\beta\) inhibitor U73122 or with 50 \(\mu M\) ryanodine. Cells were preincubated for 18 h with 100 ng/ml pertussis toxin (PTX) for inhibition of G\(\alpha\)-protein mediated receptor responses. Control experiments in the presence of inhibitors, except for inhibition of ryanodine-sensitive calcium stores, were performed using BK as agonist. HOE-140 (1 \(\mu M\)) was preincubated with the cells for 30 min for specific BK-B2 receptor inhibition prior to agonist addition. *P < 0.05, compared to Bj-PRO-10c control data obtained in the absence of inhibitors; n.d. = not determined. The shown data are mean values ± S.E. of five independent experiments.

**Figure 7.** Inhibition of forskolin-induced cAMP accumulation in Neu-W cells. Neu-W or C6 glioma cells were preincubated for 10 min in the absence (control) or presence of 1 \(\mu M\) Bj-PRO-10c. Where indicated, 50 \(\mu M\) forskolin was added and cells were incubated for 1 h. Data were compared to those obtained in control measurements in the absence of any drug (normalized to 100%). Data (mean ± S.E.) are representative for three independent experiments. *P < 0.05, comparison of cAMP levels in cells treated with forskolin or Bj-PRO-10c + forskolin.
Our results indicate that Bj-PRO-10c-induced signal transduction in neuronal cells leading to calcium influx was mediated by G_{i/o}-proteins, as calcium responses were significantly reduced in the presence of PTX.

This mechanism has also been reported for some metabolic receptors, such as for the head-activator receptor in neuroblastoma X glioma double hybrid cells, the recombinant orphan receptor GPR37 in COS-7 cells and the thyrotropin-releasing hormone (TRH) in pituitary cells (32,34,35). ELISA assays for determination of intracellular cAMP concentration revealed that forskolin-promoted cAMP production was significantly inhibited by Bj-PRO-10c in Neu-W cells suggesting also the participation of G_{i/o}-proteins in the signaling pathway of Bj-PRO-10c, as this G-protein subtype is the dominant mediator of adenylate cyclase (AC) inhibition (36).

Moreover, in agreement with the detection of [Ca^{2+}]_{i}, elevations after stimulation by Bj-PRO-10c only in neuroblasto- toma and Neu-W cells and not in C6 glial cells, the inhibitory activity of Bj-PRO-10c on forskolin-promoted cAMP accumulation was observed in Neu-W cells but not in C6 glial cells suggesting that in the brain Bj-PRO-10c preferentially acts on neuronal cells.

Aiming to identify the putative receptor for this peptide, binding assays for Bj-PRO-10c to 105 different G-coupled receptors (listed in Table 1 Supp. Info.) were performed. However, Bj-PRO-10c did not reveal high affinity to any of the assayed receptors. Consequently, Bj-PRO-10c-induced calcium fluxes are mediated by a still unknown/unidentified receptor. Attempts to determine the putative Bj-PRO-10c receptor are underway.

Although we have not yet identified the receptor that participates of Bj-PRO-10c-induced CICR, it is possible to predict that Bj-PRO-10c-evoked signaling pathway may play key roles to promote the release of neurotransmitters by neuronal cells. CICR regulates a wide variety of neuronal functions, including the Ca^{2+}-associated mechanism of neurotransmitter exocytose (30). Neurotransmitter release depends on action potential evoked influx of Ca^{2+} through voltage-operated channels, an effect amplified by CICR from Ca^{2+}-dependent ryanodine-sensitive store (37). Moreover, Mori et al. (37) showed that a ryanodine receptor agonist enhanced release of both glutamate and GABA in dose-dependent manner. In agreement, Bj-PRO-10c promoted an increase in GABA and glutamate release in Neu-W cells. The amino acid glutamate is the major excitatory neurotransmitter in the CNS of mammals, whereas GABA is the main mediator of sympathetic inhibitory currents. Both glutamate and GABA play key roles in the control of cardiovascular function in the CNS (38). Excitatory amino acid neurotransmitters, like glutamate and aspartate, generally cause pressure responses and tachycardia, while inhibitory amino acid neurotransmitters, GABA and glycine are responsible for question and answer depressing bradycardia (39). It is well established that the excitatory amino acid glutamate is considered the main neurotransmitter of primary afferent fibers of baroreceptors to the nucleus tractus solitarii (NTS) (40).

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Figure 8. Effect of Bj-PRO-10c on neurotransmitter release by Neu-W cells. The assay was realized using 3 × 10⁶ Neu-W cells. (A) The basal amount and the amount of inhibitory (glycine and GABA) or (B) excitatory neurotransmitters (aspartate and glutamate) after exposure to 1 μM Bj-PRO-10c for 3 min was determined by HPLC analysis, as described in the Methods section. The data shown are the mean values ± S.E. of two to four independent experiments. n.d. = nondetectable, *P < 0.05 compared to basal neurotransmitter release levels.
from the pit viper *Bothrops jararaca* venom, confirming previous suggestions for the participation of \([\text{Ca}^{2+}]_i\), mobilization in antihypertensive actions of ACE-inhibitors (42) and describing a novel mechanism of action involving the release of neurotransmitters from neuronal cells.

We conclude that *Bj*-PRO-10c acts through activation of an unidentified Gi/o-coupled receptor, independent from ACE inhibition and from BK-B2 receptor activation. A possible signal transduction pathway for *Bj*-PRO-10c-induced \([\text{Ca}^{2+}]_i\), elevations and neurotransmitter release is schematically presented in Figure 9. Peptide–receptor binding results in the activation of calcium influx and release of intracellular calcium by CICR mechanism involving the activation of the ryanodine- or IP3-sensitive calcium stores together with the inhibition of adenylate cyclase. The induced elevations in \([\text{Ca}^{2+}]_i\), trigger release of glutamate and GABA acting on sympathetic activity and baroreflex sensitivity control. Further studies will reveal details of the mechanisms of the CNS effects exerted by *Bj*-PRO-10c and possibly reveal novel approaches for the development of antihypertensive drugs.

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**LITERATURE CITED**


**Figure 9.** Schematic representation of *Bj*-PRO-10c-induced signal transduction. *Bj*-PRO-10c activates a metabotropic Gi/o-protein-coupled seven-transmembrane receptor resulting in inhibition of AC and activation of a calcium channel in the cell membrane. The stimulated calcium influx promotes CICR by activation of ryanodine (RyR)– or intracellular IP3-sensitive receptors. *Bj*-PRO-10c-induced \([\text{Ca}^{2+}]_i\) elevations may participate in the mechanism of release of GABA and glutamate, which exert essential roles in the control of cardiovascular function in the central nervous system.
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