# Effects of okadaic acid on voltage-gated potassium and calcium channels in cultured rat trigeminal neurons

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**Abstract**: **AIM** To investigate the effects of serine/threonine protein phosphatases in regulation of cell signal transduction on voltage-gated potassium and calcium channels in cultured rat trigeminal ganglion (TRG) neurons. **METHODS** Whole-cell patch clamp technique was used to record the potassium and calcium currents from adult rat TRG neurons before and after perfusion of okadaic acid, a potent inhibitor of the serine/threonine protein phosphatases 1 and 2A. **RESULTS** Okadaic acid 1 μmol·L$^{-1}$ inhibited transient outwards potassium currents ($I_A$) by 28.6%, increased delay rectified potassium currents ($I_K$) and calcium currents ($I_{Ca}$) by 22.7% and 20.0%, respectively. okadaic acid 1 μmol·L$^{-1}$ produced significant hyperpolarizing shifts in the conductance-voltage (*G-V*) curves and inactivation curves of $I_A$, also produced significant hyperpolarizing shifts in the *G-V* curves of $I_K$, while it had no effect on the activation and inactivation kinetics of $I_{Ca}$. **CONCLUSION** Serine/threonine protein phosphatases 1 and 2A may be involved in the modulation of voltage-gated potassium and calcium channels on rat TRG neurons. In addition, voltage-gated potassium and calcium channels show different dependence on the dephosphorylation reactions of PP1 and PP2A phosphatases.

**Key words**: okadaic acid; phosphoprotein phosphatase; patch-clamp technique; trigeminal ganglion; neurons; ion chnnels

**CLC number**: R966, R972
**Document code**: A
**Article ID**: 1000-3002(2007)05-0369-08

Protein phosphorylation and dephosphorylation reactions, catalyzed by kinases and phosphatases, are involved in the regulation of a wide variety of physiological processes. They regulate the activity of many proteins that participate insynaptic transmission processes in the central nervous system, such as ion channels, receptors and transporters for neurotransmitters[1-4]. Serine/threonine-specific phosphatases, together with mitogen-activated protein kinase phosphatases and tyrosine phosphatases, are the main types of protein phosphatases[1]. Serine/threonine protein phosphatases can be divided into type 1 (PP1), type 2A (PP2A), type 2B, type 2C, type 4, type 5, type 6 and type 7[1]. Among them, PP2A is the most abundant phosphatase in mammalian cells and is expressed at high levels in the central nervous system[5-6].

Voltage-gated ion channels are integral membrane proteins that enable the passage of selected inorganic ions across membranes. They open and close in response to changes in transmembrane voltage, and play a key role in electrical signaling on excitable cells such as neurons. Receptors for chemical, thermal and me-

**Received date**: 2006-12-26 **Accepted date**: 2007-05-19

**Foundation item**: The project supported by National Natural Science Foundation of China (30271500); National Natural Science Foundation of China (04510002)

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chanical stimuli can be found on the terminals of several types of trigeminal ganglion (TRG) neurons, including polymodal nociceptors, the most prevalent nociceptor that is activated by all chemical, thermal and mechanical stimuli[7]. The activation of nociceptors is the initial step in eliciting a painful stimulus. These neurons contain, in addition to transient potential receptor vanilloid receptors, tetrodotoxin (TTX)-resistant sodium channels, and a variety of potassium and calcium channels that are important in action potential (AP) generation and propagation[8].

Nearly all aspects of cell function involve phosphorylation of the amino acids serine/threonine. It has been estimated that one-third of cellular proteins are reversibly phosphorylated[9]. Phosphorylation can regulate proteins that induce very short-term or very long-term effects like ion channels and transcription factors. Specifically, cell division, cell differentiation, neuronal activity, muscle contraction, and metabolic functions are regulated by phosphorylation. The studies on the physiological role of phosphatases have made rapid progress with the advent and widespread experiments using new inhibitors as pharmacological tools in the past decade. But the dephosphorylation reactions of PP1 and PP2A on voltage-gated potassium and calcium channels in TRG neurons are unclear.

The purpose of this study was therefore to investigate the effects of PP1 and PP2A antagonist okadaic acid on voltage-gated $K^+$ and $Ca^{2+}$ ion channels in cultured adult rat TRG neurons using whole-cell patch clamp.

## 1 MATERIALS AND METHODS

### 1.1 Solutions and drugs

The intra- and extra-cellular solutions used to isolate the different ion channels and for the voltage-clamp experiments are given in Tab 1 and Tab 2. Okadaic acid (Sigma, St Louis, Mo, USA) at the final concentration of 1 $\mu mol \cdot L^{-1}$ was added to the intracellular solution, and then introduced into the cytoplasm by simple diffusion from the pipette milieu to the cytosol[10]. Unless stated otherwise, all other chemicals came from Sigma. Cell culture materials were purchased from Gibco (Life Technologies, Rockville, MD, USA).

**Tab 1. Composition of extracellular solutions**

| Current | Concentration/$mmol \cdot L^{-1}$ | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | KCl | $CaCl_2$ | $MgCl_2$ | TEA-Cl | Chol-Cl | Glu | HEPES | $CdCl_2$ | 4-AP | $BaCl_2$ |
| $I_K$ | 5 | 2 | 1 | 0 | 137 | 10 | 10 | 0.1 | 3 | 0 |
| $I_A$ | 5 | 2 | 1 | 70 | 70 | 10 | 10 | 0.1 | 0 | 0 |
| $I_{Ca}$ | 0 | 0 | 2 | 20 | 110 | 20 | 10 | 0 | 0 | 10 |

TEA-Cl: tetraethylammonium chloride; Chol-Cl: choline chloride; Glu: glucose; 4-AP: 4-aminopyridine; $I_K$: delay rectified potassium currents; $I_A$: transient outwards potassium currents; $I_{Ca}$: calcium currents.

**Tab 2. Composition of intracellular solutions**

| Current | Concentration/$mmol \cdot L^{-1}$ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | KAsp | CsCl | KCl | $MgCl_2$ | $CaCl_2$ | EGTA | HEPES | CsF | Tris-ATP |
| $I_K$ | 118 | 0 | 20 | 2 | 1 | 10 | 10 | 0 | 5 |
| $I_A$ | 118 | 0 | 20 | 2 | 1 | 10 | 10 | 0 | 5 |
| $I_{Ca}$ | 0 | 64 | 0 | 2 | 0.1 | 10 | 10 | 64 | 5 |

KAsp: potassium aspartic acid; EGTA: ethylene glycol-bis (β-aminoethyl ether)-tetraacetic acid.

## 1.2 Cell culture

TRG neurons from adult Sprague-Dawley rats (Faculty of Laboratory Animal Science, Huazhong University of Science and Technology, weighing from 150 - 250 g, irrespective of sex) were cultured as described previously[11]. Briefly, trigeminal ganglia were dissected aseptically and collected in modified Hank's balanced salt solution (mHBSS). After washing in mHBSS, the ganglia were diced into small pieces and incubated for 30 - 50 min at 37℃ in 0.1% collagenase (Type XI-S) in mHBSS. Individual cells were dissociated by triturating the tissue through a fire-polished glass pipette, followed by a 10 min incubation at 37℃ in 10 $mg\cdot L^{-1}$ DNase Ⅰ (Type Ⅳ) in F-12 medium (Life Technologies, Gaithersburg, MD, USA). After washing three times with F-12, the cells were cultured in DMEM supplemented with 10% fetal bovine serum. The cells were plated on poly-*D*-lysine coated glass coverslips (15 mm diameter) and cultured overnight at 37℃ in a water saturated atmosphere with 5% $CO_2$. Only neurons without visual processed or with short processes were used. All experiments were carried out at room temperature (22 - 24℃).

## 1.3 Patch clamp recordings

Glass pipettes (R-6 borosilicate, Drummond Scientific Company, Broomall, PA., USA) with resistances between 1 - 2 MΩ were used for whole-cell voltage-clamp experiments. Recordings were obtained using an Axopatch-200B patch clamp amplifier (Axon Instruments, Foster City, CA, USA) and the output was digitized with a Digidata 1322A converter (Axon Instruments). The sampling rate was 10 kHz (without filtering). For voltage-clamp experiments, the capacitance and the series resistance were compensated ≥90% and the junction potentials were adjusted to 0 mV just before patching. When the voltage-clamp errors were ≥5 mV after being compensated the data were not included in the analysis.

Transient outwards potassium currents ($I_A$) were obtained from the total outward current using tetraethylammonium chloride (TEA-Cl) to block delay rectified potassium currents ($I_K$), and $I_K$ was obtained from the total outward current using 4-aminopyridine (4-AP) to block $I_A$[12] (Tab 1). The $I_A$ and $I_K$ activation-voltage protocol measurements consisted of 250 ms depolarizing pulses from -80 to +60 mV stepping 10 mV every 2 s. The holding potential was -80 mV. The $I_A$ inactivation-voltage measurements consisted of using 250 ms precondition pulses from -120 to +40 mV in 10 mV steps before returning to the holding potential of -80 mV after 250 ms. There was 4 s delay between pulses.

Calcium currents ($I_{Ca}$) were recorded from rat TRG neurons under conditions that ① suppressed $I_K$ by the replacement of internal $K^+$ with $Cs^+$ and 20 $mmol\cdot L^{-1}$ TEA-Cl included in external solution, ② suppressed sodium currents ($I_{Na}$) with choline replacing external $Na^+$, and ③ enhanced $I_{Ca}$ with 10 $mmol\cdot L^{-1}$ $Ba^{2+}$ as a charge carrier. The $I_{Ca}$ in current traces evoked using step pulses (450 ms) to test potentials ranging from -50 mV to +50 mV in 10 mV increments from the holding potential ($V_h$) of -80 mV. The $I_{Ca}$ inactivation-voltage protocol consisted of 3 s precondition pulses ranging from -80 to +20 mV in 10 mV increments followed by 200 ms test pulses depolarizing to +10 mV. In all experiments including the experiments administered with drugs, there was a little decrease in the amplitude of $I_{Ca}$ in a time-dependent manner, and the data were measured only after the amplitudes and time courses of currents remaining constant with further perfusion of drugs, which were judged by the amplitude of $I_{Ca}$ decreased no more than 10% by the end of 10 min after recording.

## 1.4 Statistics and curve fitting

Data were analyzed and fitted using pClamp (Axon Instruments, Foster City, CA, USA) and SigmaPlot (SPSS Inc., Chicago, IL, USA) software. Conductance-voltage (*G*-*V*) and inactivation-voltage parameters were fit

to the Boltzmann equation: $X = C + \{X_{max}/[1 + \exp(V_{0.5} - V_m)/k]\}$, where X is $G/G_{max}$. The peak conductance ($G$) of $I_A$, $I_K$ and $I_{Ca}$ at each potential is calculated from the corresponding peak current by using the equation: $G = I/(E - E_{Rev})$, where $E_{Rev}$ is the reversal potential of $I_A$, $I_K$ and $I_{Ca}$, $I$ is the peak current amplitude of $I_A$, $I_K$ and $I_{Ca}$, and $E$ is the membrane potential. $V_{0.5}$ is the membrane potential ($V_m$) at which 50% of activation or inactivation was observed, $k$ is the slope of the function, and C is a constant ( =0 in the $G$-$V$ relation). All data were presented as $\bar{x} \pm s$, and analyzed for statistical significance using, where appropriate, the paired and unpaired $t$-test. Statistical significance is defined as $P < 0.05$.

## 2 RESULTS

### 2.1 Effects of okadaic acid on $I_A$

A representative response of an $I_A$ to 1 $\mu mol \cdot L^{-1}$ okadaic acid was showed in Fig 1.

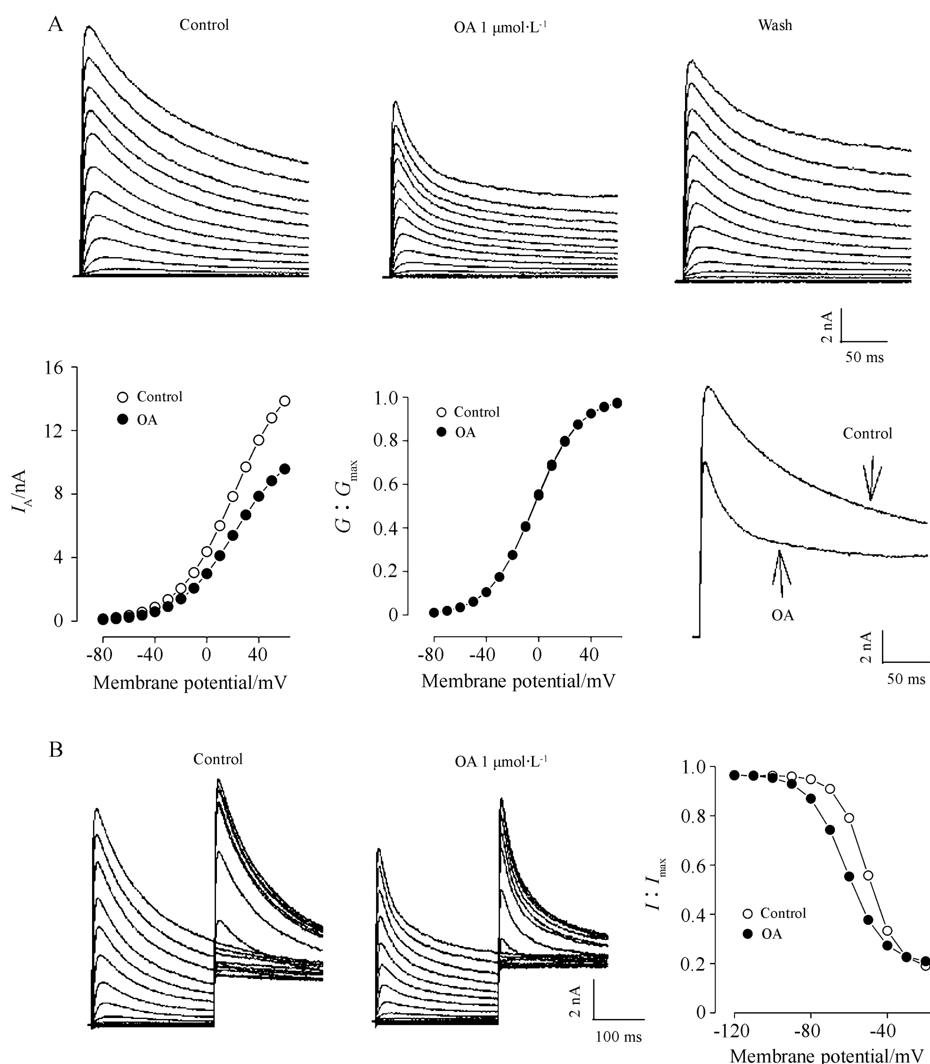


**Fig 1. Effects of okadaic acid (OA) on $I_A$ in trigeminal ganglion (TRG) neurons.** A: a representative voltage-clamp experiment showed that after 3 min incubation, OA 1 $\mu mol \cdot L^{-1}$ decreased the $I_A$ significantly. The second row shows the current-voltage ($I$-$V$) and conductance-voltage ($G$-$V$) relations. The solid lines in the $G$-$V$ curves were fit to the Boltzamann equation (control: $V_{0.5} = -3.3$ mV, $k = 14.9$; OA: $V_{0.5} = -4.0$ mV, $k = 16.1$). The activation and inactivation kinetics of the maximal $I_A$ for control and OA were also shown. B: inactivation-voltage protocol was performed in the same neuron. The shows are the control and response obtained after 3 min incubation with 1 $\mu mol \cdot L^{-1}$ OA. The solid lines were fit to the Boltzamann equation (control: $V_{0.5} = -50.3$ mV, $k = -7.5$, C = 0.18; OA: $V_{0.5} = -61.5$ mV, $k = -7.4$, C = 0.20). Holding potential −80 mV.

On average, 1 μmol·L$^{-1}$ okadaic acid inhibited $I_A$ by (28.6 ±8.5)% ($n$ =8, $P$ <0.05) compared with that obtained in the absence of okadaic acid, induced a statistically significant 3 mV hyperpolarizing shift in the $G$-$V$ curves [control: $V_{0.5}$ = (−4.6 ± 4.4) mV, $k$ =15.9 ± 1.1; okadaic acid: $V_{0.5}$ = (−8.0 ±3.5) mV, $k$ =16.8 ±0.9, $n$ =8, $P$ <0.05 for $V_{0.5}$]. At inactivation, the inactivation curve was shifted to more negative potentials by 1 μmol·L$^{-1}$ okadaic acid [control: $V_{0.5}$ = (−51.1 ±1.1) mV, $k$ = −7.1 ±1.1; okadaic acid: $V_{0.5}$ = (−60.8 ± 3.2) mV, $k$ = −8.2 ±1.3; $n$ =8, $P$ <0.05 for $V_{0.5}$].

## 2.2 Effects of okadaic acid on $I_K$

Fig 2 shows a representative response of an $I_K$ to 1 μmol·L$^{-1}$ okadaic acid. Okadaic acid increased $I_K$ by (22.7 ±10.7)% ($n$ =7, $P$ < 0.05). In addition, 1 μmol·L$^{-1}$ okadaic acid produced 4 mV hyperpolarizing shift in $G$-$V$ curves of $I_K$ (control: $V_{0.5}$ = (6.71 ± 1.61) mV, $k$ = 17.2 ± 0.56; okadaic acid: $V_{0.5}$ = (2.76 ±1.73) mV, $k$ =18.14 ±0.90; $n$ =7, $P$ <0.01 for $V_{0.5}$).

## 2.3 Effects of okadaic acid on $I_{Ca}$

Fig 3 shows representative traces that 1 μmol·L$^{-1}$ okadaic acid increased $I_{Ca}$ inward current significantly compared with that obtained in the absence of okadaic acid. The increased effect of 1 μmol·L$^{-1}$ okadaic acid on $I_{Ca}$ appeared near 1.0 nA [from (5.5 ± 2.9) to (6.5 ± 3.2) nA]. On average, the increase

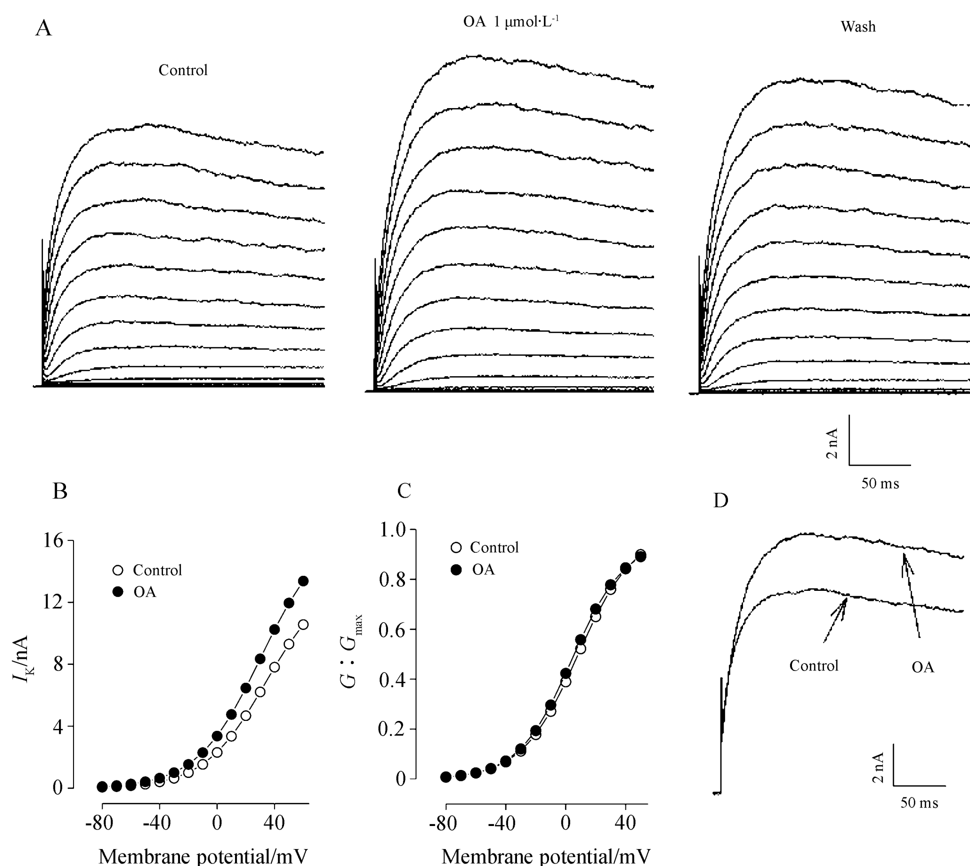


**Fig 2. Effects of okadaic acid on $I_K$ in trigeminal ganglion neurons.** A: a representative voltage-clamp experiment showed that after 3 min incubation, OA 1 μmol·L$^{-1}$ increased the $I_K$ by 24.1%. B: $I$-$V$ relation. C: $G$-$V$ relation. The solid lines in the $G$-$V$ curves were fit to the Boltzamann equation (control: $V_{0.5}$ =8.1 mV, $k$ =16.8; OA: $V_{0.5}$ =4.0 mV, $k$ =17.9). D: the activation kinetics of the maximal currents for the control and 1 μmol·L$^{-1}$ OA. Holding potential −80 mV.

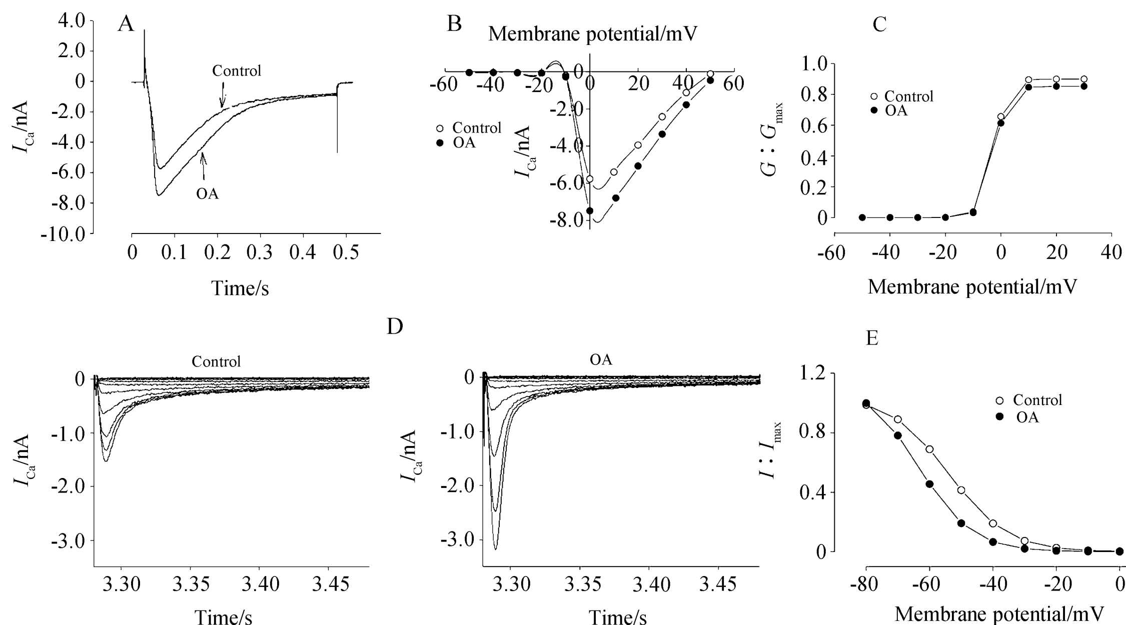


**Fig 3. Effects of okadaic acid on voltage-gated $I_{Ca}$ in trigeminal ganglion neurons.** A: a representative voltage-clamp experiment showed that after 3-min incubation, OA 1 μmol·L$^{-1}$ increased the $I_{Ca}$ significantly. B: *I-V* relation. OA increased the peak amplitude of $I_{Ca}$ but did not shift the peak inward current along its voltage axis. C: *G-V* relation. The solid lines in the *G-V* curves were fit to the Boltzamann equation (control: $V_{0.5}$ = −2.29 mV, $k$ = 2.32; OA: $V_{0.5}$ = −2.34 mV, $k$ = 2.49). D and E: the shows were inactivation-voltage curves of the control and responses obtained after 3 min incubation with 1 μmol·L$^{-1}$ OA. Holding potential −80 mV. The solid lines in inactivation-voltage curves were fit to the Boltzamann equation (control: $V_{0.5}$ = −53.92 mV, $k$ = −9.26; OA: $V_{0.5}$ = −63.39mV, $k$ = −8.39).

rate was (20 ± 11)% ($n$ = 6, $P$ < 0.05). While okadaic acid did not evoke statistically significant changes in *G-V* curves (control: $V_{0.5}$ = (−2.4 ± 0.18) mV, $k$ = 2.38 ± 0.09; okadaic acid: $V_{0.5}$ = (−2.9 ± 0.37) mV, $k$ = 2.2 ± 0.14; $n$ = 6, $P$ > 0.05 for $V_{0.5}$) and inactivation-voltage curves (control: $V_{0.5}$ = (−60.11 ± 14.89) mV, $k$ = −11.89 ± 2.26; okadaic acid: $V_{0.5}$ = (−60.53 ± 2.48) mV, $k$ = −10.27 ± 3.45; $n$ = 6, $P$ > 0.05 for $V_{0.5}$).

## 3 DISCUSSION

Okadaic acid, monocarboxylic polyether isolated from marine sponges, is known to inhibit several types of phosphatases[13]. It is a very potent blocker of PP1 and PP2A[14]. Taking account of the pretrial and literature, we used 1 μmol·L$^{-1}$ okadaic acid as a pharmacologic tool[15].

In our study, we reported systematically the effects of okadaic acid on voltage-gated sodium, potassium and calcium currents in cultured rat TRG neurons by patch-clamp technique. Our previous study[16] reported that okadaic acid inhibited total sodium currents ($I_{Na}$-T) significantly and tetrodotoxin-resistant sodium currents ($I_{Na}$-TTX-R) slightly. The results suggest that PP1 and PP2A play a role in the regulation of cellular signaling on sodium channels.

Primary sensory neurons contain several types of voltage-gated potassium channels, which include $I_K$ that activate rapidly and inactivate slowly and $I_A$ that activate and inactivate rapidly[17]. In nociceptive neurons, $I_{Na}$-TTX-R are mainly responsible for the upstroke of the AP, but may also contribute to the resting potential[18]. $I_K$ are involved in the repolarization phase of the AP, and $I_A$ may regulate AP fre-

quency[19]. It follows that inhibiting sodium channels would decrease excitability, whereas the inhibition of potassium channels would increase excitability[20]. The previous study found that in frog cardiac myocytes, okadaic acid caused a decrease in the amplitude of $I_K$, which is opposite to the increase produced by cAMP-dependent phosphorylation[21]. Contradictorily, in our study, for the first time, we found that 1 μmol·L$^{-1}$ okadaic acid inhibited $I_A$, increased $I_K$ in cultured rat TRG neurons. In addition, okadaic acid produced leftward shift in the *G-V* curves of $I_A$ and $I_K$, shifted the inactivation-voltage curves of $I_A$ to more negative potentials. This indicates that PP1 and PP2A phosphatases take part in the modulation of activation and inactivation kinetics of $I_A$ and $I_K$.

$Ca^{2+}$ influx through calcium channels is involved in regulation of membrane excitability, synaptic plasticity, gene expression and release of several neuronal transmitters[22], so change of voltage-gated calcium currents can induce complicated changes. The previous study found that PP1 and PP2A phosphatases inhibitor okadaic acid increased basal $I_{Ca}$ in human chronic atrial fibrillation and sinus rhythm[23]. Similarly, in our study, 1 μmol·L$^{-1}$ okadaic acid increased the peak amplitude of $I_{Ca}$, but had no significant shift on *G-V* and inactivation-voltage curves. This indicates that PP1 and PP2A phosphatases have no significant effect on activation and inactivation kinetics of $I_{Ca}$ in TRG neurons.

In summary, our results suggest that serine/threonine protein phosphatases 1 and 2A be involved in the regulation of sodium, potassium and calcium channels, but the effects of PP1 and PP2A phosphatases on activation and inactivation kinetics of them are different. This may be associated with different molecular configuration of various voltage-gated ion channels. Therefore they have different dependence on PP1 and PP2A phosphatases. It would be interesting to investigate in detail what kinases are involved in the regulation of PP1 and PP2A phosphatases on different voltage-gated ion channels in TRG sensory neurons.

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# 冈田酸对培养的大鼠三叉神经元电压门控性钾和钙通道的调节

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**摘要: 目的** 通过研究冈田酸对大鼠三叉神经元电压门控性钾、钙电流的影响,探讨磷酸酯酶在细胞信号转导中的调节作用。**方法** 采用全细胞膜片钳方法。**结果** 冈田酸 1 μmol·L$^{-1}$对瞬时外向钾电流($I_A$)的抑制率为 28.6%,对延迟整流钾电流($I_K$)和钙电流($I_{Ca}$)的增加率分别为 22.7% 和 20.0%。冈田酸 1 μmol·L$^{-1}$使 $I_A$ 和 $I_K$ 的激活曲线以及 $I_A$ 的失活曲线发生超级化位移,对 $I_{Ca}$ 激活和失活曲线的影响没有统计学意义。**结论** ① 蛋白丝/苏氨酸磷酸酯酶 1 和 2A 可能参与了大鼠三叉神经节神经元电压门控性钾和钙通道的调节。② 电压门控性钾和钙通道对蛋白丝/苏氨酸磷酸酯酶 1 和 2A 的去磷酸化反应表现出不同的依赖性。

**关键词:** 冈田酸; 磷蛋白磷酸酶; 膜片钳技术; 三叉神经; 神经元; 离子通道

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**基金项目:** 国家自然科学基金资助项目(30271500); 国家自然科学基金资助项目(04510002)

(本文编辑 董立春)