

Title	Cyclic AMP-dependent protein kinase-independent angiotensin II-induced inhibition of calcium current in hamster submandibular ganglion neurons
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**Short Communication**

**CYCLIC AMP-DEPENDENT PROTEIN  
KINASE-INDEPENDENT ANGIOTENSIN II-INDUCED  
INHIBITION OF CALCIUM CURRENT IN HAMSTER  
SUBMANDIBULAR GANGLION NEURONS**

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**Abstract**

In a previous study, we demonstrated that angiotensin II (Ang II) inhibited voltage-dependent calcium channels (VDCCs) currents ( $I_{Ca}$ ) in hamster submandibular ganglion (SMG) neurons. In sinoatrial node cells, it has been reported that Ang II inhibits  $I_{Ca}$  by suppressing cyclic AMP production. In this study, to investigate the possible involvement of a cyclic AMP-cyclic AMP dependent protein kinase (PKA) pathway in the Ang II-induced inhibition of  $I_{Ca}$ , effects of Ang II were examined in SMG neurons after treatment with an activator and inhibitor of PKA. Neither pretreatment of neurons with membrane permeable cyclic AMP nor intracellular dialysis of PKA blocker attenuated the Ang II-induced inhibition of  $I_{Ca}$ . These results indicate that Ang II inhibited  $I_{Ca}$  via a cyclic AMP-PKA-independent mechanism in SMG neurons.

Key words: Hamster submandibular ganglion neurons—  
Voltage-dependent calcium channels—Angiotensin II—  
Cyclic AMP—Protein kinase A

**INTRODUCTION**

Angiotensin II (Ang II), one of the main active peptides of the renin-angiotensin system, exerts its multiple functions, including drinking, vasopressin and oxytocin release, natriuresis, cardiovascular, endocrine and neuronal targets. This peptide is one of the most important vasoconstrictive hormones but is also known to act as a neuromodulator and a neurotransmitter in the central and peripheral nervous systems. These actions of Ang II involve modulation of membrane ion

channel currents and neuronal activity.

It is well known that cyclic AMP-cyclic AMP dependent protein kinase (PKA) pathways are the most important second messenger pathways which mediate hormone-ion channel regulation. Intracellular  $Ca^{2+}$ , which acts as a second messenger, can interact with cyclic AMP, and diacylglycerol (DAG)-protein kinase C (PKC), which is another important second messenger, interacts with the cyclic AMP-PKA pathways.

It has been reported that Ang II receptors cause inhibition of PKA through inhibition of

adenylyl cyclase and reduction in cyclic AMP in various tissues<sup>1,2,10</sup>). Additionally, it has been demonstrated that Ang II inhibits  $I_{Ca}$  by suppressing cyclic AMP production in sinoatrial node cells<sup>3</sup>).

We previously reported that Ang II, mediated via Ang II type 1 ( $AT_1$ ) receptors, inhibits voltage-dependent calcium channels (VDCCs) currents ( $I_{Ca}$ ) in submandibular ganglion (SMG) neurons<sup>7</sup>. It remains unclear, however, whether cyclic AMP-PKA pathways are mediated in the Ang II-induced inhibition of  $I_{Ca}$  in this neuron. Thus, the purpose of the present study was to examine the contribution of cyclic AMP-PKA pathways in the Ang II-induced inhibition of  $I_{Ca}$  in the SMG neuron.

## MATERIALS AND METHODS

Experiments were conducted according to the guidelines for the treatment of experimental animals at Tokyo Dental College. SMG neurons from hamsters were acutely dissociated with a modified version of the method described previously<sup>7</sup>. In brief, SMG neurons were isolated from 4–6 week-old hamsters and maintained in  $Ca^{2+}$ -free Krebs solution of the following composition (in mM): 136 NaCl, 5 KCl, 3  $MgCl_2 \cdot 6H_2O$ , 10.9 glucose, 11.9  $NaHCO_3$  and 1.1  $NaH_2PO_4 \cdot 2H_2O$ . The neurons were treated with collagenase type I (3 mg/ml in  $Ca^{2+}$ -free Krebs solution; Sigma, St. Louis, MO, U.S.A.) for 50 min at 37°C, followed by incubation in trypsin type I (1 mg/ml in  $Ca^{2+}$ -free Krebs solution; Sigma, St. Louis, MO, U.S.A.) for an additional 10 min. The supernatant was replaced with normal Krebs solution of the following composition (in mM): 136 NaCl, 5 KCl, 2.5  $CaCl_2$ , 0.5  $MgCl_2 \cdot 6H_2O$ , 10.9 glucose, 11.9  $NaHCO_3$  and 1.1  $NaH_2PO_4 \cdot 2H_2O$ . Neurons were then plated onto poly-L-lysine (Sigma)-coated glass coverslips.

Voltage-clamp recordings were conducted using the whole-cell configuration of the patch clamp technique<sup>4</sup>). Fabricated recording pipettes (2–3 M $\Omega$ ) were filled with an

internal solution with the following composition (in mM): 100 CsCl, 1  $MgCl_2$ , 10 HEPES, 10 BAPTA, 3.6  $MgATP$ , 14 tris<sub>2</sub>phosphocreatine, 0.1 GTP, and 50 U/ml creatine phosphokinase. The pH was adjusted to 7.2 with CsOH. After the formation of a giga seal, in order to record  $I_{Ca}$ , the external Krebs solution was replaced by solution containing the following (in mM): 67 cholinechloride, 100 tetraethylammonium chloride, 5.3 KCl, 5  $CaCl_2$  and 10 HEPES. The pH was adjusted to 7.4 with Tris base. Command voltage protocols were generated with a computer software pCLAMP version 8 (Axon Instruments, Union City, CA, U.S.A.) and transformed to an analogue signal using a DigiData 1200 interface (Axon Instruments, Union City, CA, U.S.A.). The command pulses were applied to the cell through an L/M-EPC7 amplifier (HEKA Elektronik, Lambrecht, Germany). The currents were recorded with the amplifier and a computer software pCLAMP 8 acquisition system.

All data analyses were performed using the pCLAMP 8 acquisition system. Statistical analyses were made by Student *t*-test. Probability (*p*) values of less than 0.05 were considered significant.

Ang II was purchased from Peptide Institute. Losartan was a gift from Merck & Co., Inc. 8-(4-chlorophenylthio)-adenosine 3': 5'-cyclic monophosphate (CPT-cAMP) was purchased from Sigma, St. Louis, MO, U.S.A. The PKA inhibitor peptide, PKI (5–24), was purchased from BIOMOL Research Laboratories Inc. (Plymouth, PA, U.S.A.).

## RESULTS

An example of the effects of Ang II and  $AT_1$  receptor antagonist, losartan, on  $I_{Ca}$  is shown in Fig. 1 (A). Application of 1  $\mu M$  Ang II inhibited  $I_{Ca}$  from  $-2,408$  pA to  $-1,160$  pA (51.8% inhibition) in this neuron. 1  $\mu M$  losartan completely antagonized the inhibitory effect of Ang II.

To evaluate the possible contribution of PKA to the Ang II-induced inhibition of  $I_{Ca}$ ,

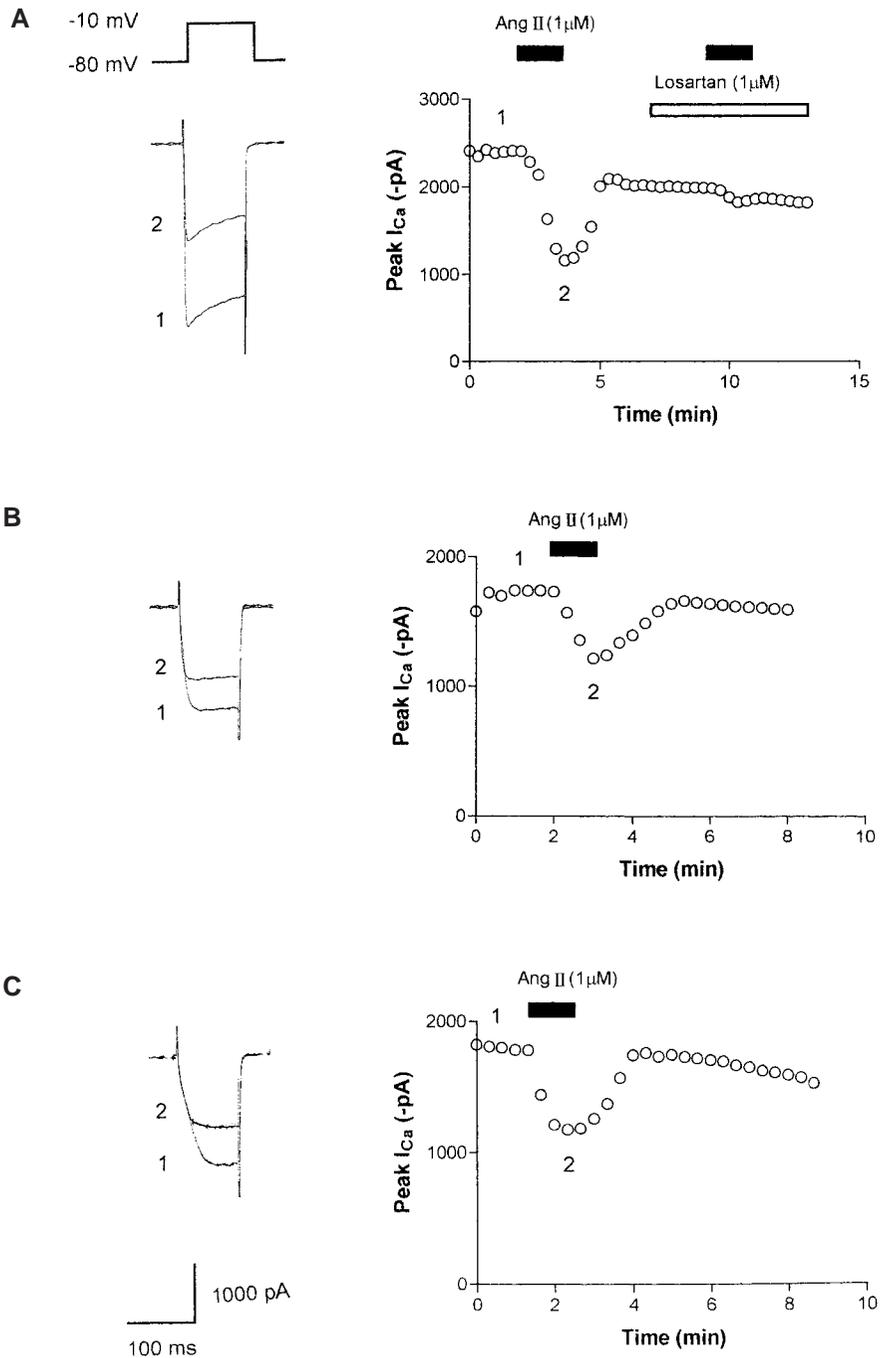


Fig. 1 (A) Effects of Ang II and losartan on I<sub>Ca</sub>. Superimposed I<sub>Ca</sub> traces were recorded using a voltage protocol at the times indicated in the time course graph (right). Open points in the graph indicate I<sub>Ca</sub>. Ang II (1 μM) and losartan (1 μM) were bath applied during the times indicated by the filled and open bars, respectively.  
 (B) Effects of Ang II on I<sub>Ca</sub> in a neuron pretreated with CPT-cAMP (10 μM for 10 min).  
 (C) Effects of Ang II on I<sub>Ca</sub> in the presence of PKI (5-24) (20 μM for 10 min) contained in the recording pipette.

the effects of Ang II on  $I_{Ca}$  in neurons treated with CPT-cAMP ( $10\mu\text{M}$  for 10 min), membrane permeable cyclic AMP, were investigated. We have previously demonstrated that this compound has a sufficient effect to cause the saturation of intracellular cyclic AMP levels<sup>11</sup>. As shown in Fig. 1 (B), Ang II-induced inhibition of  $I_{Ca}$  was not affected by CPT-cAMP treatment. In addition, intracellular dialysis of PKI (5–24) ( $20\mu\text{M}$  for 10 min), PKA inhibitor, also did not attenuate the Ang II-induced inhibition of  $I_{Ca}$  as shown in Fig. 1 (C).

The data showing the effects of altering PKA activity and antagonizing  $AT_1$  receptors are summarized in Fig. 2. On average, the Ang II-induced inhibition of  $I_{Ca}$  was  $32.1 \pm 2.7\%$  ( $n=9$ ) for control,  $7.6 \pm 1.5\%$  ( $n=7$ ) for neurons treated with losartan,  $32.7 \pm 4.7\%$  ( $n=5$ ) for the neurons treated with CPT-cAMP, and  $25.6 \pm 3.9\%$  ( $n=5$ ) when the recording pipette was filled with PKI (5–24).

## DISCUSSION

The present results show that Ang II inhibited  $I_{Ca}$  by a cyclic AMP-PKA pathway-independent mechanism. In contrast, there is considerable evidence that Ang II receptors cause inhibition of cyclic AMP-PKA through inhibition of adenylyl cyclase and reduction in cyclic AMP<sup>1–3,10</sup>. This evidence raises several questions: (1) which processes are absent in Ang II-induced inhibition of  $I_{Ca}$  in SMG?; (2) are cyclic AMP-PKA pathways between  $AT_1$  receptors and VDCCs possibly absent in SMG?; and (3) is there a possible lack of  $AT_1$  receptors that is selectively regulating cyclic AMP-PKA pathways in SMG?

Various reports have demonstrated the distinct second messenger pathways in the Ang II-induced ion channel modulations: for instance, PKC pathways and calcium/calmodulin-dependent protein kinase II (CaM KII) pathways in the central nervous system (CNS) neurons<sup>9,12</sup>, Ras/Raf/mitogen-activated protein (MAP) kinase pathways and Janus kinase/signal transducers and

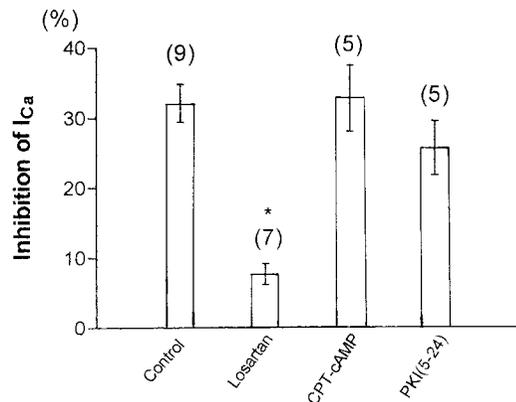


Fig. 2 Summary of Ang II-induced inhibition of  $I_{Ca}$  determined when various conditions. Histogram demonstrating the degree of  $I_{Ca}$  inhibition by  $1\mu\text{M}$  Ang II in control (untreated neurons), after losartan ( $AT_1$  receptor antagonist), after CPT-cAMP (membrane permeable cyclic AMP) and intracellular dialysis of PKI (5–24) (PKA inhibitor). Ordinates represent the Ang II-induced inhibition of  $I_{Ca}$ . Number of neurons tested is indicated in parentheses. \*Significant difference relative to control ( $p < 0.05$ , by post hoc Dunnett's test)

activators of transcription (JAK/STAT) pathways<sup>6,8</sup>, Fos-regulating kinase and c-Jun  $NH_2$ -terminal kinase<sup>5</sup>.

The present data do not allow us to deduce the exact mechanism by which intracellular pathways may alter VDCCs function. It will be important in future experiments to determine what second messenger pathways contribute to the Ang II-induced inhibition of  $I_{Ca}$  in SMG neurons.

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