Effects of Anthopleurin-Q on the Intracellular Free Ca^{2+} Concentration in Cultured Rat Cortical Neurons

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Key words

- anthopleurin-Q
- patch clamp technique
- cultured cortical neuron
- sodium current

Abstract

The present study was designed to investigate the mechanism underlying the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) modulated by Anthopleurin-Q (AP-Q), a sea anemone toxin, using whole-cell patch clamp and fluorescence digital imaging techniques. Results indicated that the overall Ca^{2+} concentration could be augmented in presence of AP-Q. The increase of $[\text{Ca}^{2+}]_i$ induced by AP-Q was eliminated in Na^+ -free solution, Ca^{2+} -free solution or in presence of TTX. However, the Ca^{2+} increase induced by AP-Q could not be influenced by cyclopiazonic acid (CPA), a specific inhibitor of the endoplasmic

reticulum Ca^{2+} -ATPase pump. We furthermore demonstrated that voltage-gated calcium channels (VGCCs) blocker verapamil, or inhibitor of the reverse operation Na^+ - Ca^{2+} exchanger NiCl_2 attenuated AP-Q-induced $[\text{Ca}^{2+}]_i$ elevation. Furthermore, the inactivation process of Na^+ current (I_{Na}) was significantly delayed with slightly change of its amplitude by AP-Q. These findings demonstrated that neuron voltage-gated Na^+ channels are also targets of AP-Q. Overall, the present results suggested that AP-Q induced calcium influx via Na^+ -dependent activation of voltage-gated sodium channels (VGSCs), VGCCs and reverse operation of the Na^+ / Ca^{2+} exchanger.

Introduction

Sea anemone toxins, which are polypeptide toxins extracted from marine invertebrate sea anemone, have been classified into 4 classes according to their structural characteristics (4): a) type 1 class, comprising long polypeptides isolated from the genera *Anthopleura* and *Anemonia*, members of the family Actiniidae; b) type 2 class, comprising long polypeptides isolated from the genera *Radianthys* and *Stichodactyla*, members of the family Stichodactylidae; c) type 3 class, comprising long polypeptides isolated from the genus *Calliactis*, and d) type 4 class, comprising short polypeptides isolated from the genus *Parasicyonis* [1,2].

Anthopleurin-Q (AP-Q) is one of the anthopleurin toxin extracted from *Anthopleurin Xanthogrammica*. It is a stable and basic polypeptide of 40 amino acid residues. In the previous study, AP-Q showed concentration-dependent positive inotropic effect in isolated atria of guinea pigs and inhibitory effect on rat myocardial hypertrophy. It also markedly prolonged the functional refractory period in isolated atria of guinea pigs [3]. It has been reported that sea anemone toxins cause

intracellular Na^+ and Ca^{2+} elevation, leading to enhance heart contraction without producing significant effect on the heart rate or blood pressure in vivo [4–6].

The pharmacological effects of some anemone peptides on the nervous system have also been studied. For example, ATX II, a sea anemone neurotoxin derives from *Anemonia sulcata*, selectively enhanced persistent Na^+ current in hippocampal and neocortical pyramidal neurons [7,8]. Granulitoxin, a neurotoxic peptide from the sea anemone *Bunodosoma granulifera*, could induce seizures and related changes of electroencephalogram in vivo [9]. However, still with respect to the pharmacological effects on the cardiovascular system, no studies on the central nervous system have been conducted thus far using AP-Q.

Ca^{2+} plays a pivotal role in regulating diverse aspects of cellular function [10]. In the central nervous system, changes in intracellular Ca^{2+} levels underlie major neuronal process, including neurotransmitter release, excitability, synaptic plasticity, neurite outgrowth and gene expression [11,12]. In recent years, accumulated evidences have indicated that sodium currents,

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alteration in intracellular calcium concentration and calcium homeostatic mechanisms play a role in development and maintenance of epilepsy [13, 14]. In the present study, the effects of AP-Q on the sodium current (I_{Na}) and intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured rat cortical neurons were determined. To our knowledge, this is the first electrophysiological characterization of the action of this toxin on neuronal cells.

Materials and Methods

Cell preparation

Primary cultured cortical neurons were initiated from neonatal Sprague-Dawley rats (the Experimental Animal Center of Tongji Medical School, Huazhong University of Science and Technology) on postnatal day 1–3. The University Animal Welfare Committee approved the animal protocol used. Neurons were isolated as previously described with some modifications [15]. In brief, cortical hemispheres of newborn rats were dissected and rinsed in ice cold Dulbecco's phosphate buffered saline. Meninges and blood vessels were removed. The cortical hemispheres were minced with forceps, and completely dissociated into a single-cell suspension using 0.125% trypsin for 15 min. Neurons were collected by centrifugation in 5 min 800 rpm and resuspended in Dulbecco's modified Eagle's medium (DMEM) and F-12 supplement (1:1) (Gibco Invitrogen Corporation) medium supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin G and 100 U/ml streptomycin. Then the cells were diluted to 1×10^5 per cm^2 and plated onto 35 mm culture dishes containing 12×10 mm glass coverslips previously coated with 0.01% poly-D-lysine, then incubated in a humidified incubator (95% air/5% CO_2 at 37 °C). After 24 h, the culture medium was changed to DMEM/F12 medium containing 2% B27 and 2 mM glutamine. 10 μ M cytarabine was added to the culture medium on day 3 after plating to prevent the proliferation of non-neuronal cells. The cells were cultured for 7–9 days and then used for the experiment. All experiments were performed at room temperature (20–22 °C).

Drugs and solutions

AP-Q (MW=4840, purity>99%) was supplied by Qingdao Marine Biology Research Institute. Aliquots of stock solution in distilled water were prepared and stored in a freezer (–20 °C). N-methyl-D-glucamine (NMDG⁺), Dimethyl Sulfoxide (DMSO) and $NiCl_2$, cyclopiazonic acid (CPA), verapamil, DL-2-amino-5-phosphovaleric acid (D-AP5) were products of Sigma Co. (St. Louis, MO, USA). Fura-2/AM ester was obtained from Biotium (Hayward, CA, USA).

Calcium imaging

Intracellular calcium level was monitored using the fluorescent Ca^{2+} indicator fura-2/AM as described in our previous study [15]. Cortical neurons plated on coverslips were loaded in extracellular solution with 2–3 μ M fura-2/AM for 30 min at 37 °C. Coverslips were then transferred to a chamber (about 1 ml) mounted on an inverted fluorescence microscope stage (IX70, Olympus, Tokyo, Japan), where they were superfused for at least 10 min with extracellular solution at a rate of 4 ml/min to wash away the remaining dye. Measurements of $[Ca^{2+}]_i$ in single cells were performed with a dual excitation fluorometric imaging system (T.I.L.L. Photonics GmbH, Germany). The illumination was generated by a 75 W Xenon bulb. The excitation wavelength was altered

between 340 and 380 nm, and the emission fluorescence of selected areas within the neuron was passed through a 510 nm long-pass filter and recorded with a video camera (T.I.L.L. Photonics GmbH, Germany). Monochromator settings, chopper frequency and complete data acquisition were controlled by TillvisION 4.0 software (T.I.L.L. Photonics GmbH, Germany). The sampling rate was 1 Hz. For the region of interest, the ratio (R) of the light intensities obtained at 340 and 380 nm excitation. The change in $[Ca^{2+}]_i$ was represented by relative fluorescent intensity: $[(FI-FI_0)/FI_0] \times 100\%$ (FI_0 : basal level; FI: administration of drugs). The extracellular solution contained (mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 10 Glucose, pH 7.4. Ca^{2+} -free extracellular solution was prepared by replacing $CaCl_2$ with equimolar amounts of $MgCl_2$ and 0.5 mM EGTA was added. Na^+ -free extracellular solution was isosmotically replaced Na^+ with NMDG⁺.

Whole-cell patch-clamp recording

The whole-cell patch clamp technique was employed to record I_{Na} in cultured rat cortical neurons. All recordings were carried out at room temperature (20–22 °C). Membrane currents were recorded with EPC10 amplifier (HEKA electronic, Germany) controlled by EPC10 data acquisition system. The microelectrodes fabricated with microelectrodes puller (PC-10, Narishige, Japan) had a resistance of approximately 2–3 M Ω when filled with pipette solutions.

In the whole cell configuration, the series resistance was partially compensated by about 70–80%. Currents were filtered at 10 kHz using a four-pole low-pass Bessel filter. Linear leak and capacitive currents were subtracted using the P/N protocol as implemented in Pulse. The bath solution contained (in mM): 120 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 20 glucose, 10 HEPES (pH 7.3 with NaOH). Depending upon the type of experiment, 4-aminopyridine (4-AP, 2 mM) and Cd^{2+} (0.2 mM) were also added to the bath solution. The pipette solution was composed of (mM): 140 CsCl, 2 tetraethylammonium (TEA)-Cl, 10 EGTA, 10 glucose, 10 HEPES, 2 $MgCl_2$, 2 Na_2ATP (pH 7.3 with CsOH). During the experiment, the cells were continuously superfused with the bath solution with a flow rate of 0.5–1 ml/min.

Data analysis

Electrophysiological data were analyzed using pClamp8.0 (Axon Instruments, Foster City, CA) and SigmaPlot 9.0 (SPSS Inc, Chicago, IL) software. All averaged and normalized data was presented as mean $\pm$ S.E.M. The statistical significance of the differences was evaluated using the *t*-test. $P < 0.05$ was considered to be significant.

Results

AP-Q increased $[Ca^{2+}]_i$ of rat cortical neurons in extracellular solution

The $[Ca^{2+}]_i$ responses triggered by 600 nM AP-Q varied depending on the duration of drug application. Short applications of about 30 s resulted in a transient elevation of $[Ca^{2+}]_i$ (○ Fig. 1a). A fast initial increase in $[Ca^{2+}]_i$ was followed by a slower decline to the resting level. Treatment with 600 nM AP-Q could increase $[Ca^{2+}]_i$ by $69.21 \pm 6.80\%$ ($n=62$, $P < 0.01$). When the application of AP-Q was prolonged (2–6 min), two different response patterns could be distinguished. The majority of the cells (69%) showed a biphasic Ca^{2+} response consisting of an initial, large transient

Fig. 1 AP-Q induced $[Ca^{2+}]_i$ increase in cultured rat cortical neurons. Representative traces from a single cell show the time course of changes in $[Ca^{2+}]_i$ in the presence of external Ca^{2+} . Different response patterns obtained with application of 600 nM AP-Q. (n = 62). **a** Transient response occurred in short application of 600 nM AP-Q. **b** Transient $[Ca^{2+}]_i$ increase can not return to the basal level in the presence of AP-Q. The latency was about 30 s⁻¹ min. Washout of toxin could restore $[Ca^{2+}]_i$ to basal level. **c** Calcium oscillatory responses occurred upon application of AP-Q. The experiments were repeated at least 3 times and representative data are shown for each treatment.

Fig. 2 Calcium influx through VGCCs, Na^+/Ca^{2+} exchanger was required for AP-Q-induced $[Ca^{2+}]_i$ rise in cultured cortical neurons. **a** AP-Q-induced $[Ca^{2+}]_i$ elevation ceased in Ca^{2+} -free medium and restarted after re-addition of 2 mM Ca^{2+} to the extracellular medium (n = 20). **b** Preincubation with SERCA Ca^{2+} pump inhibitor CPA (10 μ M) for 10 min in calcium-containing solution did not suppress AP-Q-induced $[Ca^{2+}]_i$ elevation. **c** Preincubation with voltage-gated calcium channels (VGCCs) blocker verapamil (10 μ M) partly inhibited the AP-Q response (n = 28). **d** Preincubation of Na^+/Ca^{2+} exchanger blocker $NiCl_2$ (5 mM) greatly attenuated AP-Q-induced $[Ca^{2+}]_i$ elevation (n = 13). **e** Preincubation with NMDA receptor blocker D-AP5 (100 μ M) had no effect on AP-Q-induced $[Ca^{2+}]_i$ elevation (n = 14). **f** AMPA receptor antagonist CNQX (20 μ M) had no effect on AP-Q-induced $[Ca^{2+}]_i$ elevation (n = 20). Re-addition of 600 μ M AP-Q was usually applied at the end of protocols without using the corresponding antagonist. Data represented percent of peak level of increases in $[Ca^{2+}]_i$, compared with the controls shown in (○ Fig. 1a). **g** Summary data revealed that respectively application of verapamil (10 μ M), $NiCl_2$ (10 μ M) partly decreased the AP-Q-evoked $[Ca^{2+}]_i$ increase by themselves. * indicate significant different means as calculated by Student's t-test with $P < 0.05$, compared with the AP-Q.

component and sustained component (○ Fig. 1b). $[Ca^{2+}]_i$ failed to return to basal level in the presence of AP-Q, but washout of toxin could restore $[Ca^{2+}]_i$ to basal level, indicating that AP-Q-induced the increase in $[Ca^{2+}]_i$ in cortical neurons is reversible (○ Fig. 1b). Finally, in 31 % of the cells, the initial $[Ca^{2+}]_i$ peak was followed by $[Ca^{2+}]_i$ oscillations (○ Fig. 1c).

Extracellular Ca^{2+} contributed to AP-Q-induced $[Ca^{2+}]_i$ increase in cortical neurons

To test whether the AP-Q-induced $[Ca^{2+}]_i$ elevation was due to Ca^{2+} influx across the plasma membrane, the extracellular solution was replaced by Ca^{2+} -free solution. 600 nM AP-Q failed to increase $[Ca^{2+}]_i$ anymore in Ca^{2+} -free solution (n = 20, ○ Fig. 2a,

Fig. 3 Extracellular sodium was essential for AP-Q-induced $[Ca^{2+}]_i$ elevation in cultured rat cortical neurons. **a** In Na^+ -free solution, in which Na^+ was replaced with NMDG $^+$, AP-Q – induced $[Ca^{2+}]_i$ elevation was prevented. While AP-Q still induced a transient $[Ca^{2+}]_i$ increase in sodium – containing solution ($n=21$). **b** 1 μM TTX prevented AP-Q – induced $[Ca^{2+}]_i$ elevation, While AP-Q still induced a transient $[Ca^{2+}]_i$ increase without TTX ($n=17$). Data were summarized from 3 independent experiments and representative data are shown for each treatment.

Fig. 4 Electrophysiological recording of Na^+ currents in rat cortical neurons was modulated by AP-Q. **a** Representative tracings of Na^+ currents were evoked by depolarization ranging from -80 mV to -30 mV by from a holding potential of -80 mV in the absence or presence of 600 nM AP-Q. Inactivation phase of Na^+ currents was prolonged significantly by 600 nM AP-Q. The increase in I_{Na} by AP-Q 600 nM could be reversed after washout AP-Q. **b** $I-V$ curve of I_{Na} in the absence or presence of 600 nM AP-Q. $n=6$. mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$ vs. control.

$P<0.01$ vs. extracellular solution). The $[Ca^{2+}]_i$ level was then increased when 2 mM Ca^{2+} was reintroduced. To further investigate whether $[Ca^{2+}]_i$ increase induced by AP-Q was due to intracellular Ca^{2+} release, the effect of cyclopiazonic acid (CPA) was examined. CPA is a specific inhibitor of endoplasmic reticulum Ca^{2+} -ATPase pump, which can deplete the endoplasmic reticulum Ca^{2+} store. Pre-perfusion with 10 μM CPA alone caused $[Ca^{2+}]_i$ increase, but failed to affect AP-Q-induced $[Ca^{2+}]_i$ elevation. When 10 μM CPA was applied with calcium-free solution to completely discharge intracellular calcium store, AP-Q can not induce the $[Ca^{2+}]_i$. When calcium was reintroduced in the extracellular environment, a transient $[Ca^{2+}]_i$ elevation was triggered (Fig. 2b), which further demonstrated that calcium influx accounts for AP-Q – induced $[Ca^{2+}]_i$.

To further determine whether voltage-gated calcium channels (VGCCs) involves SKF83959-induced $[Ca^{2+}]_i$ elevation in these experiments, verapamil was employed to block VGCC. As shown in Fig. 2c, preincubation of verapamil 10 μM for 10 min greatly attenuated AP-Q – induced $[Ca^{2+}]_i$ from $69.21 \pm 6.80\%$ to $32.18 \pm 4.11\%$ ($n=28$, $P<0.05$). The possible effect of reverse operation of Na^+/Ca^{2+} exchanger was also tested by $NiCl_2$, a blocker of Na^+/Ca^{2+} exchanger. Prior to exposure to 600 nM AP-Q, cortical neurons were incubated with $NiCl_2$ 5 mM for 3 min. $NiCl_2$ reduced the responses of neurons to AP-Q significantly. The increase of $[Ca^{2+}]_i$ was only $20.69 \pm 3.32\%$ of the basal level (Fig. 2d, $n=13$). NMDA receptor antagonist D-AP5 (50 μM) (Fig. 2e, $n=14$) and AMPA receptor antagonist CNQX (20 μM) (Fig. 2f, $n=20$) had no effect on AP-Q – induced $[Ca^{2+}]_i$. These results indicated that AP-Q – induced $[Ca^{2+}]_i$ rise was

dependent on extracellular calcium. Activation of VGCCs and Na^+/Ca^{2+} exchanger played an important role in AP-Q induced response.

AP-Q – induced $[Ca^{2+}]_i$ rise was dependent on extracellular sodium

As shown in Fig. 3a, AP-Q induced $[Ca^{2+}]_i$ increase was completely abolished in the absence of external Na^+ , but increased after reestablishment to external Na^+ in the bath solution. To further test this phenomenon, the cells were preincubated with 1 μM TTX, a specific voltage-gated Na^+ channel blocker, for 10 min. The result showed that 1 μM TTX almost inhibited $[Ca^{2+}]_i$ elevation induced by 600 nM AP-Q (Fig. 3b). This result indicated that AP-Q – induced $[Ca^{2+}]_i$ rise was dependent on extracellular sodium.

AP-Q delayed the inactivation process of I_{Na}

To further confirm AP-Q – induced $[Ca^{2+}]_i$ rise was dependent on extracellular sodium, we employed the whole-cell recording of patch-clamp technology to examine the effect of AP-Q on I_{Na} elicited by a depolarizing pulse in cultured rat cortical neurons. Sodium currents were evoked by a 50 ms depolarizing test pulses to -30 mV from a holding potential of -80 mV. Na^+ currents inactivated rapidly after activation in control condition (Fig. 4a, $n=6$). In the presence of 600 nM AP-Q, the inactivation kinetics were potently slowed down at all tested pulse with the increase of peak Na^+ currents (Fig. 4b, $n=6$). I_{Na} in the cortical neurons was increased $21.98 \pm 3.21\%$ after exposure to 600 nM AP-Q (Fig. 4b, $n=6$, $P<0.05$). The onset was achieved

within 1–2 min after addition of AP-Q, and about 3 min, the effect reached the steady state. This effect could be reversed after washout for 3 min and blocked completely by 1 μ M TTX. In the presence of 600 nM AP-Q, the inactivation time constant (τ_h) increased about 4 times from 2.47 ± 0.71 ms to 11.78 ± 0.68 ms ($n=6$, $P<0.05$).

Current-voltage (I-V) relation was determined using 50 ms depolarizing steps every 10 s from -70 mV to $+60$ mV with a holding potential of -80 mV (Fig. 4b, $n=6$). In order to allow for differences in cortical neurons size, current density versus voltage curves were obtained by normalizing ionic current amplitudes as a function of membrane capacitance. Cell capacitance of cortical neuron used in this experiment was 16.72 ± 2.68 pA/pF, the series resistance (R_s) was 5.41 ± 1.37 M Ω , during recording process. The amplitude of I_{Na} was increased at the membrane potentials of -50 mV to -30 mV ($P<0.05$) and reached the maximum at -30 mV. I-V curve shifted to the leftward and downward.

Discussion

In the present study, we demonstrated that AP-Q enhanced $[Ca^{2+}]_i$ in cultured rat cortical neurons. Extracellular Ca^{2+} contributed to AP-Q-induced $[Ca^{2+}]_i$ increase. The AP-Q strongly delayed the inactivation phase of I_{Na} , slightly increased I_{Na} and modulated calcium entry through VGCCs and Na^+/Ca^{2+} exchanger.

We found that AP-Q increased $[Ca^{2+}]_i$ of cultured rat cortical neurons. This increase was significantly inhibited in Na^+ -free solution and prevented by TTX, suggesting that the change of $[Ca^{2+}]_i$ by AP-Q was dependent on extracellular Na^+ . This is consistent with results that the whole cell patch clamp recordings showed the inactivation of TTX-S sodium currents was prolonged significantly by AP-Q. AP-Q belongs to one of the anthopleurin toxin extracted from *Anthopleurin Xanthogramica*. Anemone peptide neurotoxins act on site 3 of sodium channels. AP-Q had significant effect on the inactivation process of TTX-S sodium current in rat cortical neurons. This result was in agreement with our previous study that AP-Q increased Na^+ current via modulating the change of inactivation characteristics of sodium channel in guinea ventricular myocytes [16]. Thus, this is suggested that VGSC was essential for AP-Q-induced $[Ca^{2+}]_i$ elevation in cultured rat cortical neurons. However, the concentration of AP-Q which increased the amplitude of I_{Na} in cortical neurons was higher than that in guinea pig ventricular myocytes. Several studies have revealed that the sea anemone toxins bind with higher affinity to cardiac sodium channels than to neuronal ones [17, 18]. The discrepancies between the actions of AP-Q on cardiac and neuronal cells could be the results of tissue or species differences.

Two major intracellular sources contribute to $[Ca^{2+}]_i$ mobilization: an intracellular influx through plasma membrane channels, and an internal reservoir in the endoplasmic reticulum (ER) and mitochondria. Our data showed that the AP-Q induced $[Ca^{2+}]_i$ increase was not observed in Ca^{2+} -free solution, and was not abolished in cells that depleted of intracellular calcium stores by CPA, which demonstrated that Ca^{2+} influx was necessary and sufficient for AP-Q-induced $[Ca^{2+}]_i$ elevation.

Three main routes have been reported to mediate Ca^{2+} entry from extracellular space: VGCCs, receptor-gated calcium chan-

nels and the reversed mode of Na^+/Ca^{2+} exchanger function. In the previous study, it was found that AP-Q had no effect on VGCCs. However, it could not rule out the possibility that Ca^{2+} influx induced by AP-Q was mediated by VGCCs. In excitable cells, VGCCs are mainly responsible for the influx of extracellular Ca^{2+} due to membrane depolarization [19]. Increasing Na^+ permeability can induce the depolarization of plasma membrane [20, 21]. In addition, this $[Ca^{2+}]_i$ elevation could be reduced by $NiCl_2$, which is a nonspecific Na^+/Ca^{2+} exchanger blocker that could bind to the Ca^{2+} -binding sites on protein [22]. Under normal physiological conditions, the Na^+/Ca^{2+} exchanger will operate in the direct mode, with Na^+ entering the cell and extruding Ca^{2+} against its electrochemical gradient. After Na^+ loaded, the ion fluxes through Na^+/Ca^{2+} exchanger will revert, and calcium will accumulate inside the cell [21, 23]. AP-Q maybe selectively bound to voltage-activated Na^+ channels and lead to the accumulation of intracellular $[Na^+]_i$ in cortical neurons. The increased $[Na^+]_i$ triggered the movement of Na^+ out of the cell for Ca^{2+} taken in via reverse operation of the Na^+/Ca^{2+} exchanger system. Combined above observations that AP-Q-induced increase $[Ca^{2+}]_i$ was significantly inhibited in Na^+ -free solution, suggesting that Na^+/Ca^{2+} exchanger did play an important role in $[Ca^{2+}]_i$ increase. This is consistent with reports in other laboratories that some neurotoxins from sea anemone, such as Palytoxin, can enhance the persistent Na^+ currents and modulate calcium entry through VGCCs and Na^+/Ca^{2+} exchanger in neuron cells [24, 25].

In conclusion, the data presented here show that AP-Q delayed inactivation phase of I_{Na} , increased the amplitude of I_{Na} and induced calcium influx in cultured rat cortical neurons. Ca^{2+} enters AP-Q treated cultured cortical neurons primarily through three routes: VGSCs, VGCCs and reverse operation of the Na^+/Ca^{2+} exchanger. To our knowledge, this is the first study on the effects of AP-Q in the central nervous system. This toxin may constitute a valuable tool for the investigation of mammalian brain function.

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Conflict of Interest

The authors declare that they have no conflict of interest in relation to this study.

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