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Tyrosine kinase participates in α_{1A}-adrenoceptor-mediated increase of intracellular calcium in human embryo kidney 293 cells[1]

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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 α_{1A}-adrenoceptor-mediated increase of $[Ca^{2+}]_i$ (intracellular calcium) in human embryo kidney (HEK) 293 cells expressed α_{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 ± 31) nmol·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 ± 0.72)-fold over the control by norepinephrine 10 μmol·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 α_{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[1]. 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[2,3].

The α_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 α_{1A}-adrenoceptor contributed to the exogenous norepinephrine-induced vasopressor response in rat hindlimb[4]. 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[5]. These results suggest that the PTK participates in the signal transduction of α_{1A}-adrenoceptor in vascular smooth muscles of rat mesentery and hindlimb.

Although α_{1A}-adrenoceptors are the major functional α_1-adrenoceptor mediating the vasoconstriction in the perfused mesentery and hindlimb of rat, there might exist other subtypes of α_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 α_1-adrenoceptors in native tissue, on the signaling of α_{1A}-adrenoceptors, we used

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the HEK 293 cells expressed α_{1A}-adrenoceptors to determine if PTK participate in the signaling of α_{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 α_{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-azaspirol[4,5]decane-7,9-dione (BMY 7378) (Research Biochemical International, Natick MA, USA); 2-β (4-hydroxyphenyl)-ethylaminomethyl)-tetralone (BE 2254) (Beiersdorf, Hamburg, Germany); $Na^{125}I$ and [γ-^{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[14]. For measurements of [^{3}H]InsPs formation, cells of 4×10^5 cells·L^{-1} were seeded in the 24-well plates. For studies involving radioligand binding, Ca^{2+}, and PTK activity measurements, cells of 6×10^5 cells per dish were seeded in the 90-mm^2 dishes. Cells were grown to confluence before use.

Stably transfected cell lines cDNA for the bovine α_{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 μg per 90-mm^2 plate), as described previously[16].

^{125}I-BE binding ^{125}I-BE binding was performed in membrane preparation[14]. BE was radioiodinated to theoretical specific activity and stored in methanol at −20 ℃. 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·L^{-1}) in the presence or absence of 14 to 16 concentration of drug, as described previously[14,16]. 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 (2×10^5 cells·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·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 ℃ (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·L^{-1}. Wells were sonicated for 10 s each and [^{3}H]InsPs were isolated by anion exchange chromatography[17].

$[Ca^{2+}]_i$ determinations $[Ca^{2+}]_i$ transients were determined by using Fura-2[17]. 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·L^{-1}), 0.1 % BSA. Cells were washed with HBSS and detached by incubation with 0.05 % trypsin and edetic acid 0.53 mmol·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·L^{-1}) in DMEM containing 0.05 % BSA and

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

Extraction of plasma-associated tyrosine kinase and assays of tyrosine kinase activity Most experiments were performed in 75-cm^2 flasks. On the day of the experiments, when cells were propagated well, flasks were washed by Krebs' buffer: *NaCl* 110, *KCl* 5, $MgCl_2$ 1, $CaCl_2$ 1.5, $NaHCO_3$ 20, glucose 11, and edetic acid 0.029 (mmol·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 ℃ (in 95 % O_2 + 5 % CO_2) for 30 min and were stimulated with norepinephrine 10 μmol·L^{-1} at 37 ℃ 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 (mmol·L^{-1}), leupeptin 5 mg·L^{-1}. The cells were homogenized with an Ultrasonic Processor (amplitude 15, 10 s, 2 times). The homogenate was first centrifuged at 2000 × g for 10 min to remove nuclei, and the resulting supernatant was homogenized and clarified by centrifugation at 100 000 × g at 4 ℃ 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[8]. In brief, assays were performed at 37 ℃ for 10 min in a 50 μL final reaction, using a reactive buffer 37 μL (pH 7.0, Tirs-HCl 50 mmol·L^{-1}, $MgCl_2$ 50 mmol·L^{-1}, Na_3VO_4 50 μmol·L^{-1}, *p*-nitrophenyl phosphate 7 g·L^{-1}, and [γ-^{32}P]ATP 37 kBq (> 18.5 PBq·mol^{-1}). The substrates, when present, were added at concentration of 840 mg·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 mmol·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}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}I-BE labeled apparently homogeneous populations of binding sites in membrane prepared from subclones of HEK 293 cells transfected with the bovine α_{1A}-adrenoceptors cDNA-containing vectors. Saturation binding isotherms analyzed by the method of Scatchard showed that the density of binding sites (B_{max}) was (1.26 ± 0.16) nmol·g^{-1} of protein (n = 5) and that the K_d values (163 ± 34) pmol·L^{-1} (n = 5). Inhibition of specific ^{125}I-BE (40 – 50 pmol·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 ± 0.22 (n_H = 0.85 ± 0.21, $P < 0.01$ *vs* unity, n = 4), 6.11 ± 0.20 (n_H = 1.10 ± 0.20, $P < 0.01$ *vs* unity, n = 4), and 9.28 ± 0.26 (n_H = 0.75 ± 0.11, $P < 0.01$ *vs* unity, n = 4), receptively.

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

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

Drugs/μmol·L^{-1}	Peaks	Plateau
Control	371 ± 31	15.3 ± 2.1
Quercetin 100	285 ± 22^b	15.4 ± 1.5
Tyrphostin A_{47} 100	294 ± 12^b	14.8 ± 2.4

Effects of norepinephrine and phentolamine on PTK activity The synthetic peptide Poly (Glu^{80}/Tyr^{20}) 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 μmol·L^{-1}. However, the increase of activity of PTK induced by norepinephrine 10 μmol·L^{-1} was inhibited to (0.99 ± 0.12)-fold by pretreatment with phentolamine 10 μmol·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_{1A}-adrenoceptors. $\bar{x} \pm s$. $^bP < 0.05$ *vs* the basal; $^eP < 0.05$ *vs* control.

Drugs/μmol·L^{-1}	n	Activity of tyrosine kinases/fold
Basal	5	1.00
Norepinephrine 10	5	1.73 ± 0.72^b
Phentolamine 10	3	1.03 ± 0.16
Norepinephrine 10 + Phentolamine 10	4	1.03 ± 0.33^e

Effects of calphostin C and depletion of intra- and extracellular Ca^{2+} on PTK activity of tyrosine kinase induced by norepinephrine When the cells were pretreated with calphostin C 0.1 μmol·L^{-1} (a PKC inhibitor) and CPA 10 μmol·L^{-1} in Ca^{2+}-free Krebs' solution containing egtazic acid 100 μmol·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).

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

Drugs/μmol·L^{-1}	n	Activity of tyrosine kinases/fold
Basal	4	1.00
Norepinephrine 10	4	2.00 ± 0.23^b
Calphostin C 0.1	3	1.04 ± 0.12
Calphostin C 0.1 + Norepinephrine 10	4	1.05 ± 0.32^e
Cyclopiazonic acid 10	3	1.05 ± 0.21
Cyclopiazonic acid 10 + Norepinephrine 10	4	1.00 ± 0.07^e

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

Drugs/μmol·L^{-1}	PI Accumulation/Bq
Basal	9.4 ± 1.1
Norepinephrine 10	45.4 ± 10.5^c
Genistein 30	9.6 ± 1.5
Genistein 30 + Norepinephrine 10	48.9 ± 11.4^{cd}
Genistein 100	12.2 ± 1.2
Genistein 100 + Norepinephrine 10	44.5 ± 8.7^{cd}

Effects of genistein and tyrphostin A_{47} on the [^{3}H]InsPs formation induced by norepinephrine The [^{3}H]InsPs formation induced by norepinephrine 10 μmol·L^{-1} were not inhibited by the pretreatment with tyrosine kinase inhibitor genistein (30 and 100 μmol·L^{-1}) and tyrphostin A_{47} (30 and 100 μmol·L^{-1}) for 30 min, as shown in Tab 4 and Tab 5.

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

Drugs/μmol·L^{-1}	PI Accumulation/Bq
Basal	9.3 ± 3.6
Norepinephrine 10	36.9 ± 7.4^c
Tyrphostin 30	11.8 ± 3.4
Tyrphostin 30 + Norepinephrine 10	44.6 ± 8.5^{cd}
Tyrphostin 100	9.6 ± 1.7
Tyrphostin 100 + Norepinephrine 10	42.2 ± 5.6^{cd}

DISCUSSION

In the present study, the binding affinity (K_i values) for α_{1A}-adrenoceptor-selective antagonists, 5-methyl-urapidil and WB 4101, were consistent with their high affinities at the bovine α_{1A}-adrenoceptors. and BMY 7378, an α_{1D}-adrenoceptor-selective antagonist, was consistent with its low affinity at the bovine α_{1A}-adrenoceptors[9,10]; 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 α_1-adrenoceptors existing in the HEK 293 cells. Together with the saturation binding, the results suggested that the transfected bovine α_{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 α_{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 α_{1A}-adrenoceptors. Our finding was consistent with the results of Semenchuk *et al*[11]. But the finding was inconsistent with the results of Meucci *et al*[12], 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 α_{1A}-adrenoceptors mediated the norepinephrine-induced PTK activation. Since the phosphatidylinositol-Ca^{2+} pathway is a classical signal pathway of α_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[13]. Thus, when the cells were incubated in Ca^{2+}-free Krebs' solution containing CPA 10 $\mu mol \cdot L^{-1}$ and egtazic acid 100 $\mu mol \cdot L^{-1}$ for 30 min, the intra- and extra-cellular Ca^{2+} were depleted. Calphostin C was an inhibitor of PKC[14]. 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*[15] reported that the activation of PYK_2 (a proline-rich tyrosine kinase) in PC12 cells were PKC-dependent and independent pathways, but the activity of PYK_2 induced by bradykinin were associated with the increase of $[Ca^{2+}]$ concentration. Whether the PYK_2 was one of the PTK activated by α_A-adrenoceptor should be determined.

The steps where α_{1A}-adrenoceptor-mediated PTK action in the Ca^{2+} mobilization are very interesting and complex. In the present study, the norepinephrine-induced $[^3H]$InsP formation were observed. The fact that $[^3H]$InsP formation mediated by α_{1A}-adrenoceptor were not inhibited by the pretreatment with the PTK inhibitors suggested that the PTK inhibitors were after IP_3 formation. It meant α_{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: α subunit of $G_{q/11}$, PI-3 kinase, and PLC_γ[2,16,17]. 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[5]. We suspect that the step where PTK acted is at one sides between the G-protein and PKC.

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酪氨酸激酶参与 α_{1A} 肾上腺素受体介导的人胚胎肾细胞浆游离钙浓度升高[1]

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关键词 α-1肾上腺素受体；蛋白质酪氨酸激酶；钙；calphostin C；槲皮素；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 α_{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 ± 31) $nmol \cdot L^{-1}$，酪氨酸激酶抑制剂槲皮素和 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 ± 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 α_{1A} 肾上腺素受体介导的人胚胎肾细胞内游离钙浓度升高。

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