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Involvement of Phosphatidylcholine-Selective Phospholipase C in Activation of Mitogen-Activated Protein Kinase Pathways in Imidazoline Receptor Antisera-Selected Protein

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Abstract Imidazoline receptor antisera-selected protein (IRAS) is considered as a candidate for the I_1-imidazoline receptor (I_1R), but the signaling pathway mediated by IRAS remains unknown. In our study, the signal transduction pathways of IRAS were investigated in CHO cells stably expressing IRAS (CHO-IRAS), and compared to the native I_1R signaling pathways. Rilmenidine or moxonidine (10 nM–100 μM), I_1R agonists, failed to stimulate [^{35}S]-GTPγS binding in CHO-IRAS cell membrane preparations, suggesting that G protein may not be involved in IRAS signaling pathway. However, incubation of CHO-IRAS with rilmenidine or moxonidine for 5 min could induce an upregulation of phosphatidylcholine-selective phospholipase C (PC-PLC) activity, and an increase in the accumulation of diacylglycerol (DAG), the hydrolysate of PC-PLC, in a concentration-dependent manner. The elevated activation of PC-PLC by rilmenidine or moxonidine (100 nM) could be blocked by efaroxan, a selective I_1R antagonist. Cells treated with rilmenidine or moxonidine showed an increased level of extracellular signal-regulated kinase (ERK) phosphorylation in a concentration-dependent manner, which could be reversed by efaroxan or D609, a selective PC-PLC inhibitor. These results suggest that the signaling pathway of IRAS in response to I_1R agonists coupled with the activation of PC-PLC and its downstream signal transduction molecule, ERK. These findings are similar to those in the signaling pathways of native I_1R, providing some new evidence for the relationship between I_1R and IRAS. J. Cell. Biochem. 98: 1615–1628, 2006.

Key words: imidazoline receptor antisera-selected protein; I_1-imidazoline receptor; Nischarin; G protein; phosphatidylcholine-selective phospholipase C; diacylglycerol; extracellular signal-regulated kinase

The concept of imidazoline receptors was first set up by Bousquet et al. [1984] when they studied antihypertension effect of clonidine. It is now accepted that there are at least two subtypes of imidazoline receptors, I_1-imidazoline receptor (I_1R) and I_2-imidazoline receptor (I_2R). I_1R is characterized by a high affinity to a group of agents including clonidine, rilmenidine, and moxonidine, which act on the brain stem to reduce blood pressure [Ernsberger et al., 1995a; Eglen et al., 1998]. I_2R shows high affinity to other imidazolines or guanidine, which presents a novel recognition site on monoamine oxidase [Ernsberger et al., 1995a; Eglen et al., 1998]. In 2000, a gene encoding an I_1R candidate protein, named imidazoline receptor antisera-selected protein (IRAS), was cloned from human hippocampus. It was identified that IRAS mRNA encodes a protein containing 1,504-amino acid residues, yield a 167 kDa protein, which could be broken down into 85 kDa and smaller protein molecules [Piletz et al., 2000].

Evidence supported that IRAS is a candidate for I_1R. First, IRAS mRNA was shown to be appropriately localized in brain neurons as expected for I_1R-binding sites [Ivanov et al., 1998], and a positive correlation ($r = 0.7$) was established between the mRNA for IRAS and

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membranous I_1-binding sites (B_{max}) over a range of native rat tissues [Piletz et al., 1999]. Second, transfection of IRAS cDNA into the Chinese hamster ovary (CHO) cells resulted in high affinity I_1-like binding sites without the appearance of α_2-AR or the other major subtype of imidazoline binding sites [Piletz et al., 2000, 2003]. Third, it has been revealed that IRAS could function on promoting cell survival [Dontenwill et al., 2003a], anti-apoptosis [Dontenwill et al., 2003b], and proliferation [Sano et al., 2002], which is similar to the intracellular functions of I_1R [Dupuy et al., 2004]. Recent studies have also found that the transfection activation of human IRAS is able to inhibit a cellular model of opioid dependence (cAMP overshoot) in morphine-dependent CHO cells stably co-expressing both IRAS and μ opioid receptor [Wu et al., 2005], which parallels is similar to the findings in vivo that the activation of I_1R inhibits tolerance and dependence on morphine [Georges and Aston-Jones, 2003; Su et al., 2003]. These findings collectively suggest that IRAS is a strong candidate for I_1R.

However, the above similarities between IRAS and I_1R are not enough to prove that IRAS expressed from the cloned gene is a native I_1R. It is well known that besides such characters as distribution and functions, the foremost is the identity in the signal transduction pathways between a receptor and its cloned protein. Little evidence has been shown to prove that IRAS is coupled with the same signaling pathways as I_1R. Although the signal pathways of I_1R have been studied systemically, those of IRAS remain unclear.

Previous studies have shown that the activation of I_1R increases the accumulation of diacylglycerol (DAG) [Liedtke and Ernsberger, 1995; Separovic et al., 1996; Separovic et al., 1997], and the release of arachidonic acid (AA) [Ernsberger, 1998], and eicosanoids [Ernsberger et al., 1995; Separovic et al., 1997]. DAG or AA may activate protein kinase C (PKC) [Edward et al., 2001] which phosphorylates and activates mitogen-activated protein kinases (MAPK) [Edward et al., 2001; Zhang et al., 2001]. The phosphorylation of MAPK can be blocked by phosphatidylcholine-selective phospholipase C (PC-PLC) inhibitor D609, suggesting the involvement of PC-PLC in I_1R signaling pathway. The phospholipid metabolism signaling pathway of I_1R has been identified in rat pheochromocytoma (PC12) cells and some other tissues expressing native I_1R. However, it is not clear if I_1R acts via the most common G-protein linked systems. Classical GTP shift analyses in bovine brainstem, human platelets, and rat PC12 cells showed that the binding of agnists to I_1R was sensitive to the hydrolysis-resistant guanine nucleotide $G_{pp(NH)p}$ or GTPγS, suggesting I_1R is a G-protein coupled receptor (GPCR) [Moldering et al., 1993; Ernsberger and Shen, 1997; Takada et al., 1997]. In contrast, similar GTP shift studies in other labs failed to prove that I_1R is a GPCR [Piletz and Sletten, 1993; Bricca et al., 1994]. The paradox is probably due to the influence of other receptors that cannot be excluded in the experimental model, especially α_2-AR (α_2-adrenoceptors).

Although IRAS gene has been cloned and expressed in some cell lines, the signaling pathways through IRAS are not well defined. The mouse homologue of IRAS, previously identified as Nischarin, has been shown in the absence of imidazolines to interact with the α_5 subunit of integrin and inhibit cell migration [Alahari et al., 2000], but the relevance of this has been disputed [Lim and Hong, 2004]. Therefore, we chose to use human IRAS transfected into CHO, a host cell line that completely lacks native I_1R [Piletz et al., 2000]. Previous studies have shown that I_1R agonists can bind to the I_1-like sites encoded by transfected human IRAS in CHO cells. Considering the similarities between IRAS and I_1R, we hypothesized that I_1R agonists may trigger signaling transduction through IRAS in a way similar to I_1R. Herein, we have investigated IRAS-mediated signaling pathways in CHO cells stably expressing IRAS (CHO-IRAS) and compared the IRAS signaling pathways with the native I_1R pathways.

MATERIALS AND METHODS

Materials

IRAS-pcDNA 3.1 (+) plasmid was a kindly gift of Dr. J.E. Piletz (Jackson State University, Jackson, MS). CHO and CHO cell stably expressing μ opioid receptor (CHO-μ) cells were provided by Dr. L.Y. Liu-Chen (Temple University School of Medicine, Philadelphia, PA). [^{3}H]clonidine (55.5 Ci/mmol) and [^{35}S]-GTPγS (1250 Ci/mmol) were purchased from NEN Life Sciences (Boston, MA). Geneticin, lipofectamine, and RPMI 1640 medium were purchased

from Invitrogen Corporation (Gibco™, Grand Island, NY). Fetal bovine serum was purchased from HyClone-Pierce (HyClone ®, South Logan, UT). Guanosine 5′-O-(3-thiotriphosphosate) (GTPγS), guanosine 5′-diphosphate (GDP), moxonidine, rilmenidine, clonidine, efaroxan, D609, 1,2-dioleoyl-sn-glycerol, leupeptin, pepstatin, aprotinin, 1.10-phenanthroline monohydrate, were purchased from Sigma Chemical Co. (St. Louis, MO). Anti-extracellular signal-regulated kinase (ERK) antibody, anti-phospho-ERK antibody, anti-rabbit and anti-mouse horseradish peroxidase antibodies, and enhanced chemiluminesence detection (ECL). The western blotting detection reagent was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [D-Ala^2, N-Me-Phe^4, Gly-ol]-enkephalin (DAMGO), GF/C filters were purchased from Whatman (Whatman, UK). High-performance thin-layer chromatography (HPTLC) plates (10 × 10 cm, glass plate of silica gel 60) were purchased from Merck (Darmstadt, Germany).

Generation of Cell Lines Expressing Human IRAS and Cell Culture

IRAS stably expressing CHO cell lines were generated by transfecting Human IRAS expression vector (hIRAS-pcDNA3.1(+)) into CHO cells with Lipofectamine reagent following the manufacturer's instructions. Transfected cells were selected using 1 mg/ml geneticin for 4–6 weeks. Clones were obtained and one of the clones was used as the representative in this study.

CHO cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with humidified atmosphere consisting of 95% air and 5% CO_2. Medium for CHO-IRAS cells was the same as that for CHO cells except for the 200 μg/ml geneticin contained.

Membrane Protein Preparation

Membrane proteins were isolated following the method of Zhu et al. [1997]. Isolated membrane protein was diluted with ice-cold assay buffer (5.0 mM HEPES, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM EDTA, pH 7.5) and the protein concentration was determined using Brad-ford method. All membrane protein samples used in the experiments were freshly prepared.

Radioligand-Binding Assay

HME assay buffer (5.0 mM HEPES, 0.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM EDTA, freshly added with 100 μM ascorbic acid and 100 μM PMSF, pH 7.5) was used to optimize the binding condition for I_1R [Ernsberger et al., 1995b]. For saturation analyses, [^{3}H]clonidine ranging from 1.5 to 48 nM was added to 20 μg membrane proteins in HME assay buffer with a final volume of 500 μl and incubated in a 21°C waterbath for 1 h. Nonspecific binding was defined with idazoxan (100 μM). For the competition analyses, 20 μg membrane protein samples were incubated with 30 nM [^{3}H]clonidine ($2 \times K_d$ value) and the competitor ligands at various concentrations in a 21°C waterbath for 1 h. Reactions were terminated by adding 5 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.5), and rapid vacuum filtration through GF/C glass fiber filters using a cell harvester, followed by washing with cold Tris-HCl buffer (pH 7.5) for three times. GF/C glass fiber filters were presoaked in 0.2% BSA at room temperature for 30 min to lower the nonspecific binding. Radioactivity in filters was determined by liquid scintillation counting.

[^{35}S]-GTPγS-Binding Assay

[^{35}S]-GTPγS-binding assay was carried out as described previously [Zhu et al., 1997]. Briefly, membrane proteins of CHO-IRAS and CHO-μ cells were prepared as described above. Binding of [^{35}S]-GTPγS to the CHO-μ cell membrane protein was in the assay buffer containing 15 μM GDP, 50 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaCl, pH 7.4 (plus 0.1 mM ascorbic acid, 0.1 mM DTT, 0.1 mM PMSF, 1 μg/ml leupeptin, pepstatin and aprotinin just before the experiments), incubated at 25°C for 1 h. Stimulated binding of [^{35}S]-GTPγS to CHO-μ cell membrane preparation was determined using DAMGO (10 μM). Different concentrations of GDP (0.1–50 μM), $MgCl_2$ (0–20 mM), and NaCl (0–100 mM), different temperatures (21, 25, 30°C), and time needed of reaction (30 min, 1, 1.5, 2 h) were tested to optimize the [^{35}S]-GTPγS-binding assay condition of IRAS. Stimulated binding of [^{35}S]-GTPγS to IRAS was determined using moxonidine or rilmenidine (10 nM–100 μM). Nonspecific binding was defined by GTPγS (40 μM). Each reaction was

set up in the following order: [^{35}S]-GTPγS (0.2 nM); agonists at different concentrations or GTPγS and membrane proteins (20 μg protein/tube) in a total volume of 500 μl assay buffer per tube. The increased percentage over basal binding level of [^{35}S]-GTPγS was calculated as follows: $100 \times$ [(mean total sample cpm−mean basal sample cpm)/mean basal sample cpm]. Basal binding was defined as [^{35}S]-GTPγS binding in the absence of agonists. Because μ opioid receptor is a classical GPCR [Burford et al., 2000], we used CHO-μ cells as experimental control in this assay in order to make sure our experiment is correct.

PC-PLC Experiment

The PC-PLC experiment was prepared according to the method of Greney [Greney et al., 2000] with some modifications. CHO-IRAS cells were seeded at 1×10^6 cells/well in a 6-well plate in RPMI1640 containing 10% FBS. After 24 h of culture, cells were rinsed twice with serum-free RPMI1640 followed by the addition of the agonists. Cells were washed three times with serum- and drug-free RPMI1640, and were lysed by adding 1.0 ml of ice-cold buffer [3 mM 1,4-piperazinediethanesulfonic acid (PIPES), 0.6 mM EDTA, 0.03% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) (pH 7.4)]. Samples were frozen at −20°C followed by thawing at the room temperature. Lysed cells were scraped off the plate and the PC-PLC activity of cell lysates was measured according to the protocol of the Amplex Red PC-PLC kit (Molecular Probes, Interchim, France). The free choline generated by PC-PLD was also determined using the Amplex Red PC-PLC assay kit.

Measurement of DAG Accumulation

Extraction of DAG was carried out as described previously [Lee et al., 1991]. CHO-IRAS cells were detached from culture flasks by adding 2 mM EDTA in 0.01 M PBS buffer (pH 7.4) when they reached 90% confluence. Cells were collected by centrifugation at 1,500 rpm for 10 min, and washed with the buffer (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose, and 30 mM HEPES, pH 7.4). Cells were resuspended in the buffer and incubated with 1 ml I_1R agonists at various concentrations for indicated time. Reactions were terminated by adding 3 ml of chloroform plus methanol (1:2) to reaction tubes. The cells were sonicated for 20 s in an ultrasonic bath followed by the centrifugation at 1,500*g* for 10 min. The supernatant was transferred to another tube and the pellet was resuspended in 2 ml chloroform: methanol (1:1) followed by centrifugation at 1,500*g* for 10 min. The supernatant was combined to the previous supernatant, and 1 ml of chloroform and 1.4 ml of 0.9% NaCl solution were added, mixed well, and centrifuged (1,000*g*, 5 min). The upper layer was removed and the lower layer was separated again by adding 0.5 ml $CHC1_3$, mixing well and centrifuging (1,000*g*, 5 min) once again. Finally, the lower layer was dried under N_2 and the residue was dissolved in a small volume of chloroform.

DAG was purified and identified using HPTLC assay. 1,2-dioleoyl-sn-glycerol (R_F 0.67) was used as the standard. 1,2-dioleoyl-sn-glycerol (2 μg) was spotted on each plate and used to determine the material of identical R_F present in the cell extracts. HPTLC plates (full height) were initially pretreated with chloroform: methanol (1:1) to remove impurities, and activated by incubating at 110°C for 1 h. The lipids were separated using the method described by Yao and Rastetter [1985] with some modifications. The plate was first developed in solvent system I containing benzene:-diethyl ether:ethanol:acetic acid (65:40:1:0.5). The solvent front was allowed to migrate 5.5 cm above the origin (<10 min). The plate was then dried by directing hot air towards the glass side (~5 min) to remove acetic acid completely. The plate was cooled to the room temperature and developed in solvent system II (hexane: diethyl ether 94:6) to a 7 cm above the preadsorbent layer. The plate was thoroughly dried under hot air, and cooled to the room temperature. The charring reagent was a mixture of 100 g/L $CuSO_4$ and 80 g/L H_3PO_4. The density of the spots was analyzed by TLC scanner (CS-930) under UV light (365 nM).

Western Blotting

CHO-IRAS cells were grown on Φ 100 mm sterile dishes and treated with drugs (0.01 nM–10 μM) for indicated time. Afterward the cells were washed twice with cold 0.01 M PBS and lysed at 4°C with 200 μl/well of ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% (v/v) NP40, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin,

pepstatin, and aprotinin, pH 7.5) for 30 min. Cell lysates were centrifuged at 12,000*g* for 20 min at 4°C. Equal amount of proteins (20 μg) were subjected to 12% SDS–PAGE gels, transferred onto nitrocellulose membranes. The membranes were blocked with TBST/5% dried milk solution for 2 h at the room temperature before being incubated with rabbit anti-ERK (1:1,000 diluted) and mouse anti-phosphor-ERK (1:1,000 diluted) antibodies at 4°C overnight. Immunoreactive bands were visualized by incubating membranes with HRP-conjugated anti-rabbit (1:5,000 diluted) and anti-mouse secondary antibodies (1:2,000 diluted) for 1 h at the room temperature and detected by ECL reagents. Film images were quantified using a scanning densitometer. Results were expressed as a ratio between anti-phospho-ERK and anti-ERK blots.

Data Analysis

Data were presented as mean ± SEM. All experiments were performed at least three times, each on a different culture. Statistical analyses were performed by ANOVA followed by Student–Newman–Keuls test to analyze the variance. Densitometric quantification of the Western blotting signals was performed by the Beta 4.0.2 of Scion Image software.

RESULTS

Ligand Binding Studies in CHO-IRAS Cells Membrane

Saturation binding experiments were performed with [^{3}H]clonidine to determine the density (B_{max}) and affinity (K_d) of IRAS in CHO-IRAS cells (Fig. 1A). No [^{3}H]clonidine-specific binding was detected in parental CHO cells (data not shown). The B_{max} and K_d values in the membrane fraction of CHO-IRAS cells were 713.3 ± 102 fmol/mg protein and 13.84 ± 1.75 nM, respectively (n = 3). The affinity of the expressed IRAS in CHO-IRAS cells is similar to that of wild-type I_1R in the bovine adrenal membrane ($K_d = 16 \pm 3$ nM in the bovine adrenal membranes), but the level of IRAS expression ($B_{max} = 713.3 \pm 102$ fmol/mg protein) is higher than that of wild-type I_1R in the bovine adrenal membrane ($B_{max} = 44 \pm 8$ fmol/mg protein) [Moldering et al., 1993].

Competition binding experiments were performed using two I_1R agonists, moxonidine and clonidine, and α_2-AR agonist norepinephrine.

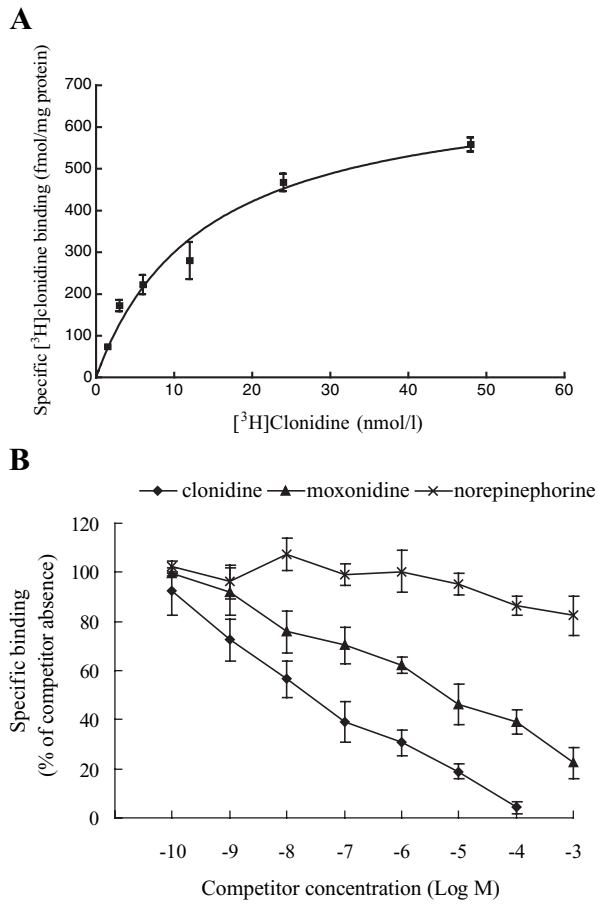

Fig. 1. [^{3}H]clonidine binding to IRAS. **A**: Saturation curve for specific [^{3}H]clonidine binding to IRAS. Specific binding of [^{3}H]clonidine was determined by subtraction of nonspecific binding in the presence of idazoxan (100 μM) from total binding. B, competition experiments were performed with 30 nM [^{3}H]clonidine for IRAS. The ligands used as competitors were moxonidine, clonidine, and norepinephrine. The values shown are at least four separate experiments.

The I_1R agonists, moxonidine and clonidine, were able to inhibit [^{3}H]clonidine binding in CHO-IRAS cells membrane preparation, while the α_2-AR agonist norepinephrine failed (Fig. 1B). This result is consistent with a previous report that parental CHO cells lack α_2-AR [Fraser et al., 1989].

[^{35}S]-GTPγS-Binding Assay

Initially, CHO-μ was used as a classical GPCR experimental control [Burford et al., 2000]. The μ opioid receptor agonist DAMGO (10 μM) increased [^{35}S]-GTPγS binding in CHO-μ cells membrane preparation to about 200% of the basal binding value (Fig. 2A). However, under the same assay condition with experimental control, moxonidine or rilmenidine

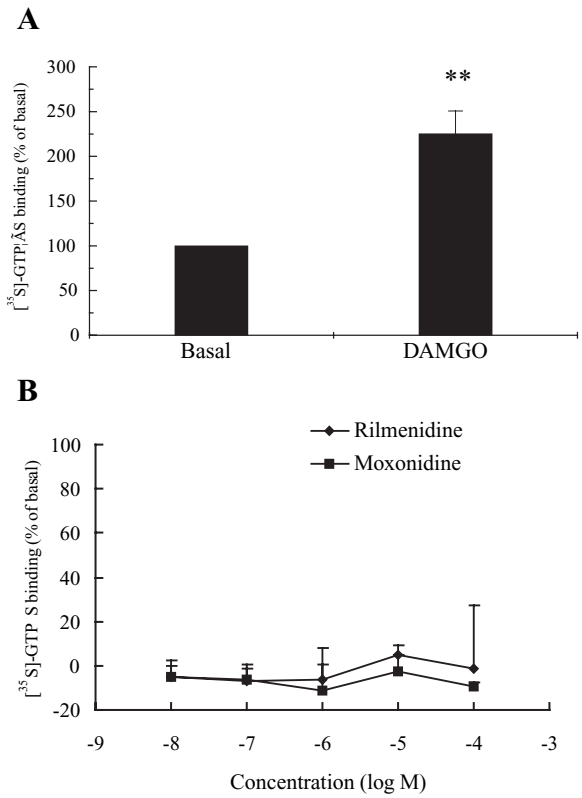

Fig. 2. [^{35}S]-GTPγS binding in CHO-μ cells and CHO-IRAS cells membrane preparation. **A**: The level of [^{35}S]-GTPγS binding in CHO-μ cells membrane preparation was determined in the presence of DAMGO (10 μM) in the assay buffer containing 50 mM HEPES, 5 mM $MgCl_2$, 1 mM EDTA, 15 μM GDP, 100 mM NaCl (add 0.2 nM [^{35}S]-GTPγS and 20 μg membrane protein in buffer, pH 7.4, 25°C) for 1 h, 40 μM GTPγS to define nonspecific binding. **B**: the level of [^{35}S]-GTPγS binding in CHO-IRAS cells membrane preparation was determined in the presence of moxonidine or rilmenidine (10 nM–100 μM) in the assay buffer containing 20 mM HEPES, 10 mM $MgCl_2$, 0.2 mM EGTA, 2 mM EDTA, 15 μM GDP (add 0.2 nM [^{35}S]-GTPγS and 20 μg membrane protein in buffer, pH 7.4), 21°C for 1.5 h, 40 μM GTPγS to define nonspecific binding. Rusults are expressed as the agonists stimulated binding increase over basal [^{35}S]-GTPγS binding. The values shown represent at least four separate experiments. Basal binding in the experiments shown was approximately 4,000 cpm. $^{**}P < 0.01$ versus basal binding value.

(10 nM–100 μM) failed to stimulate [^{35}S]-GTPγS binding in CHO-IRAS cell membrane preparation, suggesting IRAS might not be coupled with G-protein. Given some influence on [^{35}S]-GTPγS-binding assay [Harrison and Traynor, 2003] and I_1R-binding assay [Ernsberger et al., 1995b], such as the concentrations of Mg^{2+}, Na^+, and GDP, optimal conditions for measuring [^{35}S]-GTPγS binding in CHO-IRAS cells were determined. For the [^{35}S]-GTPγS-binding assay in CHO-IRAS cells, we found that the basal binding and stimulated binding decreased with increasing concentrations of GDP and NaCl, but basal binding altered almost as much as stimulated binding (data not shown). We selected the assay buffer which contained 20 mM HEPES, 10 mM $MgCl_2$, 0.2 mM EGTA, 2 mM EDTA, 15 μM GDP (pH 7.4), incubation at 21°C for 1.5 h as a relative suitable assay condition. The assay buffer has no NaCl which is known to markedly diminish the affinity of imidazolines for I_1R [Ernsberger et al., 1995b], but 100 mM NaCl is needed in the assay buffer of [^{35}S]-GTPγS binding in CHO-μ cells. Under this assay condition, moxonidine or rilmenidine could bind to CHO-IRAS, and the nonspecific binding for [^{35}S]-GTPγS binding was very low. Nevertheless, under this assay condition, moxonidine or rilmenidine (10 nM–100 μM) still failed to stimulate [^{35}S]-GTPγS binding in CHO-IRAS cell membrane preparation (Fig. 2B).

Effect of Rilmenidine or Moxonidine on PC-PLC Activation

The time course of PC-PLC activation after being treated with rilmenidine (1 μM) or moxonidine (1 μM) in CHO-IRAS cells was shown in Figure 3. The significant increase in PC-PLC activity stimulated by rilmenidine or moxonidine was peaked at 5 min and remained at the plateau level for 10 min, with a decline towards baseline after 30 min after administration of the drugs (Fig. 3A,B). Compared with the vehicle-treated control, the maximal increase percentages in PC-PLC activity stimulated by rilmenidine or moxonidine were about $17.4 \pm 6.2\%$ and $20.2 \pm 4.9\%$, respectively.

According to the above results, the period of preincubation of CHO-IRAS cells with rilmenidine or moxonidine (0.01 nM–10 μM) were determined at 5 min after administration of the drugs in concentration-response curves (Fig. 4). Rilmenidine or moxonidine led to concentration-dependent increase of PC-PLC activity. Rilmenidine significantly increased PC-PLC activity at the concentration from 100 nM to 10 μM (Fig. 4A), and reached the peak at 1 μM to $117.2 \pm 1.7\%$ compared with vehicle-treated control. Similar to rilmenidine, pretreatment of the CHO-IRAS cells with moxonidine also increased PC-PLC activation at the concentration from 10 nM to 10 μM (Fig. 4B), and reached the peak at 100 nM to $119.3 \pm 2.9\%$ compared with vehicle-treated control.

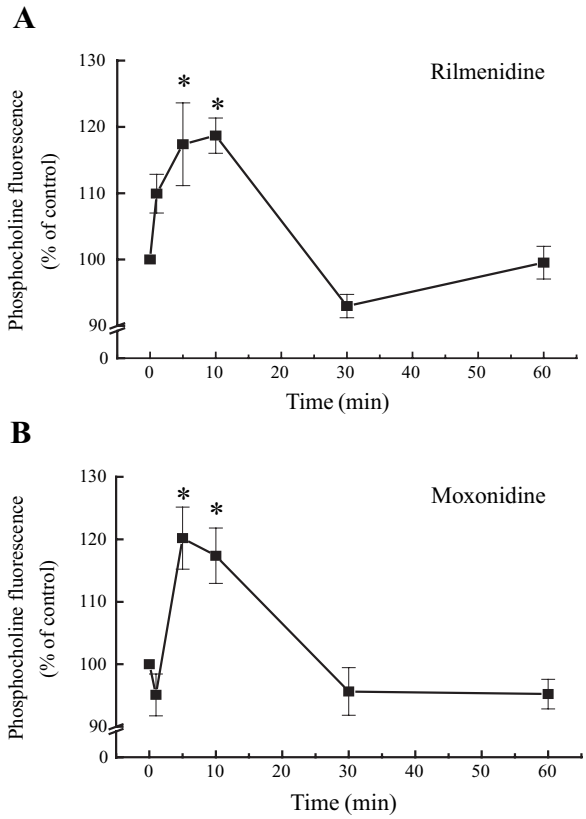

Fig. 3. Time course of PC-PLC activation in CHO-IRAS cells treated by rilmenidine or moxonidine. The cells (1×10^6 cells/well) were incubated with rilmenidine (1 μM) for indicated time. Cells were collected and lysed. The PC-PLC activity was determined by measuring the value of fluorescence (excitation at 535 nm and emission detection at 590 nm), which was red after 45 min development of the enzymatic reaction as described under Amplex Red phosphatidylcholine-specific PLC assay kit Experimental Procedures. The results represent at least six experiments, each performed with separate cell cultures, and shown as increase percentage of fluorescence compared with vehicle-treated control. *$P < 0.05$ versus vehicle-treated control (by Student–Newman–Keuls test). **A**: Rilmenidine-treated group. **B**: Moxonidine-treated group.

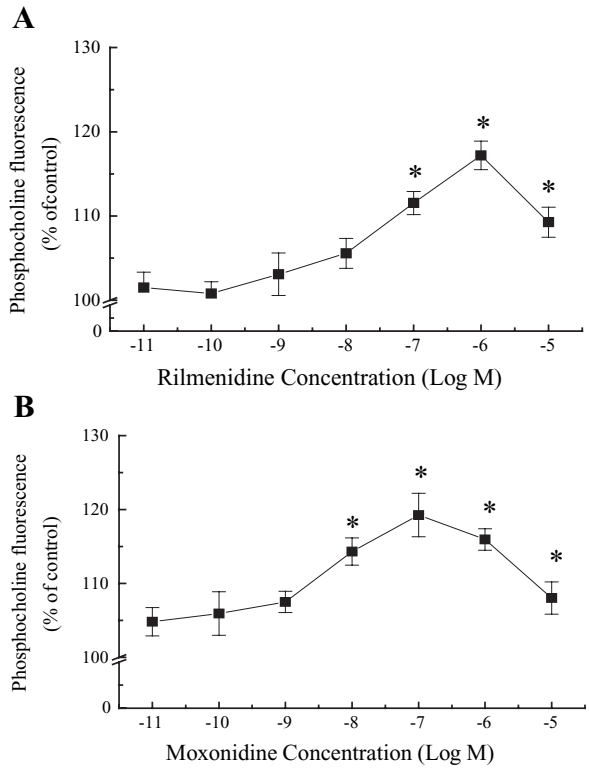

Fig. 4. Concentration-dependent curves of PC-PLC activation stimulated by rilmenidine or moxonidine in CHO-IRAS cells. The cells (1×10^6 cells/well) were incubated with different concentration of rilmenidine or moxonidine (0.01 nM–10 μM) for 5 min. Cells were collected and lysed. The PC-PLC activity was determined by measuring the value of fluorescence (excitation at 535 nm and emission detection at 590 nm), which was red after 45 min development of the enzymatic reaction as described under Amplex Red phosphatidylcholine-specific PLC assay kit Experimental Procedures. The results represent at least six experiments, each performed with separate cell cultures, and shown as increase percentage of fluorescence compared with vehicle-treated control. *$P < 0.05$ versus vehicle-treated control (by Student–Newman–Keuls test). **A**: Rilmenidine-treated group. **B**: Moxonidine-treated group.

In order to test whether the upregulation of rilmenidine or moxonidine on PC-PLC activity was mediated by IRAS, the cells were pretreated with efaroxan for 15 min, an I_1R antagonist. Efaroxan (10 μM) abolished the increase in PC-PLC activity induced by moxonidine (1 μM) or rilmenidine (1 μM), but had no significant effect on PC-PLC activity when given alone (Fig. 5A). Furthermore, no change in PC-PLC activity was detected in parental CHO cells treated by rilmenidine (1 μM) or moxonidine (1 μM) for 5 min (data not shown). These results indicated that IRAS mediates the PC-PLC activation. In addition to PC-PLC activation, the PLD activation could also cause positive results measured by this assay kit. To confirm that IRAS activates PC-PLC rather than PC-PLD, we added D609, a putative PC-PLC inhibitor, to the cells to see if it could block the lipase activity in response to moxonidine or rilmenidine (1 μM). Figure 5B indicated that pretreatment with D609 (1 μM) for 30 min completely inhibited the lipase activity, suggesting that the binding of moxonidine or rilmenidine to IRAS causes the activation of PC-PLC but not PC-PLD. These results are consistent with previous studies of native I_1R in PC12 cells [Separovic et al., 1996; Ernsberger, 1999].

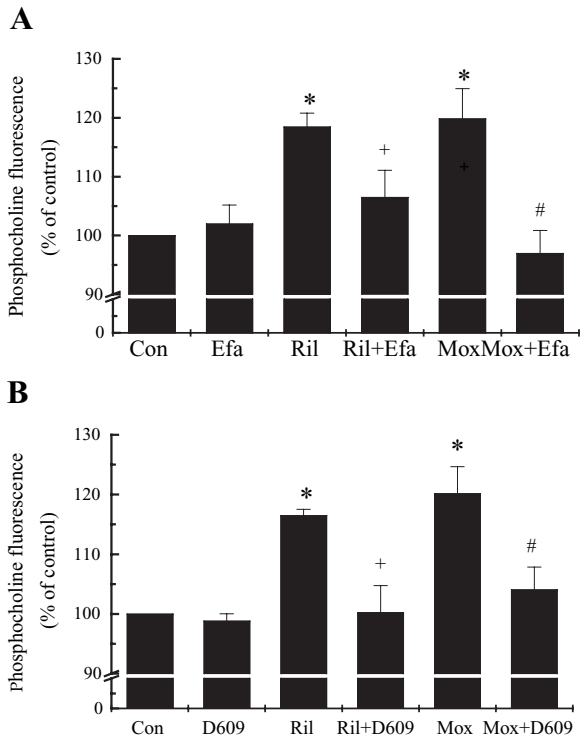

Fig. 5. Effect of efaroxan or D609 on PC-PLC activation stimulated by rilmenidine or moxonidine. **A**: CHO-IRAS cells were pretreated with efaroxan (10 μM) or vehicle alone for 15 min, followed by the treatment of rilmenidine or moxonidine (1 μM) for 5 min. **B**: CHO-IRAS cells were pretreated with D609 (10 μM) or vehicle alone for 30 min, followed by the treatment of rilmenidine or moxonidine (1 μM) for 5 min. The enzymatic reaction was performed as described under Amplex Red phosphatidylcholine-specific PLC assay kit Experimental Procedures. $^{*}P < 0.05$ versus vehicle-treated control, $^{+}P < 0.05$ versus rilmenidine-treated group, $^{\#}P < 0.05$ versus moxonidine-treated group (by Student–Newman–Keuls test).

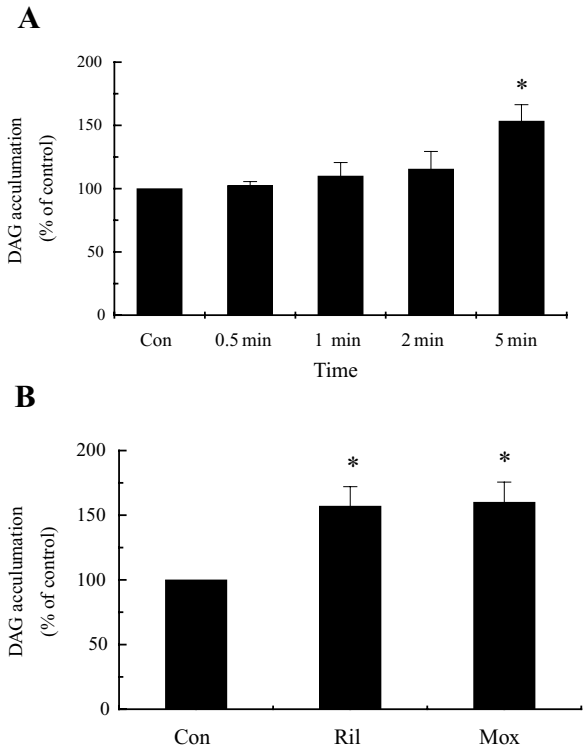

Fig. 6. The level of DAG accumulation stimulated by moxonidine or rilmenidine in CHO-IRAS cells. **A**: CHO-IRAS cells were incubated with rilmenidine (1 μM) for 30 s, 1, 2, and 5 min. **B**: CHO-IRAS cells were incubated with moxonidine or rilmenidine (1 μM) for 5 min. Then DAG was extracted and quantified. The density of the spots was analyzed by dual wavelength TLC scanner (CS-930) under UV light (365 nM). The results were determined from at least five experiments, each performed with separate cell cultures. $^{*}P < 0.05$ versus vehicle-treated control.

Effect of Rilmenidine or Moxonidine on DAG Accumulation

The DAG was isolated and analyzed by HPTLC. As DAG is the hydrolysate of PC, we assumed that DAG accumulation may be changed at 5 min. In order to obtain the optimal reaction time, the cells were treated with rilmenidine (1 μM) or moxonidine (1 μM) for 0.5, 1, 2, and 5 min, respectively. Pretreatment of CHO-IRAS with rilmenidine or moxonidine for 0.5–2 min had no significant influence on DAG accumulation, but when the incubation period was prolonged to 5 min, the DAG accumulation was significantly raised (Fig. 6A,B). Treatment with moxonidine (1 μM) or rilmenidine (1 μM) for 5 min in CHO-IRAS cells led to an obvious increase in DAG accumulation by $57.1 \pm 15.0\%$ and $60.1 \pm 15.6\%$, respectively.

Effect of Rilmenidine or Moxonidine on ERK Activation

Exposure of CHO-IRAS cells to rilmenidine or moxonidine (0.01 nM–10 μM) for 15 min resulted in a concentration-dependent elevation of phosphorylated ERK (Fig. 7). Rilmenidine significantly increased ERK phosphorylation at the concentration from 10 nM to 1 μM and reached the peak at 100 nM in CHO-IRAS cells. Compared with vehicle-treated control, ERK phosphorylation was increased by $74.5 \pm 18.6\%$ at 100 nM (Fig. 7A). Like rilmenidine, moxonidine significantly increased ERK phosphorylation at the concentration from 10 nM to 10 μM, and reached the peak at 100 nM by $66.8 \pm 11.1\%$ compared with vehicle-treated control (Fig. 7B). High

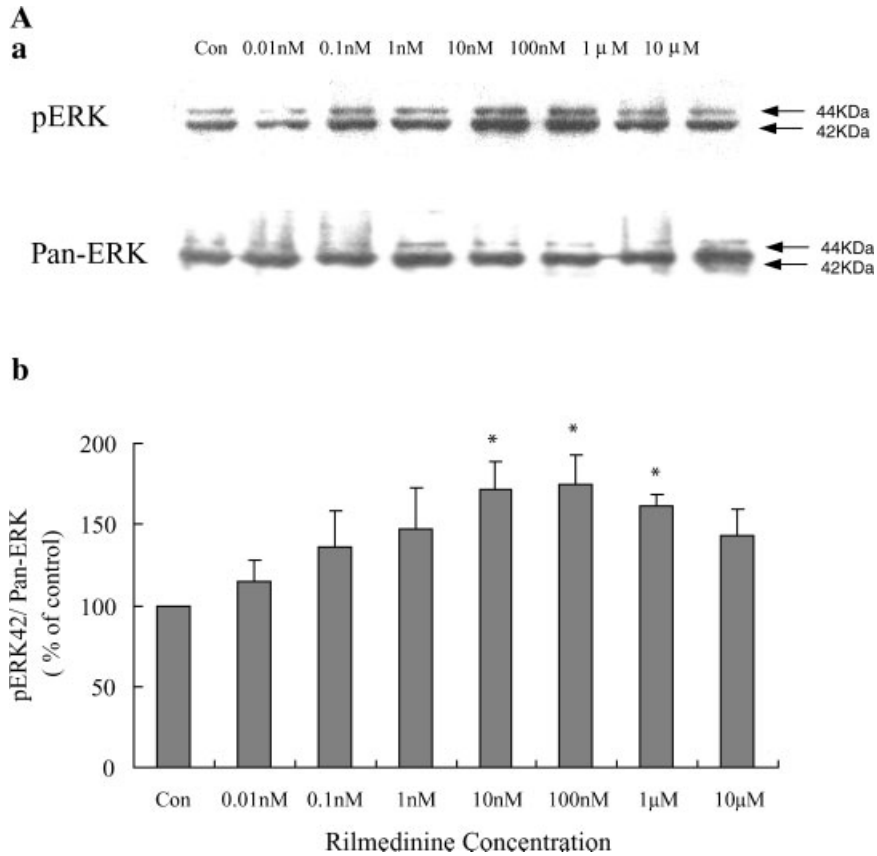

Fig. 7. Concentration-dependent curve of ERK phosphorylation stimulated by rilmenidine or moxonidine in CHO-IRAS cells. The cells were treated with rilmenidine or moxonidine (0.01 nM–10 μM) for 15 min. Then the cells were lysed, separated by SDS–PAGE and the protein levels of phosho-ERK and ERK were detected with anti-phospho-ERK antibody or anti-ERK antibody. Immunoreactive bands were visualized by incubation of membranes with an HRP-conjugated anti-mouse (1:2,000 dilution) and anti-rabbit secondary antibody (1:5,000 dilution) for 1 h at room temperature and detected by chemiluminescence. a: protein levels of pERK and total ERK. **b:** Data from a analyse of determining the radio of optical density between the pERK42 and Pan-ERK42 blot. The results represent at least five experiments, each performed with separate cell cultures. $^*P < 0.05$ versus vehicle-treated control (by Student–Newman–Keuls test). **A**: Rilmenidine-treated group. **B**: Moxonidine-treated group.

concentrations of rilmenidine or moxonidine (10 μM) may elicit an attenuated response, suggesting this concentration may be supramaximal. By the high concentration of rilmenidine or moxonidine, other signaling pathways may be induced which would interfere with IRAS signaling response or induce cytotoxicity. The similar biphasic concentration-response curves had been observed in DAG accumulation and ERK activation in native I_1R of PC12 cells [Separovic et al., 1996; Edward et al., 2001].

To further investigate whether the effect of rilmenidine or moxonidine on ERK stimulation was mediated by IRAS, we pretreated the cells with efaroxan, an I_1R inhibitor, for 15 min. Efaroxan (10 μM) abolished ERK phosphorylation stimulated by moxonidine (100 nM) or rilmenidine (100 nM), but had no significant effect on ERK phosphorylation when given alone (Fig. 8A). Furthermore, we also found that no change in phosphorylated ERK was detected in parental CHO cells treated with rilmenidine or moxonidine (0.01 nM–10 μM) for 15 min (data not shown), indicating that the phosporylation of ERK in response to rilmenidine or moxonidine was mediated by IRAS. The PC-PLC inhibitor, D609, was also used in this study to determine the relationship between PC-PLC and ERK activation. The cells were pretreated with D609 (10 μM) for 30 min and then incubated with moxonidine (100 nM) or rilmenidine (100 nM) for 15 min. Figure 8B showed that D609 blocked the increase of ERK phosphorylation induced by moxonidine or rilmenidine, but had no significant effect on ERK phosphorylation when given alone.

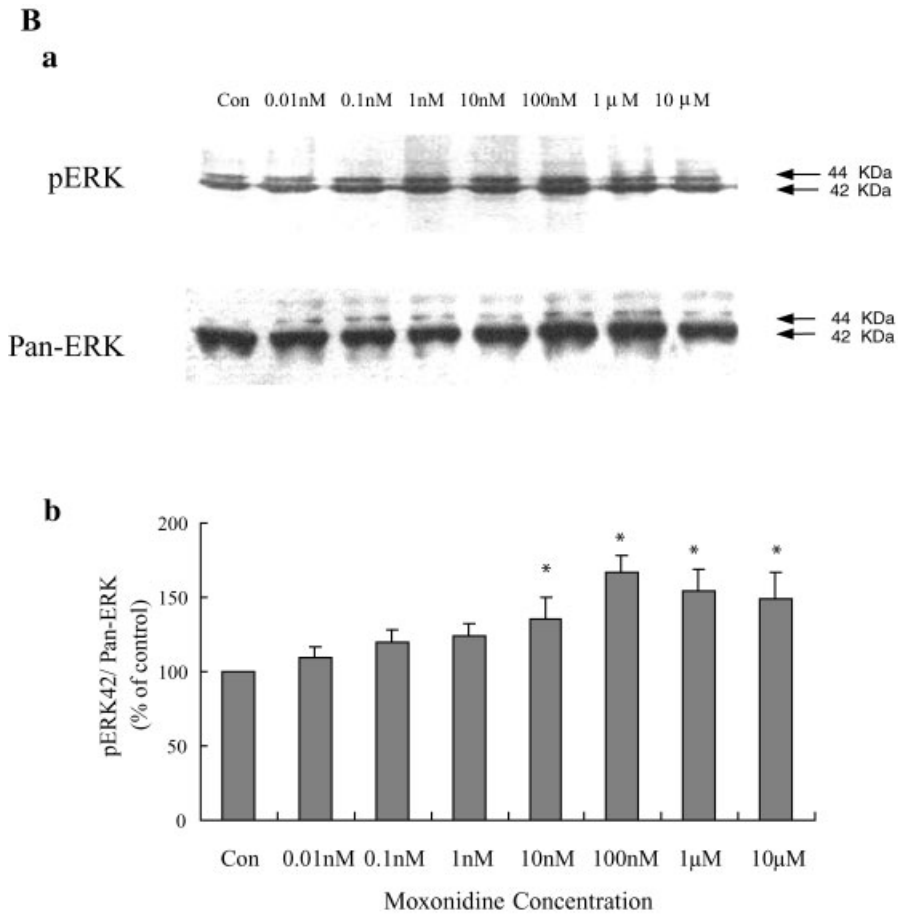

Fig. 7. (*Continued*)

DISCUSSION

Our study demonstrated that PC-PLC is involved in IRAS mediating ERK activation induced by the I_1R agonists, rilmenidine or moxonidine. These results provide direct evidence that the signaling pathways mediated by IRAS are similar to those mediated by I_1R.

In our study, we found that IRAS activation by rilmenidine or moxonidine significantly increased the PC-PLC activity at 5 min and lasted for at least 10 min. DAG, the hydrolysate of PC-PLC, could be detected after 5 min of rilmenidine or moxonidine treatment. It is generally accepted that DAG may be formed by two phases. The initial phase is transient ($\sim$1 min) and primarily derived from the hydrolysis of phosphatidylinositol phospholipase C (PI-PLC). The sustained phase is more prolonged, which is mediated by PC-PLC or PC-PLD [Billah and Anthes, 1990; Exton, 1990, 1994; Lee and Severson, 1994]. Either PC-PLC or PC-PLD can hydrolyze phosphatidylcholine, yielding DAG and phosphocholine, or phosphatidic acid and choline, respectively. Phosphatidic acid is subsequently converted into DAG by phosphatide phosphohydrolase [Billah et al., 1989; Murthy and Makhlouf, 1995]. In our study, DAG accumulation could not be observed within initial phase, which suggests PI-PLC may not contribute to DAG accumulation, but possibly PC-PLC or PC-PLCD mediates DAG accumulation. Our results indicate that PC-PLC, but not PC-PLD, is involved in IRAS signaling. First, the PC-PLC activity increased by rilmenidine or moxonidine could be reversed by D609, a PC-PLC inhibitor. Second, according to manufacturer's instructions of Amplex Red PC-PLC assay kit, phosphocholine produced by PC-PLC action on PC is hydrolyzed by alkaline phosphatase to generate choline. In the non-alkaline phosphated state, the enzyme-dependent fluorescent product would therefore result from PC-PLD. In order to determine whether PLD is involved, we used the assay kit in the absence of alkaline phosphatase, and found that

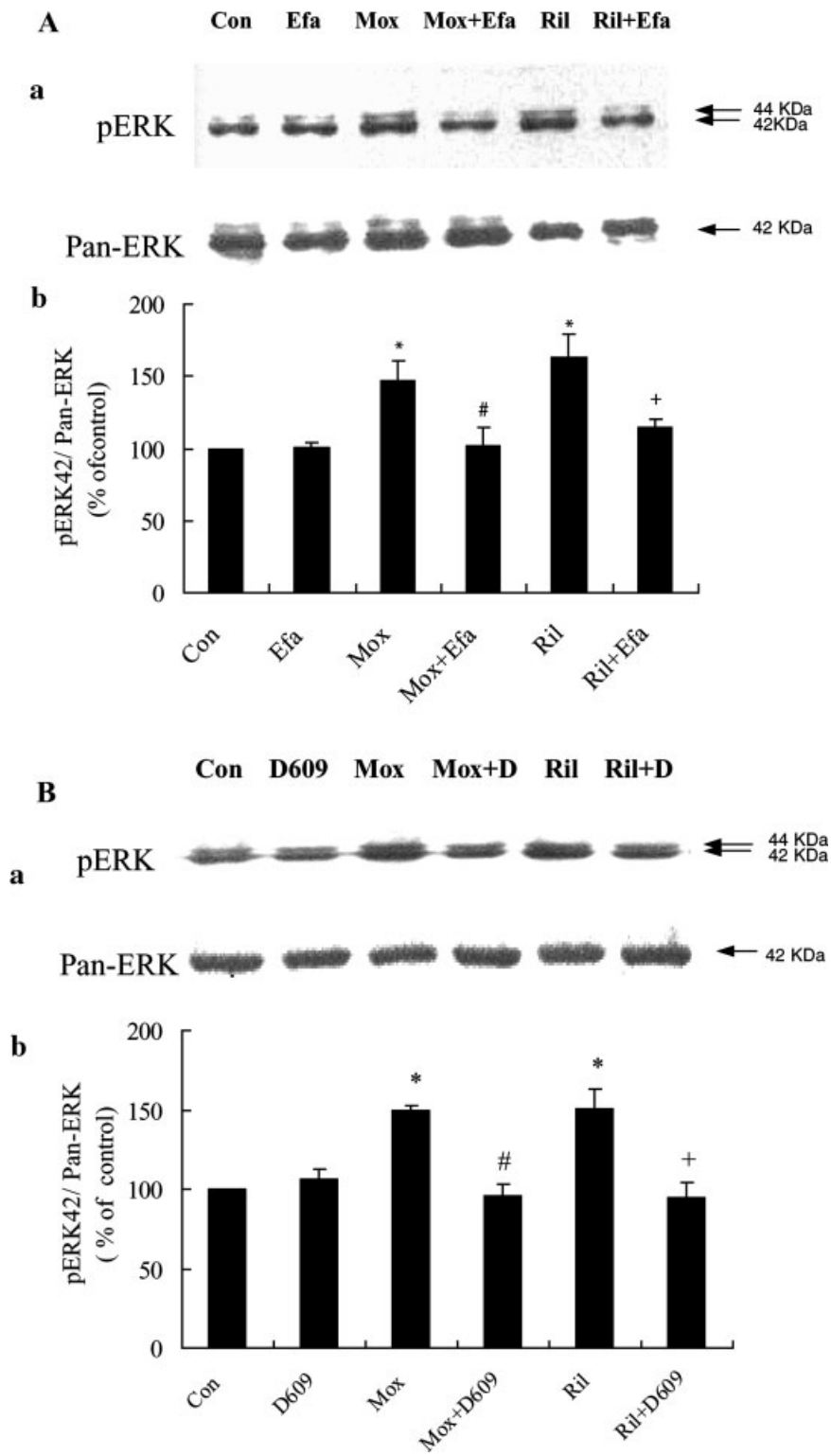

Fig. 8. Efaroxan or D609 inhibits rilmenidine or moxonidine induced ERK phosphorylation in CHO-IRAS cells. **A**: Cells were pretreated with efaroxan (10 μM) or vehicle alone for 15 min, and then the cells were treated with moxonidine (100 nM) or rilmenidine (100 nM) for 15 min. **B**: Cells were pretreated with D609 (10 μM) or vehicle alone for 30 min, followed by moxonidine (100 nM) or rilmenidine (100 nM) treatment for 15 min. **a:** The protein levels of pERK and total ERK **b:** Data from a analyse of determining the radio of optical density between the pERK42 and Pan-ERK42 blot. The results represent at least six experiments, each performed with separate cell cultures. $^{*}P < 0.05$ versus vehicle-treated control, $^{\#}P < 0.05$ versus moxonidine-treated group, $^{+}P < 0.05$ versus rilmenidine-treated group (by Student–Newman–Keuls test).

the final fluorescence sharply decreased to the value which is equal to the negative control (data not shown), and the fluorescence in agonists-treated groups had no significant difference compared with that in vehicle-treated control, suggesting that PLD was not stimulated in CHO-IRAS cells treated by rilmenidine or moxonidine. Third, PC-PLC has been reported to be involved in cell growth [Johansen et al., 1994] and death [Yonghong et al., 1998], which is consistent with known IRAS-mediated intracellular functions [Sano et al., 2002; Dontenwill et al., 2003a]. Our findings thus illuminate PC-PLC and its hydrolysate DAG involved in the signal pathway of IRAS.

However, what stimulates PC-PLC remains unknown. Our study demonstrated that the IRAS signaling pathway might not be coupled with G proteins, suggesting that PC-PLC may not be stimulated by G proteins. According to the structure of IRAS, which has integrin-binding motif plus a proline-rich region and a PX domain [Piletz et al., 2000], it is likely that IRAS may bind to a tyrosine kinase like Src and stimulate a signaling cascade leading to the activation of PC-PLC. However, further studies need to be done to identify this.

ERK typically controls cellular processes, such as proliferation, differentiation, development, stress response, and apoptosis [Gutkind, 1998; Aplin and Juliano, 1999; Davis, 2000]. Previous studies have shown that a modest level of IRAS by co-transfection into the HEK293 cells with insulin receptor substrate proteins (IRSs) led to a twofold rise in the activated state of ERK by the stimulation of insulin [Sano et al., 2002], and that NGF treatment resulted in a fivefold increase in phospho-ERK level in PC12 cells stably transfected with IRAS compared to that in the nontransfected cells [Piletz et al., 2003]. These results indicate that IRAS might work on ERK in the absence of I_1R agonists. In our study, we proved that ERK phosphorylation was activated in the presence of I_1R agonists. Rilmenidine or moxonidine induced a concentration-dependent increase in the phosphorylation of ERK, and this increase was reversed by efaroxan, the selective I_1R antagonist, suggesting the involvement of ERK in the signaling pathway of IRAS. In order to identify the relationship between PC-PLC and ERK activation, we pretreated CHO-IRAS cells with D609, the selective PC-PLC antagonist. Results showed that D609 attenuated the ERK phosphorylation induced by rilmenidine or moxonidine. The findings indicate that PC-PLC mediates the activation of ERK in response to IRAS activation.

Although the sequence analysis of the IRAS cDNA indicated that the product might not be a GPCR [Piletz et al., 2000], there is no direct functional evidence to show this. Because of the interference of other receptors, especially α_2-AR, whether native I_1R couples to G protein is controversial. In order to investigate if IRAS is coupled with G protein, we used [^{35}S]-GTPγS-binding assay in CHO-IRAS cells. In this cell line, the interference of α_2-AR is ruled out. The advantage of [^{35}S]-GTPγS-binding assay is that it measures a functional consequence of the receptor occupancy at one of the earliest events mediated by the receptor [Harrison and Traynor, 2003]. Moreover, the assay is the most direct method to test the relationship between a receptor and the G protein [Lazareno, 1997; Sovago et al., 2001]. Considering [^{35}S]-GTPγS-binding assay and I_1R-binding assay are influenced by many factors, including the concentration of Mg^{2+}, Na^{+}, GDP, temperature, and time of reaction [Ernsberger et al., 1995b; Lazareno, 1997; Harrison and Traynor, 2003], we optimized the assay condition based on the influencing factors. However, under any condition, moxonidine or rilmenidine failed to stimulate [^{35}S]-GTPγS binding in CHO-IRAS cell membrane preparation. Our data did not support IRAS coupling with G protein, which is consistent with the sequence analysis of the IRAS cDNA.

Compared to previous studies of I_1R in rat PC12 cells, we found that clonidine had slightly higher affinity than moxonidine for the transfected IRAS (Fig. 1B), which is the opposite rank order reported [Greney et al., 2000]. In addition to this, our findings that IRAS activation by rilmenidine increased the PC-PLC activity, DAG acculumation and ERK phosphorylation in CHO-IRAS cells are consistent with previous reported findings on I_1R activated by rilmenidine in NGF-induced differentiated PC12 cells [Zhang et al., 2001]. However, our results are different from Edward et al. [2001] and Separovic et al. [1997] findings in the time course of DAG accumulation and ERK activation. They reported that DAG accumulation was significantly increased in 15 s and ERK phosphorylation peaked at 90 min by moxonidine in NGF-induced differentiated PC12 cells. Additionally,

our result of PC-PLC activity elevation induced by moxonidine in CHO-IRAS cells (19%) was lower than Greney's result in PC12 cells (37%) [Greney et al., 2000]. We think that these differences maybe due to species differences or unknown factors.

In summary, we found that IRAS activation by imidazolines causes PC-PLC hydrolysis and DAG accumulation, apparently leading to ERK phosphorylation. These results are similar to those reported for native I_1R [Liedtke and Ernsberger, 1995; Separovic et al., 1996; Edward et al., 2001; Zhang et al., 2001], including some reports that I_1R do not couple to G proteins [Piletz and Sletten, 1993; Bricca et al., 1994]. Our findings therefore support the hypothesis that IRAS is an I_1R protein.

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