

# Tyrosine kinase participates in $\alpha_{1A}$ -adrenoceptor-mediated increase of intracellular calcium in human embryo kidney 293 cells<sup>1</sup>

ZHU Wei-Zhong<sup>2</sup>, GAO Ben-Bo, LI He-Wang, ZHANG You-Yi, HAN Qi-De<sup>3</sup>

(Institute of Vascular Medicine, The Third Hospital, Beijing Medical University, Beijing 100083, China)

**KEY WORDS** alpha-1 adrenergic receptors; protein-tyrosine kinase; calcium; calphostin C; quercetin; genistein; tyrphostin; phentolamine

## ABSTRACT

**AIM:** To determine the role of protein-tyrosine kinase (PTK) in  $\alpha_{1A}$ -adrenoceptor-mediated increase of  $[Ca^{2+}]_i$  (intracellular calcium) in human embryo kidney (HEK) 293 cells expressed  $\alpha_{1A}$ -adrenoceptor. **METHODS:** Effects of two PTK inhibitors: genistein and tyrphostin, were investigated on the increase of  $[Ca^{2+}]_i$ ; by using Fura-2. The activity of PTK was measured and the accumulation of  $[^3H]$ InsPs were observed. **RESULTS:** Norepinephrine stimulated a rapid increase in  $[Ca^{2+}]_i$  to  $(371 \pm 31) \text{ nmol} \cdot \text{L}^{-1}$  in HEK 293 cells. Norepinephrine-induced increase of  $[Ca^{2+}]_i$  was inhibited by the tyrosine kinase inhibitors quercetin and tyrphostin by 23.8% and 21.4%, respectively, but the accumulation of  $[^3H]$ InsPs induced by norepinephrine was not. The activity of the plasma-associated tyrosine kinase was increased to  $(1.73 \pm 0.72)$ -fold over the control by norepinephrine  $10 \mu\text{mol} \cdot \text{L}^{-1}$ . The norepinephrine-activated PTK was inhibited by calphostin C and depletion of intra- and extra-cellular  $Ca^{2+}$ . **CONCLUSION:** The PTK participates in mobilization of  $Ca^{2+}$  mediated by  $\alpha_{1A}$ -adrenoceptors in HEK 293 cell lines.

## INTRODUCTION

Protein tyrosine kinase (PTK) plays an important role in the biological responses mediated by G protein-coupled receptors (GPCR) after the discovery of various cytosolic nonreceptor PTK. Both the GPCR-mediated vasoconstriction and the protein-tyrosine phosphorylation in vascular smooth muscle cells were suppressed by PTK inhibitors<sup>[1]</sup>. In the subclone cells expressing GPCR, the GPCR-mediated increase of cytoplasmic  $Ca^{2+}$  concentration and protein-tyrosine phosphorylation can be inhibited by the PTK inhibitors and by the deficiency of PTK<sup>[2,3]</sup>.

The  $\alpha_1$ -adrenoceptor-mediated activation of PLC results in the phosphodiesteratic cleavage of phosphatidylinositol 4,5-diphosphate, yielding the second messengers,  $IP_3$  and diacylglycerol. However, our previous data showed that only the  $\alpha_{1A}$ -adrenoceptor contributed to the exogenous norepinephrine-induced vasopressor response in rat hindlimb<sup>[4]</sup>. Later, our data displayed that PTK inhibitors, genistein and tyrphostin  $A_{47}$ , inhibited the norepinephrine-induced vasopressor response in rat hindlimb, but KCl-induced vasopressor responses was not inhibited by the same concentration PTK inhibitors<sup>[5]</sup>. These results suggest that the PTK participates in the signal transduction of  $\alpha_{1A}$ -adrenoceptor in vascular smooth muscles of rat mesentery and hindlimb.

Although  $\alpha_{1A}$ -adrenoceptors are the major functional  $\alpha_1$ -adrenoceptor mediating the vasoconstriction in the perfused mesentery and hindlimb of rat, there might exist other subtypes of  $\alpha_1$ -adrenoceptors in these tissues which were not functional receptors. Thus, in order to exclude the effect of other factors in perfused tissues, especially other subtypes of  $\alpha_1$ -adrenoceptors in native tissue, on the signaling of  $\alpha_{1A}$ -adrenoceptors, we used

<sup>1</sup> Project supported by the National Natural Science Foundation of China (No 93470268) and the grant from China Medical Board of New York Inc (# 93-951)

<sup>2</sup> Now in Department of Pharmacology, Nantong Medical College, Nantong 226001, China

<sup>3</sup> Correspondence to Prof HAN Qi-De. Ptn 86-10-6209-2306. Fax 86-10-6201-5681. E-mail hanqd@mail.bjmu.edu.cn  
Received 1998-11-06 Accepted 1999-04-06

the HEK 293 cells expressed  $\alpha_{1A}$ -adrenoceptors to determine if PTK participate in the signaling of  $\alpha_{1A}$ -adrenoceptors. Since the level of  $[Ca^{2+}]_i$  plays an important role in the contraction of vascular smooth muscles, the effects of PTK inhibitors on the vasopressor response induced by norepinephrine might be associated with the change of  $[Ca^{2+}]_i$ . Thus, we investigated the effects of PTK inhibitors on the free  $Ca^{2+}$  concentration in the expressing  $\alpha_{1A}$ -adrenoceptor HEK 293 cells in the present study. To investigate the mechanism of PTK activated by norepinephrine, we examined the effects of PKC inhibitors and intracellular and extracellular  $Ca^{2+}$  depletion on PTK activity. To determine whether the PTK inhibitors act before or after  $IP_3$  formation, we examined the formation of  $IP_3$  in the presence or absence of PTK inhibitors.

## MATERIALS AND METHODS

**Materials** Drugs were obtained from the following sources: norepinephrine, phentolamine, Triton X-100, egtazic acid, edetic acid, Fura-2/AM, histidinol, HEPES, bovine serum albumin (BSA), quercetin, tyrphostin, calphostin C, cyclopiazonic acid (CPA), poly (Glu/Tyr), and *p*-nitrophenyl phosphate (Sigma); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) (Gibco); 2-(2,6-dimethoxyphenoxyethyl) aminomethyl-1,4 benzodioxane (WB 4101), 5-methyl-urapidil, 8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione (BMY 7378) (Research Biochemical International, Natick MA, USA); 2- $\beta$  (4-hydroxyphenyl)-ethylaminomethyl)-tetralone (BE 2254) (Beiersdorf, Hamburg, Germany);  $Na^{125}I$  and  $[\gamma\text{-}^{32}P]$  ATP (China Institute of Atomic Energy); myo- $^{3}H$  inositol (Amersham).

**Cell culture** Human embryo kidney (HEK) 293 cells were propagated in that as described previously<sup>(4)</sup>. For measurements of  $^{3}H$ InsPs formation, cells of  $4 \times 10^6$  cells  $\cdot L^{-1}$  were seeded in the 24-well plates. For studies involving radioligand binding,  $Ca^{2+}$ , and PTK activity measurements, cells of  $6 \times 10^6$  cells per dish were seeded in the 90-mm<sup>2</sup> dishes. Cells were grown to confluence before use.

**Stably transfected cell lines** cDNA for the bovine  $\alpha_{1A}$ -adrenoceptors was kindly provided by Dr K P Minneman. Full length inserts were subcloned into

the Epstein-Barr virus-based vectors pREP8, containing a resistance marker for selection of mammalian cells. HEK 293 cells were maintained as mentioned above. Cells were transfected by calcium phosphate precipitation with the cDNA-containing vectors (20  $\mu g$  per 90-mm<sup>2</sup> plate), as described previously<sup>(16)</sup>.

**$^{125}I$ -BE binding**  $^{125}I$ -BE binding was performed in membrane preparation<sup>(17)</sup>. BE was radioiodinated to theoretical specific activity and stored in methanol at  $-20\text{ }^\circ C$ . Saturation curves were obtained by incubating cell membranes with increasing concentrations of  $^{125}I$ -BE and were analyzed by the method of Scatchard. Displacement by competitive antagonists was determined by incubating a single concentration of  $^{125}I$ -BE (40–50 pmol  $\cdot L^{-1}$ ) in the presence or absence of 14 to 16 concentration of drug, as described previously<sup>(14,6)</sup>. Saturation and displacement curves were analyzed by nonlinear regression analysis, and Hill coefficients were determined from a Hill plot.

**$^{3}H$ InsPs formation** Most experiments were performed in 24-well plates. Transfected subclones were seeded at low density ( $2 \times 10^5$  cells  $\cdot L^{-1}$ ) into each well of 24-well plates. Myo- $^{3}H$  inositol was added to grow to confluence (2–3 d) before experiments. On the day of the experiments, plates were washed by submersion into 1 mL of Lithium-containing Krebs Ringer bicarbonate buffer: NaCl 110, LiCl 10, KCl 5,  $MgCl_2$  1,  $CaCl_2$  1.5,  $NaHCO_3$  20, glucose 11, and edetic acid 0.029 (mmol  $\cdot L^{-1}$ ), where they were gently shaken for 30–60 s. Plates fluid was replaced by lithium-containing Krebs Ringer bicarbonate buffer containing drugs. Incubations were continued at  $37\text{ }^\circ C$  (in 95%  $O_2$  + 5%  $CO_2$ ) for 1 h and were then stopped by addition of 0.1 mL of formic acid 20 mmol  $\cdot L^{-1}$ . Wells were sonicated for 10 s each and  $^{3}H$ InsPs were isolated by anion exchange chromatography<sup>(17)</sup>.

**$[Ca^{2+}]_i$  determinations**  $[Ca^{2+}]_i$  transients were determined by using Fura-2<sup>(17)</sup>. In brief, confluent 90-mm plates were washed with HBSS: NaCl 130, KCl 5,  $MgCl_2$  1,  $CaCl_2$  1.5, HEPES 20, glucose 10 (mmol  $\cdot L^{-1}$ ), 0.1% BSA. Cells were washed with HBSS and detached by incubation with 0.05% trypsin and edetic acid 0.53 mmol  $\cdot L^{-1}$  in HBSS for 1 min, followed by incubation in HBSS for 5–10 min. Cells were centrifuged, resuspended ( $3 \times 10^9$ – $4 \times 10^9$  cells  $\cdot L^{-1}$ ) in DMEM containing 0.05% BSA and

incubated with  $5 \mu\text{mol} \cdot \text{L}^{-1}$  Fura-2/acetoxymethyl ester at  $37^\circ\text{C}$  for 30 min. The pellet was resuspended in 3 mL of oxygenated BSS and placed in a Perkin-Elmer LS 50 luminescence spectrofluorometer with  $\lambda_{\text{ex}}$  340 and 380 nm and  $\lambda_{\text{em}}$  510 nm (all with 5-nm bandwidths). Calibration of the fluorescence signals for calculation of  $[\text{Ca}^{2+}]_i$  was performed for each aliquot by equilibrating intracellular and extracellular  $\text{Ca}^{2+}$  with digitonin  $30 \mu\text{mol} \cdot \text{L}^{-1}$  ( $R_{\text{max}}$ ), followed by addition of egtazic acid  $300 \text{mmol} \cdot \text{L}^{-1}$ , Tris  $1 \text{mol} \cdot \text{L}^{-1}$ , pH 9.0 ( $R_{\text{min}}$ ), and using a  $K_d$  of Fura-2  $225 \text{nmol} \cdot \text{L}^{-1}$ .

#### Extraction of plasma-associated tyrosine kinase and assays of tyrosine kinase activity

Most experiments were performed in  $75\text{-cm}^2$  flasks. On the day of the experiments, when cells were propagated well, flasks were washed by Krebs' buffer; NaCl 110, KCl 5,  $\text{MgCl}_2$  1,  $\text{CaCl}_2$  1.5,  $\text{NaHCO}_3$  20, glucose 11, and edetic acid  $0.029 \text{mmol} \cdot \text{L}^{-1}$ , where they were gently shaken for 30–60 s. Flasks fluid was replaced by Krebs' buffer containing drugs. Incubations were continued at  $37^\circ\text{C}$  (in 95%  $\text{O}_2$  + 5%  $\text{CO}_2$ ) for 30 min and were stimulated with norepinephrine  $10 \mu\text{mol} \cdot \text{L}^{-1}$  at  $37^\circ\text{C}$  for 3 min. Cells were lysed with ice-cold lysis buffer 3 mL: Tris-HCl 20, pH 7.5, edetic acid 2, sucrose 0.25, DTT 20, PMSF  $0.5 \text{mmol} \cdot \text{L}^{-1}$ , leupeptin  $5 \text{mg} \cdot \text{L}^{-1}$ . The cells were homogenized with an Ultrasonic Processor (amplitude 15, 10 s, 2 times). The homogenate was first centrifuged at  $2000 \times g$  for 10 min to remove nuclei, and the resulting supernatant was homogenized and clarified by centrifugation at  $100\,000 \times g$  at  $4^\circ\text{C}$  for 60 min. The supernatant was the extraction of plasma-associated PTK, which activity was measured, using poly (Glu/Tyr) (molar ratio 4/1) as substrate<sup>[8]</sup>. In brief, assays were performed at  $37^\circ\text{C}$  for 10 min in a  $50 \mu\text{L}$  final reaction, using a reactive buffer  $37 \mu\text{L}$  (pH 7.0, Tris-HCl  $50 \text{mmol} \cdot \text{L}^{-1}$ ,  $\text{MgCl}_2$   $50 \text{mmol} \cdot \text{L}^{-1}$ ,  $\text{Na}_3\text{VO}_4$   $50 \mu\text{mol} \cdot \text{L}^{-1}$ , *p*-nitrophenyl phosphate  $7 \text{g} \cdot \text{L}^{-1}$ , and  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$   $37 \text{kBq}$  ( $> 18.5 \text{PBq} \cdot \text{mol}^{-1}$ ). The substrates, when present, were added at concentration of  $840 \text{mg} \cdot \text{L}^{-1}$ . The assay was begun by adding reactive buffer to extraction of plasma-associated PTK and was terminated by spotting the reaction mixture on a Whatman 3-MM paper strip. The washed (7 mL of 10% TCA containing ATP  $1 \text{mmol} \cdot \text{L}^{-1}$  followed by acetone and

then air dried) paper was counted for radioactivity. Less than 3% of the substrate was consumed in the reaction. Routinely, the enzyme activity was expressed as  $^{32}\text{P}$  incorporated per sample over a 10-min time period.

**Statistics** Results were expressed as  $\bar{x} \pm s$ . Comparisons were made using ANOVA and *t* test.

## RESULTS

**Radioligand binding**  $^{125}\text{I}$ -BE labeled apparently homogeneous populations of binding sites in membrane prepared from subclones of HEK 293 cells transfected with the bovine  $\alpha_{1A}$ -adrenoceptors cDNA-containing vectors. Saturation binding isotherms analyzed by the method of Scatchard showed that the density of binding sites ( $B_{\text{max}}$ ) was  $(1.26 \pm 0.16) \text{nmol} \cdot \text{g}^{-1}$  of protein ( $n=5$ ) and that the  $K_d$  values  $(163 \pm 34) \text{pmol} \cdot \text{L}^{-1}$  ( $n=5$ ). Inhibition of specific  $^{125}\text{I}$ -BE ( $40\text{--}50 \text{pmol} \cdot \text{L}^{-1}$ ) binding by selective competitive antagonists was performed in membrane from the transfected cell lines. The  $K_i$  of 5-methylurapidil, BMY 7378, and WB 4101 were  $8.24 \pm 0.22$  ( $n_H = 0.85 \pm 0.21$ ,  $P < 0.01$  vs unity,  $n=4$ ),  $6.11 \pm 0.20$  ( $n_H = 1.10 \pm 0.20$ ,  $P < 0.01$  vs unity,  $n=4$ ), and  $9.28 \pm 0.26$  ( $n_H = 0.75 \pm 0.11$ ,  $P < 0.01$  vs unity,  $n=4$ ), respectively.

**Inhibitory effects of quercetin and tyrphostin  $A_{47}$  on  $[\text{Ca}^{2+}]_i$  response induced by norepinephrine** The basal level of  $[\text{Ca}^{2+}]_i$  in HEK 293 cells expressed  $\alpha_{1A}$ -adrenoceptors in Krebs' buffer was  $(93 \pm 10) \text{nmol} \cdot \text{L}^{-1}$  ( $n=4$ ). Norepinephrine stimulated a rapid increased in  $[\text{Ca}^{2+}]_i$  in the presence of extracellular  $\text{Ca}^{2+}$   $1.5 \text{mmol} \cdot \text{L}^{-1}$ . After this rapid increase, the  $[\text{Ca}^{2+}]_i$  was maintained at a sustained level for  $(8 \pm 5) \text{s}$ , which slowly declined for  $(87 \pm 12) \text{s}$ . The peak of rapid increased  $[\text{Ca}^{2+}]_i$  was induced by norepinephrine in a concentration-dependent manner. The maximal response and  $pD_2$  were  $(572 \pm 26) \text{nmol} \cdot \text{L}^{-1}$  and  $6.45 \pm 0.24$  ( $n=4$ ). The peak of rapid increased  $[\text{Ca}^{2+}]_i$  induced by norepinephrine  $10 \mu\text{mol} \cdot \text{L}^{-1}$  was  $(371 \pm 31) \text{nmol} \cdot \text{L}^{-1}$  ( $n=3$ ). When the cells were treated with quercetin  $100 \mu\text{mol} \cdot \text{L}^{-1}$  or tyrphostin  $A_{47}$   $100 \mu\text{mol} \cdot \text{L}^{-1}$  for 30 min, the  $[\text{Ca}^{2+}]_i$  were reduced by 23.8% and 21.4% respectively, but the value of  $[\text{Ca}^{2+}]_i$  plateau were not changed (Tab 1).

**Tab 1. Effects of quercetin and tyrphostin A<sub>47</sub> on the increase of [Ca<sup>2+</sup>] in HEK 293 cells expressed α<sub>1A</sub>-adrenoceptors (nmol·L<sup>-1</sup>). n = 3,  $\bar{x} \pm s$ . <sup>b</sup>P < 0.05 vs the control.**

Drugs/ $\mu\text{mol}\cdot\text{L}^{-1}$	Peaks	Plateau
Control	371 ± 31	15.3 ± 2.1
Quercetin 100	285 ± 22 <sup>b</sup>	15.4 ± 1.5
Tyrphostin A <sub>47</sub> 100	294 ± 12 <sup>b</sup>	14.8 ± 2.4

**Effects of norepinephrine and phentolamine on PTK activity** The synthetic peptide Poly (Glu<sup>80</sup>/Tyr<sup>20</sup>) was the substrate of PTK. The activity of the plasma-associated tyrosine kinase was increased to (1.73 ± 0.72)-fold over the control (n = 5, P < 0.05) by norepinephrine 10  $\mu\text{mol}\cdot\text{L}^{-1}$ . However, the increase of activity of PTK induced by norepinephrine 10  $\mu\text{mol}\cdot\text{L}^{-1}$  was inhibited to (0.99 ± 0.12)-fold by pretreatment with phentolamine 10  $\mu\text{mol}\cdot\text{L}^{-1}$  (Tab 2).

**Tab 2. Effects of norepinephrine and phentolamine on the activity of plasma-associated tyrosine kinases in the HEK 293 cell lines expressed α<sub>1A</sub>-adrenoceptors.  $\bar{x} \pm s$ . <sup>b</sup>P < 0.05 vs the basal; <sup>c</sup>P < 0.05 vs control.**

Drugs/ $\mu\text{mol}\cdot\text{L}^{-1}$	n	Activity of tyrosine kinases/fold
Basal	5	1.00
Norepinephrine 10	5	1.73 ± 0.72 <sup>b</sup>
Phentolamine 10	3	1.03 ± 0.16
Norepinephrine 10 + Phentolamine 10	4	1.03 ± 0.33 <sup>c</sup>

**Effects of calphostin C and depletion of intra- and extracellular Ca<sup>2+</sup> on PTK activity of tyrosine kinase induced by norepinephrine** When the cells were pretreated with calphostin C 0.1  $\mu\text{mol}\cdot\text{L}^{-1}$  (a PKC inhibitor) and CPA 10  $\mu\text{mol}\cdot\text{L}^{-1}$  in Ca<sup>2+</sup>-free Krebs' solution containing egtazic acid 100  $\mu\text{mol}\cdot\text{L}^{-1}$  for 30 min, the basal activity of plasma-associated PTK were not altered. However, the norepinephrine-induced increase of activity of PTK were inhibited by the pretreatment mentioned above (Tab 3).

**Effects of genistein and tyrphostin A<sub>47</sub> on the [<sup>3</sup>H]InsPs formation induced by norepinephrine** The [<sup>3</sup>H]InsPs formation induced by

**Tab 3. Effects of calphostin C and CPA in the free Ca<sup>2+</sup> Krebs' solution containing egtazic acid on the activation of tyrosine kinase induced by norepinephrine in the HEK 293 cell lines expressed α<sub>1A</sub>-adrenoceptors. <sup>b</sup>P < 0.05 vs the basal; <sup>c</sup>P < 0.05 vs the control.  $\bar{x} \pm s$ .**

Drugs/ $\mu\text{mol}\cdot\text{L}^{-1}$	n	Activity of tyrosine kinases/fold
Basal	4	1.00
Norepinephrine 10	4	2.00 ± 0.23 <sup>b</sup>
Calphostin C 0.1	3	1.04 ± 0.12
Calphostin C 0.1 + Norepinephrine 10	4	1.05 ± 0.32 <sup>c</sup>
Cyclopiazonic acid 10	3	1.05 ± 0.21
Cyclopiazonic acid 10 + Norepinephrine 10	4	1.00 ± 0.07 <sup>c</sup>

**Tab 4. Effect of genistein on the accumulation of [<sup>3</sup>H]InsPs induced by norepinephrine in the HEK 293 cell lines expressed α<sub>1A</sub>-adrenoceptors. n = 5.  $\bar{x} \pm s$ . <sup>c</sup>P < 0.01 vs the basal; <sup>d</sup>P > 0.05 vs the control.**

Drugs/ $\mu\text{mol}\cdot\text{L}^{-1}$	PI Accumulation/Bq
Basal	9.4 ± 1.1
Norepinephrine 10	45.4 ± 10.5 <sup>c</sup>
Genistein 30	9.6 ± 1.5
Genistein 30 + Norepinephrine 10	48.9 ± 11.4 <sup>cd</sup>
Genistein 100	12.2 ± 1.2
Genistein 100 + Norepinephrine 10	44.5 ± 8.7 <sup>cd</sup>

norepinephrine 10  $\mu\text{mol}\cdot\text{L}^{-1}$  were not inhibited by the pretreatment with tyrosine kinase inhibitor genistein (30 and 100  $\mu\text{mol}\cdot\text{L}^{-1}$ ) and tyrphostin A<sub>47</sub> (30 and 100  $\mu\text{mol}\cdot\text{L}^{-1}$ ) for 30 min, as shown in Tab 4 and Tab 5.

**Tab 5. Effect of tyrphostin on the accumulation of [<sup>3</sup>H]InsPs induced by norepinephrine in the HEK 293 cell lines expressed α<sub>1A</sub>-adrenoceptors. n = 5.  $\bar{x} \pm s$ . <sup>c</sup>P < 0.01 vs the basal; <sup>d</sup>P > 0.05 vs the control.**

Drugs/ $\mu\text{mol}\cdot\text{L}^{-1}$	PI Accumulation/Bq
Basal	9.3 ± 3.6
Norepinephrine 10	36.9 ± 7.4 <sup>c</sup>
Tyrphostin 30	11.8 ± 3.4
Tyrphostin 30 + Norepinephrine 10	44.6 ± 8.5 <sup>cd</sup>
Tyrphostin 100	9.6 ± 1.7
Tyrphostin 100 + Norepinephrine 10	42.2 ± 5.6 <sup>cd</sup>

## DISCUSSION

In the present study, the binding affinity ( $K_i$  values) for  $\alpha_{1A}$ -adrenoceptor-selective antagonists, 5-methyl-urapidil and WB 4101, were consistent with their high affinities at the bovine  $\alpha_{1A}$ -adrenoceptors, and BMY 7378, an  $\alpha_{1D}$ -adrenoceptor-selective antagonist, was consistent with its low affinity at the bovine  $\alpha_{1A}$ -adrenoceptors<sup>[9,10]</sup>; further, the fact that  $n_H$  of Hill plot for all antagonists mentioned above were not different from unity also supported the assumption that there was a single subtype of  $\alpha_1$ -adrenoceptors existing in the HEK 293 cells. Together with the saturation binding, the results suggested that the transfected bovine  $\alpha_{1A}$ -adrenoceptors were stably expressed in HEK 293 cells and the presence of a single homogenous population of binding sites in HEK 293 cell lines with properties that resembled those of the  $\alpha_{1A}$ -adrenoceptors.

A rapid increase of  $[Ca^{2+}]_i$  induced by norepinephrine, which was phasic, was caused by intracellular  $Ca^{2+}$  release. After this rapid increase, the  $[Ca^{2+}]_i$  maintained at a sustained level, which was a tonic phase, was caused by  $Ca^{2+}$  influx. The data showed that the increase of phasic  $[Ca^{2+}]_i$  were inhibited by PTK inhibitors, which suggested that PTK participated in the mobilization of  $Ca^{2+}$  in the HEK 293 cell which expressed  $\alpha_{1A}$ -adrenoceptors. Our finding was consistent with the results of Semenchuk *et al*<sup>[11]</sup>. But the finding was inconsistent with the results of Meucci *et al*<sup>[12]</sup>, because the responses induced by the activation of receptors were probably different in different cell lines.

To determine the mechanisms of PTK activated by norepinephrine, the PTK activity were further measured. We found that the increase of plasma-associated PTK activity induced by norepinephrine were inhibited by phentolamine. It implied that the  $\alpha_{1A}$ -adrenoceptors mediated the norepinephrine-induced PTK activation. Since the phosphatidylinositol- $Ca^{2+}$  pathway is a classical signal pathway of  $\alpha_1$ -adrenoceptor which causes the biological responses, we first considered that both  $Ca^{2+}$  and PKC were the mediators in the PTK activation. CPA, an inhibitor of  $Ca^{2+}$ -ATPase at sarcoplasmic and endoplasmic reticulum, depleted the intracellular  $Ca^{2+}$  pools<sup>[13]</sup>. Thus, when the cells were incubated in  $Ca^{2+}$ -free Krebs' solution

containing CPA  $10 \mu\text{mol} \cdot \text{L}^{-1}$  and egtazic acid  $100 \mu\text{mol} \cdot \text{L}^{-1}$  for 30 min, the intra- and extra-cellular  $Ca^{2+}$  were depleted. Calphostin C was an inhibitor of PKC<sup>[4]</sup>. The present data had shown that the norepinephrine-induced increase of PTK activity were inhibited by PKC inhibitor and the depletion of intra- and extra-cellular  $Ca^{2+}$ . These results implied that the activity of plasma-associated PTK were mediated by PKC and  $Ca^{2+}$ . Lev *et al*<sup>[15]</sup> reported that the activation of PYK<sub>2</sub> (a proline-rich tyrosine kinase) in PC12 cells were PKC-dependent and independent pathways, but the activity of PYK<sub>2</sub> induced by bradykinin were associated with the increase of  $[Ca^{2+}]$  concentration. Whether the PYK<sub>2</sub> was one of the PTK activated by  $\alpha_A$ -adrenoceptor should be determined.

The steps where  $\alpha_{1A}$ -adrenoceptor-mediated PTK action in the  $Ca^{2+}$  mobilization are very interesting and complex. In the present study, the norepinephrine-induced  $[^3\text{H}]\text{InsP}$  formation were observed. The fact that  $[^3\text{H}]\text{InsP}$  formation mediated by  $\alpha_{1A}$ -adrenoceptor were not inhibited by the pretreatment with the PTK inhibitors suggested that the PTK inhibitors were after  $\text{IP}_3$  formation. It meant  $\alpha_{1A}$ -adrenoceptor-mediated PTK did not act as the signal transduction pathway before phospholipase C (PLC). The effect of PTK on the metabolism of phosphatidylinositol showed the specificity of the tissue and their expression of GPCR. So far, the PTK causes alterations in the production and metabolism of phosphatidylinositol via at least three pathway as follows:  $\alpha$  subunit of  $G_{q/11}$ , PI-3 kinase, and PLC <sub>$\gamma$</sub> <sup>[2,16,17]</sup>. Our previous study showed that NaF (an agonist of G-protein) -induced the vasopressor responses in rat perfused hindlimb were inhibited by PTK inhibitors, but the PMA (an agonist of PKC) -induced vasopressor responses were not inhibited<sup>[5]</sup>. We suspect that the step where PTK acted is at one sides between the G-protein and PKC.

## REFERENCES

- 1 Di Salvo J, Steusloff A, Semenchuk L, Satoh S, Kolquist K, Pfitzer G. Tyrosine kinase inhibitors suppress agonist-induced contraction in smooth muscle. *Biochem Biophys Res Commun* 1993; 190: 968-74.
- 2 Umemori H, Inoue T, Kume S, Sekiyama N, Nagao M, Itoh H, *et al*. Activation of the G protein  $G_{q/11}$  through tyrosine kinase phosphorylation of the  $\alpha$  subunit. *Science*

1997; 276: 1878-81.

- Wan Y, Kurosaki T, Huang XY. Tyrosine kinase in activation of the MAP kinase cascade by G-protein-coupled receptors. *Nature* 1996; 380: 541-44.
- Zhu WZ, Zhang YY, Han C. Characterization of subtype of  $\alpha_1$ -adrenoceptor mediating vasoconstriction in perfused rat hind limb. *Eur J Pharmacol* 1997; 329: 55-61.
- Zhu WZ, Han QD. Tyrosine kinases participate in  $\alpha_{1A}$ -adrenoceptor-mediated vasoconstriction in the perfused rat hindlimb. *Acta Pharmacol Sin* 1998; 19: 473-7.
- Minneman KP, Theroux TL, Hollinger S, Han C, Esbenshade TA. Selectivity of agonists for cloned  $\alpha_1$ -adrenergic receptor subtypes. *Mol Pharmacol* 1994; 46: 929-36.
- Esbenshade TA, Han C, Murphy TJ, Minneman KP. Comparison of  $\alpha_1$ -adrenergic receptor subtypes and signal transduction in SK-N-MC and NB41A3 neuronal cell lines. *Mol Pharmacol* 1993; 44: 76-86.
- Cheng HC, Nishio H, Hatase O, Ralph S, Wang JH. A synthetic peptide derived from p34<sup>cdc2</sup> is a specific and efficient substrate of src-family tyrosine kinases. *J Biol Chem* 1992; 267: 9248-56.
- Michel MC, Kenny B, Schwinn DA. Classification of  $\alpha_1$ -adrenoceptor subtypes. *Naunyn Schmiedeberg's Arch Pharmacol* 1995; 352: 1-10.
- Goetz AS, King HK, Ward SDC, True TA, Rimele TJ, Saussy DLJ. BMY 7378 is a selective antagonist of the D subtype of  $\alpha_1$ -adrenoceptors. *Eur J Pharmacol* 1995; 45: 703-8.
- Semenchuk LA, Di Salvo J. Receptor-activated increases in intracellular calcium and protein tyrosine phosphorylation in vascular smooth muscle cells. *FEBS Lett* 1995; 370: 127-30.
- Meucci O, Scorziello A, Avallone A, Florio T, Schettini G.  $\alpha_{1B}$ , but not  $\alpha_{1A}$ , adrenoceptor activates calcium influx through the stimulation of a tyrosine kinase/phosphotyrosine phosphatase pathway, following noradrenaline-induced emptying of  $IP_3$  sensitive calcium stores, in PC Cl3 rat thyroid cell line. *Biochem Biophys Res Commun* 1995; 209: 630-8.
- Golovina VA, Blaustein MP. Spatially and functionally distinct  $Ca^{2+}$  stores in sarcoplasmic and endoplasmic reticulum. *Science* 1997; 275: 1643-48.
- Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S. Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemia cells by pharmacological inhibitors of protein kinase C. *Cancer Res* 1994; 54: 1707-14.

- Lev S, Moreno H, Martinez R, Canoll P, Peles E, Musacchio JM. Protein tyrosine kinase PYK<sub>2</sub> involved in  $Ca^{2+}$ -induced regulation of ion channel and MAP kinase functions. *Nature* 1995; 376: 737-44.
- Berk BC, Corson M. Angiotensin II signal transduction in vascular smooth muscle. Role of tyrosine kinase. *Circ Res* 1997; 80: 607-16.
- Fukai MU, Griendling KK, Lyons PR, Akers ML, Alexander RW. G protein  $\beta$  subunits of heterotrimeric G proteins are involved in angiotensin II signaling linked to phospholipase C activation in cultured vascular smooth muscle. *Circulation* 1997; 96 Suppl 1: I-297.

酪氨酸激酶参与  $\alpha_{1A}$  肾上腺素受体介导的  
人胚胎肾细胞浆游离钙浓度升高<sup>1</sup>

朱卫忠<sup>2</sup>, 高本波<sup>1</sup>, 李和旺<sup>1</sup>, 张幼怡<sup>1</sup>, 韩启德<sup>3</sup>  
(北京医科大学第三医院血管医学研究所, 北京 100083, 中国)

关键词  $\alpha_1$  肾上腺素受体; 蛋白质酪氨酸激酶;  
钙; calphostin C; 褪皮素; 4, 5, 7-三羟异黄酮;  
tyrphostin; 酚妥拉明

目的: 研究酪氨酸激酶是否参与  $\alpha_{1A}$  肾上腺素受体介导的人胚胎肾细胞(HEK293)浆游离钙浓度调节。方法: 用 Fura-2/AM 荧光法测定细胞游离钙浓度, 底物反应法测定酪氨酸激酶活性。结果: 去甲肾上腺素(NE)可引起的 HEK293 细胞内游离钙浓度升高至  $(371 \pm 31) \text{ nmol} \cdot \text{L}^{-1}$ , 酪氨酸激酶抑制剂褪皮素和 tyrphostin 可抑制 NE 引起的细胞内游离钙浓度升高, 但不抑制 NE 引起磷酸肌醇的生成, NE 可引起 HEK293 细胞浆酪氨酸激酶升高  $1.73 \pm 0.72$  倍, 这种作用可被 PKC 的抑制剂 calphostin C 和细胞内钙耗竭所抑制。结论: 酪氨酸激酶参与  $\alpha_{1A}$  肾上腺素受体介导的人胚胎肾细胞内游离钙浓度升高。

(责任编辑 刘俊娥)