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Imidazoline I_1 receptor-induced activation of phosphatidylcholine-specific phospholipase C elicits mitogen-activated protein kinase phosphorylation in PC12 cells

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Abstract

In the present study, we tested the hypothesis that the activation of imidazoline I_1-receptor, which is coupled to phosphatidylcholine-specific phospholipase C, results in downstream activation of mitogen-activated protein kinase ($p42^{mapk}$ and $p44^{mapk}$ isoforms) in PC12 cells. PC12 cells pretreated with nerve growth factor (50 ng/ml, 48 h) to initiate neuronal differentiation were incubated with [methyl-^{3}H]choline and [^{3}H]myristate. Activation of imidazoline I_1 receptor by rilmenidine (10 μM) caused time-dependent increases in diacylglycerol accumulation and phosphocholine release. The Western blotting analysis showed that rilmenidine (10 μM) produced a time-dependent activation of $p42^{mapk}$ and $p44^{mapk}$ that reached its maximum at 15 min and returned to control levels after 30 min. This finding was confirmed by immunofluorescence labeling of activated mitogen-activated protein kinase in the same model system. Efaroxan (imidazoline I_1-receptor antagonist) or tricyclodecan-9-yl-xanthogenate (D609, phosphatidylcholine-specific phospholipase C inhibitor) attenuated the phosphorylation of $p42^{mapk}$ and $p44^{mapk}$ induced by rilmenidine. Nerve growth factor-induced phosphorylation of both mitogen-activated protein kinase isoforms was not affected by D609. These results support the hypothesis that the activation of the imidazoline I_1 receptor coupled phosphatidylcholine-specific phospholipase C results in the downstream activation of mitogen-activated protein kinase.

Keywords: MAP kinase; Phosphatidylcholine-specific phospholipase C; Imidazoline I_1 receptor; PC12 Cell; Diacylglycerol; Rilmenidine

1. Introduction

Reported findings have characterized high affinity binding sites for imidazolines that are distinct from α_2-adrenergic binding sites (Ernsberger et al., 1987). Two main subtypes of imidazoline receptors have been identified, I_1-imidazoline binding sites labeled by [^{3}H]clonidine or [^{125}I]p-iodoclonidine and I_2-imidazoline binding sites labeled by [^{3}H]idazoxan (Michael and Ernsberger, 1992). The two imidazoline receptor subtypes can also be distinguished by cellular and tissue localization. The I_2-sites are present mainly in the mitochondria (Tesson et al., 1991, 1995) whereas the I_1-sites are selectively localized to cell fractions enriched in plasma membranes from human platelets (Piletz and Sletten, 1993), the rostral ventrolateral medulla (Ernsberger and Shen, 1997), and PC12 pheochromocytoma cells (Ernsberger et al., 1995). Functionally, I_1-sites have been implicated in mediating a number of responses including the blood pressure lowering effect of centrally acting drugs such as clonidine, rilmenidine and moxonidine (Chan and Head, 1996; Chan et al., 1996).

Little information is available, however, concerning the signal transduction pathway(s) triggered by the activation of the imidazoline I_1 receptor. Preliminary reports have suggested that the imidazoline I_1 receptor may be coupled to phospholipid metabolism. Because its activation increases the accumulation of diacylglycerol in tracheal epithelial cells (Liedtke and Ernsberger, 1995) and prostaglandin E2 in PC12 cells (Ernsberger et al., 1995). Moreover, Separovic et al. (1996, 1997) have shown that moxonidine, a selective imidazoline I_1 receptor agonist, increases the formation of cellular diacylglycerol in PC12 cells. This effect of moxonidine is attenuated by efaroxan, a selective imidazoline I_1 receptor antagonist, or D609, an

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inhibitor of phosphatidylcholine-specific phospholipase C (Separovic et al., 1996). These findings suggest that diacylglycerol acts as a second messenger to mediate the pharmacological responses to imidazoline I_1 receptor activation (Separovic et al., 1996). This view is further supported by recent functional studies, which showed that microinjection of D609 into the rostral ventrolateral medulla attenuated the hypotensive response to moxonidine in spontaneously hypertensive rats (Separovic et al., 1997).

The primary goal of the present study, therefore, was to further characterize the transmembrane signaling cascade triggered by the imidazoline I_1 receptor activation. The hypothesis was tested that activation of imidazoline I_1 receptor coupled phosphatidylcholine-specific phospholipase C pathway results in mitogen-activated protein kinase activation in PC12 cells. These cells lack α_2-adrenoceptors (Ernsberger et al., 1995; Separovic et al., 1996), exhibit plasma membrane imidazoline I_1 binding sites (Ernsberger et al., 1995) and show increased imidazoline I_1 receptor density when differentiated to a neuronal phenotype by nerve growth factor (Ernsberger, 1992; Tesson et al., 1995). To achieve this goal, we employed immunofluorescence and Western blotting techniques to detect changes in mitogen-activated protein kinase ($p42^{mapk}$ and $p44^{mapk}$ isoforms) phosphorylation subsequent to imidazoline I_1 receptor activation in nerve growth factor-pretreated PC12 cells. Further, the dependence of mitogen-activated protein kinase phosphorylation on the imidazoline I_1 receptor-phospholipase C pathway was evaluated. This was achieved by investigating the effect of (i) efaroxan, a selective imidazoline I_1 receptor antagonist, and (ii) D609, an inhibitor of phospholipase C, on the imidazoline I_1 receptor modulation of mitogen-activated protein kinase activity. However, to support our hypothesis, we felt it was important to demonstrate in our model system the ability of rilmenidine to increase diacylglycerol accumulation, which has been reported when another imidazoline I_1 receptor agonist, moxonidine, was used (Separovic et al., 1996).

2. Materials and methods

2.1. Materials

Rat PC12 pheochromocytoma cells (ATCC, Rockville, MD); Dulbecco's Modified Eagle's medium (Atlanta Biologicals, Norcross, GA); fetal bovine serum (Atlanta Biological, Norcross, GA); [^{3}H]chloride (85 Ci/mmol; American Radiolabeled Chemical, St Louis, MO); [^{3}H]myristic acid (49 Ci/mmol; DuPont NEN, Boston, MA); nerve growth factor (2.5S), poly-L-lysine, choline chloride, acetylcholine chloride, phosphocholine chloride, butylated Hydroxytoluene (Sigma, St Louis, MO), 1,2-Dimyristoyl-sn-glycerol (Avanti-Polar-Lipids, Alabaster, AL), efaroxan hydrochloride, D609 potassium (Research Biochemical Int., Natick, MA), anti-active™-mitogen-activated protein kinase pAb (Rabbit, pTEpY, Promega, Madison, WI), anti-Rabbit immunoglobulin G (IgG), peroxidase-linked species-specific F(ab′) 2 fragment (from donkey, Amersham Life Science, Buckinghamshire, England), GEL/MOUNT™ (Biomeda, Foster City, CA), ECL Western blotting detection reagents (Amersham Pharmacia Biotech., Piscataway, NJ), thin layer chromatography plates (TLC, LK silica Gel, Whatman, Clifton, NJ), pure nitrocellulose membrane (Trans-bolto transfer medium, Bio-Rad Laboratories, Hercules, CA). Rilmenidine dihydrogen phosphate was a gift from Servier Pharmaceutical, France.

2.2. Cell culture

Rat PC12 cells were cultured, at 37°C with saturated air containing 5% $C0_2$, in Dulbecco's Modified Eagle's medium supplemented with fetal bovine serum (10%), horse serum (5%), penicillin (100 U/ml), and streptomycin (100 μg/ml). Culture dishes (100 mm diameter) were coated with poly-L-lysine (0.1 mg/ml) to promote the cell attachment. Culture media were changed every 2 days.

2.3. Determination of lipid metabolites

To determine the lipid metabolites (diacylglycerol, choline, acetylcholine, and phosphocholine) of phosphatidylcholine, PC12 cells were subcultured in Dulbecco's modified Eagle's medium with 1% fetal bovine serum and 50 ng/ml nerve growth factor for 48 h and radiolabeled with 1 μCi/ml [^{3}H]choline chloride (85 Ci/mmol) for 24 h and 1 μCi/ml [^{3}H]myristic acid (49 Ci/mmol) for 3 h as described (Separovic et al., 1996, 1997; van Dijk et al., 1997a). Cells were washed twice with serum free Dulbecco's modified Eagle's medium and incubated in fresh serum free medium with 10 ng/ml nerve growth factor for 1 h at 37°C. The cells were then incubated with or without the test drugs. Ice-cold methanol (2 ml) was used to stop the reaction and the cells and the supernatant were collected for extraction and determination of diacylglycerol and choline-labeled compounds, respectively (Separovic et al., 1996, 1997). The cells were scraped off with rubber policeman and then chloroform and water were added to give a final ratio of 2/1/0.8 (methanol/chloroform/water). The media were centrifuged at $2000 \times g$ for 10 min and the cell pellet was dissolved in 0.5 ml 0.5 N NaOH for protein determination by the Bio-Rad protein assay.

2.3.1. Purification and identification of water-soluble radioactive compounds

The upper methanolic water layer was evaporated to dryness under reduced pressure at 40°C and dissolved in 100 μl of 50% ethanol containing 40 mM of choline,

acetylcholine and phosphocholine. Aliquots (10 μl) were spotted on TLC plates that were activated by pre-heating at 100°C for 1 h. The mobile phase consisted of 0.9% NaCl/methanol/ammonium hydroxide (10/10/1, v/v/v) and the bands were visualized with iodine vapor. Phosphocholine was distinguished as a separate band (R_f = 0.63) whereas the bands for choline and acetylcholine were overlapped (R_f = 0.27) and, therefore, were combined for the analysis as described by others (Separovic et al., 1997). The bands were scraped into scintillation vials and counted.

2.3.2. Purification and identification of diacylglycerol

The chloroform layer was evaporated with nitrogen gas. The dry lipids were reconstituted in 100 μl of chloroform/methanol (9/1, v/v) containing 2 mM 1,2-dimyristoyl-*sn*-glycerol and 0.1% butylated hydroxytoluene as antioxidant. Aliquots 10 μl were spotted on TLC plates and developed in a mobile phase consisting of 40/60° petroleum ether/diethyl ether/formic acid (80/20/1, v/v/v) and visualized with iodine vapor. The bands of diacylglycerol (R_f = 0.22) were scraped off and counted.

2.4. Immunofluorescence labeling

As described (Seidman, 1998), PC12 cells were cultured on the Lab-Tek® Chamber Slide coated with poly-L-lysine, washed with cold phosphate-buffered saline (NaCl 137, KCl 2.7, $Na_2HP0_4 \cdot 7H_20$ 4.3, and KH_2P0_4 1.4 mM; pH 7.3) and fixed with 4% paraformaldehyde for 30 min at room temperature. The cell membranes were permeabilized with 0.3% Triton X-100 and non-specific binding was blocked by 1% bovine serum albumin. The cells were incubated for 2.5 h at room temperature with the Anti-active™-mitogen-activated protein kinase antibody (diluted 1–500 with blocking solution) to label the phosphorylated $p42^{mapk}$ and $p44^{mapk}$. The cells were then incubated with antirabbit Ig, fluorescein linked (1:50) for 1 h, mounted with GEL/MOUNT™, and the fluorescence was detected by a Nikon Diaphot 300 microscope (Nikon, Tokyo, Japan) equipped with a fluorescence detection image system (Ionoptix, Boston, MA). The fluorescence intensity of the individual cells (approximately 20–30 cells) was measured according to an arbitrary scale set by the fluorescence detection system and the background intensity was subtracted to obtain the phosphorylated mitogen-activated protein kinase signal. The average fluorescence intensity was computed and used as a measure of mitogen-activated protein kinase phosphorylation.

2.5. Western blotting

The method described in previous studies including our own (Hashimoto et al., 1994; Ran et al., 1996) was employed for the detection of mitogen-activated protein kinase phosphorylation. Freshly subcultured PC12 cells were treated with 50 ng/ml nerve growth factor (2.5S) in Dulbecco's modified Eagle's medium with 1% fetal bovine serum for 48 h. The cells were washed with serum free medium, preincubated with serum free medium containing 10 ng/ml nerve growth factor for 1 h and then processed with or without drug treatment. The cells were washed with ice-cold phosphate-buffered saline and harvested and the total lysates were prepared for the Western blotting procedure. The proteins were separated by 12% sodium dedocyl sulfate-polyacrylamiide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked for 30 min with 5% skim milk, incubated with anti-Active™-mitogen-activated protein kinase polyclonal antibody, and subsequently incubated with Anti-rabbit IgG-horseradish peroxidase as a second antibody and Western blotting detection reagents.

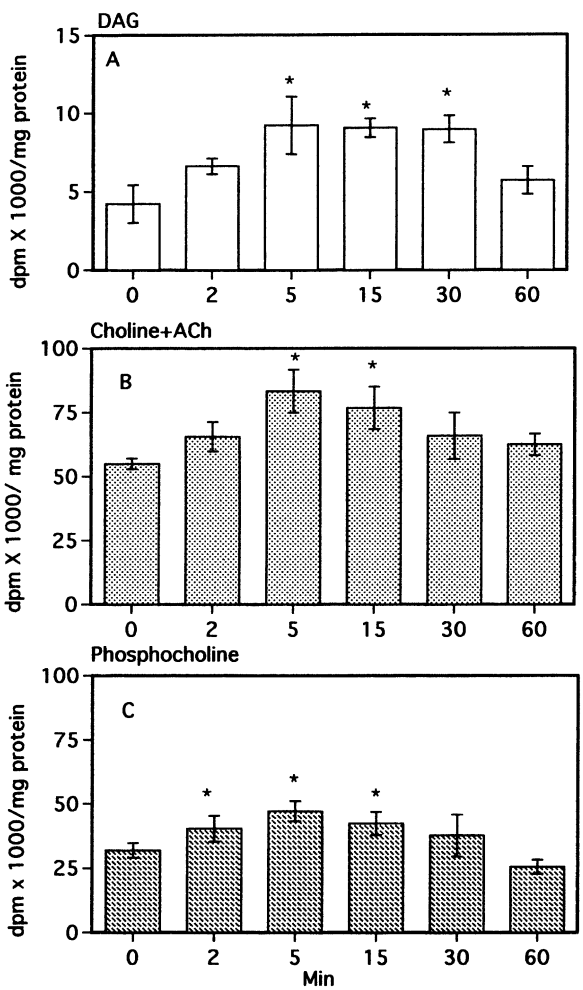

Fig. 1. The time-course of rilmenidine (10 μM) evoked accumulation of diacylglycerol (panel A), choline/acetylcholine (panel B), and phosphocholine (panel C) in nerve growth factor-differentiated PC12 cells. Values are mean ± S.E.M. of six experiments. * $P < 0.05$ vs. respective rilmenidine-untreated (zero time) values run in parallel.

2.6. Experimental protocols

2.6.1. Effect of rilmenidine on diacylglycerol accumulation

This experiment investigated the effect of imidazoline I_1 receptor activation by rilmenidine on the accumulation of diacylglycerol and other water-soluble products of the phosphatidylcholine-specific phospholipase C pathway. PC12 cells, pretreated with nerve growth factor (50 ng/ml, 48 h) and incubated with [^{3}H]myristic acid and [^{3}H]choline chloride, were incubated in serum free Dulbecco's modified Eagle's medium containing nerve growth factor (10 ng/ml) in the absence and presence (5, 15, and 30 min) of rilmenidine (10 μm). The cellular and supernatant accumulation of [^{3}H]lipid metabolites (diacylglycerol, choline, acetylcholine, and phosphocholine) were determined and expressed as dpm/mg protein.

2.6.2. Coupling of imidazoline I_1 receptors to mitogen-activated protein kinase

In this experiment, the hypothesis was tested that the activation of the imidazoline I_1 receptor coupled phosphatidylcholine-specific phospholipase C results in downstream activation of mitogen-activated protein kinase. The phosphorylation of mitogen-activated protein kinase was detected by two techniques, immunofluorescence labeling and Western blotting. The time (5–30 min)-and concentration (1 and 10 μM)-dependent effects of rilmenidine on the phosphorylation of p42^{mapk} and P44^{mapk} were evaluated. The specificity of imidazoline I_1 receptor coupled phospholipase C modulation of mitogen-activated protein kinase expression was investigated by testing the effect of efaroxan (0.1 mM), a selective imidazoline I_1 receptor antagonist, and D609 (5 μM), a phospholipase C inhibitor, on rilmenidine-evoked activation of mitogen-activated protein kinase. As a control, the effect of D609 on nerve growth factor-induced mitogen-activated protein kinase phosphorylation was also investigated to further confirm the involvement of the phospholipase C in the intracellular signaling initiated by imidazoline I_1 receptor activation.

2.7. Statistical analysis

Values are presented as mean ±S.E.M. Analysis of variance (ANOVA) followed by a Newman–Keuls post-hoc analysis was used to analyze the effects of rilmenidine, on the accumulation of phospholipid metabolites and mitogen-activated protein kinase phosphorylation. Densitometric quantification of the Western blotting signals was performed using the NIH Image software (Version 1.60) and a Power Macintosh (7300/180) as described in our previous studies (El-Mas and Abdel-Rahman, 1997, 2000). Probability levels less than 0.05 were considered significant.

3. Results

3.1. Effect of rilmenidine on the accumulation of phospholipid metabolites

The role of phosphatidylcholine-specific phospholipase C in the imidazoline I_1 receptor signaling pathway was evaluated by measuring the intracellular accumulation of diacylglycerol in PC12 cells and the release of phosphocholine, the aqueous reaction product of phosphatidylcholine-specific phospholipase C enzymatic activity, into the extracellular medium. The effects of rilmenidine on the levels of diacylglycerol, choline/acetylcholine, and phos-

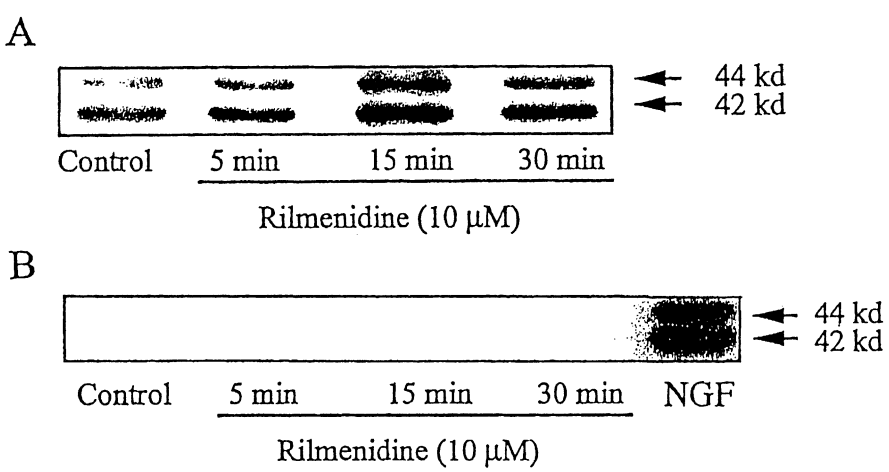

Fig. 2. Western blots showing the time-course of rilmenidine evoked p42^{mapk} and p44^{mapk} phosphorylation in nerve growth factor (NGF)-differentiated PC12 cells as identified by the anti-active™ mitogen-activated protein kinase antibody (panel A). PC12 cells were treated with NGF (50 ng/ml) for 48 h to initiate neuronal differentiation, incubated in fresh Dulbecco's modified Eagle's medium containing NGF (10 ng/ml), and mitogen-activated protein kinase activity was evaluated in the absence and presence of rilmenidine. The phosphorylation of mitogen-activated protein kinase was enhanced by rilmenidine (10 μM). No signal for mitogen-activated protein kinase was detected in the control or rilmenidine-treated undifferentiated PC12 cells (panel B). As a positive control, treatment of the undifferentiated PC12 cells with NGF (50 ng/ml) for 10 min elicited an abundant p42^{mapk} and p44^{mapk} phosphorylation (panel B).

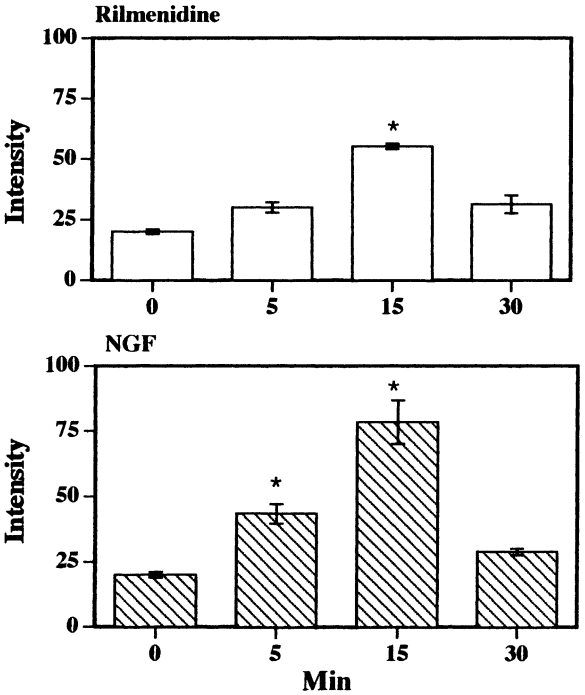

Fig. 3. The time-course of rilmenidine (10 μM, upper panel) and nerve growth factor (NGF, 50 ng/ml, lower panel) evoked mitogen-activated protein kinase phosphorylation in NGF-differentiated PC12 cells as identified by the immunofluorescence labeling technique. The fluorescence intensity was measured by a fluorescence image system. Values are mean ± S.E.M. of five to six experiments. $^{*}P < 0.05$ vs. respective rilmenidine (or NGF)-untreated (zero time) values run in parallel.

phocholine in PC12 cells, pretreated with nerve growth factor and incubated with [^{3}H]myristic acid and [^{3}H]choline chloride, are shown in Fig. 1. Activation of imidazoline I_1 receptors by rilmenidine (10 μM) produced time-dependent increases ($P < 0.05$) in cellular [^{3}H]diacylglycerol content compared with control (rilmenidine-untreated, zero time) values (Fig. 1A). The increase in diacylglycerol by rilmenidine was demonstrated at 2 min, reached its maximum at 5 min and remained at that level for 30 min. The levels of diacylglycerol in the absence and presence (5 min) of rilmenidine amounted to 4220 ± 1200 and 9220 ± 1840 dpm/mg protein, respectively. At 60 min, the diacylglycerol level was not significantly different from the control value run in parallel (Fig. 1A). Similarly, rilmenidine caused significant ($P < 0.05$) increases in [^{3}H]choline/acetylcholine (Fig. 1B) and [^{3}H]phosphocholine (Fig. 1C) released into the medium that peaked at 5 min and gradually declined thereafter. The intracellular levels of choline and phosphocholine were not affected by rilmenidine (data not shown).

3.2. Effect of rilmenidine on mitogen-activated protein kinase phosphorylation

The effects of imidazoline I_1 receptor activation by rilmenidine on p42^{mapk} and p44^{mapk} phosphorylation in PC12 cells preincubated with nerve growth factor (50 ng/ml) for 48 h are shown in Fig. 2. Rilmenidine (10 μM) produced time-related increases in the phosphorylation of mitogen-activated protein kinase in nerve growth factor-differentiated PC12 cells (Fig. 2A). The maximal increase in mitogen-activated protein kinase phosphorylation by rilmenidine was demonstrated at 15 min (Fig. 2A). Densitometric analysis showed that rilmenidine produced similar and concentration-dependent increases in the two isoforms of the phosphorylated mitogen-activated protein kinase. Compared with the 15-min control (rilmenidine-untreated) values, the percentage increase in p42^{mapk} and p44^{mapk} phosphorylation amounted to 94.8 ± 16.4% and 110.5 ± 14.5%, respectively, in case of 1 μm rilmenidine, and 253.2 ± 51.9% and 291.6 ± 28.4%, respectively, in case of 10 μM rilmenidine ($n = 4–6$ observations). No signals for the phosphorylated mitogen-activated protein kinase were detected in the control or rilmenidine-treated undifferentiated PC12 cells, i.e. cells that were not incubated with nerve growth factor (Fig. 2B).

Immunofluorescence image analysis also showed that rilmenidine (10 μM) produced time-related increases ($P < 0.05$) in mitogen-activated protein kinase phosphoryla-

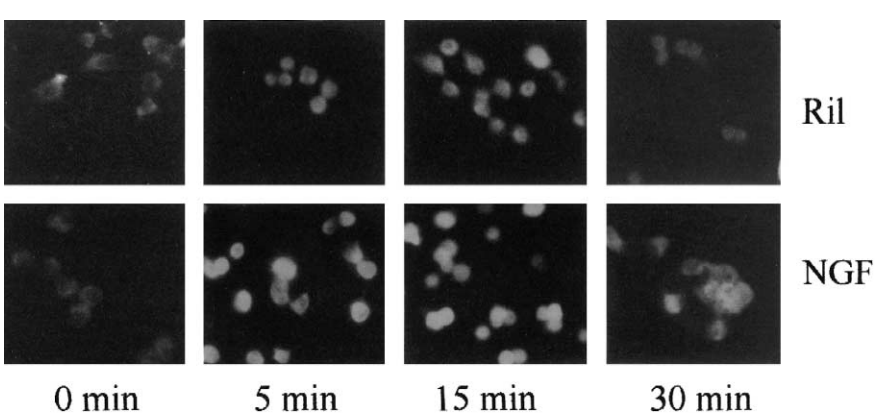

Fig. 4. Immunolabeling fluorescence images (magnification, 200×) showing the time-related increases in mitogen-activated protein kinase phosphorylation evoked by rilmenidine (10 μM) and nerve growth factor (NGF, 50 ng/ml) in NGF-differentiated PC12 cells.

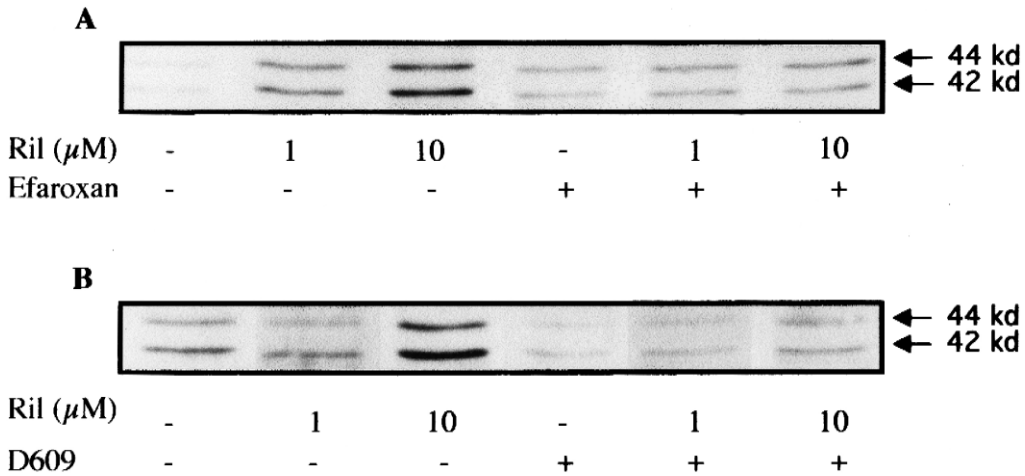

Fig. 5. Western blots showing the effect of pretreatment with efaroxan 0.1 mM (imidazoline I_1 receptor blocker, panel A) or D609 5 μM (phospholipase C inhibitor, panel B) on rilmenidine (Ril; 1 and 10 μM) evoked mitogen-activated protein kinase phosphorylation in nerve growth factor-differentiated PC12 cells. Pretreatment with efaroxan or D609 reduced the mitogen-activated protein kinase phosphorylation evoked by rilmenidine.

tion as indicated by the increased fluorescence intensity, reaching maximum at 15 min and then declined at 30 min (Fig. 3, top panel). Similar pattern of mitogen-activated protein kinase phosphorylation was obtained by nerve growth factor (50 ng/ml; Fig. 3, bottom panel). Fig. 4 shows representative fluorescence images from PC12 cells, stained with fluorescein, acquired before and 5, 15 and 30 min after treatment with rilmenidine (10 μM) or nerve growth factor (50 ng/ml). Treatment with either rilmenidine or nerve growth factor caused positive staining in more than 95% of cells.

3.3. Effect of efaroxan or D609 on rilmenidine-induced mitogen-activated protein kinase phosphorylation

The effects of pretreatment of PC12 cells with 0.1 mM efaroxan (5 min), a selective imidazoline I_1 receptor antagonist, or 5 μM D609 (1 h), a phospholipase C inhibitor, on mitogen-activated protein kinase phosphorylation induced by rilmenidine are shown in Fig. 5. As mentioned earlier, rilmenidine (1 and 10 μM) produced concentration-dependent increases in $p42^{mapk}$ and $p44^{mapk}$ phosphorylation (Fig. 5A and B). The phosphorylation of both mitogen-activated protein kinase isoforms was markedly reduced in cells pretreated with efaroxan (Fig. 5A) or D609 (Fig. 5B). Efaroxan caused $48.2 \pm 2.5\%$ and $64.4 \pm 12.5\%$ reductions in the rilmenidine (10 μM)-induced $p42^{mapk}$ and $p44^{mapk}$ phosphorylation, respectively. Similarly, pretreatment with D609 reduced the rilmenidine (10 μM)-induced $p42^{mapk}$ and $p44^{mapk}$ phosphorylation by $62.5 \pm 6.3\%$ and $49.8 \pm 7.2\%$, respectively. Nerve growth factor produced concentration (2.5–50 ng/ml)-related increases in the phosphorylation of both mitogen-activated protein kinase isoforms ($p42^{mapk}$ and $p44^{mapk}$) that were not affected by D609 pretreatment (Fig. 6).

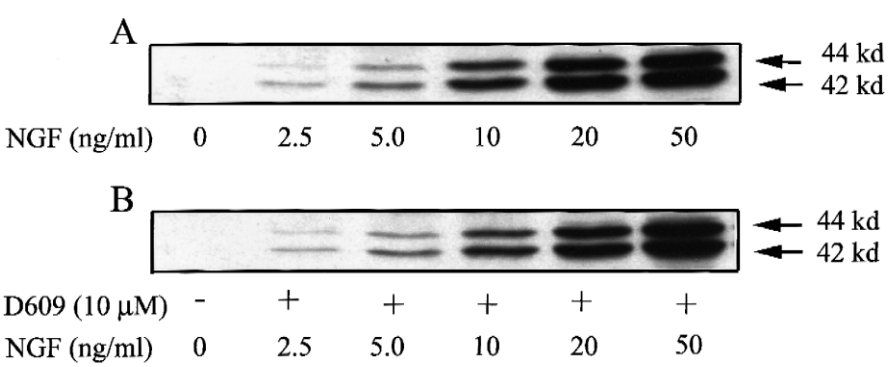

Fig. 6. Western blots showing the concentration-dependent effects of nerve growth factor (NGF, 2.5–50 ng/ml) on $p42^{mapk}$ and $p44^{mapk}$ phosphorylation in the absence (panel A) and presence (panel B) of D609 (phospholipase C inhibitor, 5 μM) in NGF-differentiated PC12 cells. The NGF-induced phosphorylation of either mitogen-activated protein kinase isoform was not affected by D609 pretreatment.

4. Discussion

The present study is the first to report on the role of mitogen-activated protein kinase in the signal transduction mechanism coupled to imidazoline I_1 receptors. The hypothesis was tested that activation of imidazoline I_1 receptor coupled to the phosphatidylcholine-specific phospholipase C results in the downstream activation of mitogen-activated protein kinase. The main findings of the study are: (i) stimulation of imidazoline I_1 receptors by rilmenidine caused an increase in intracellular diacylglycerol accumulation, (ii) rilmenidine increased the phosphorylation of $p42^{mapk}$ and $p44^{mapk}$, and this effect was time- and- concentration-dependent, and (iii) blockade of I_1-receptors with efaroxan or inhibition of phospholipase C with D609 abolished rilmenidine-induced mitogen-activated protein kinase phosphorylation. These findings provide evidence that links I_1-receptor activation to downstream phosphorylation of mitogen-activated protein kinase and the dependence of such signaling on the phospholipase C in PC12 cells.

The main objective of the present study was to further characterize the intracellular mechanisms involved in the imidazoline I_1 receptor signaling pathway. We hypothesized that diacylglycerol accumulation evoked by imidazoline I_1 receptor activation triggers the downstream phosphorylation of mitogen-activated protein kinase. This hypothesis was tested by investigating the effect of imidazoline I_1 receptor activation on diacylglycerol accumulation and mitogen-activated protein kinase phosphorylation in nerve growth factor-differentiated PC12 cells. The latter is a unique cell model for characterizing imidazoline I_1 receptor signaling pathways because it lacks α_2-adrenoceptors (Ernsberger et al., 1995; Separovic et al., 1996) and has I_1-imidazoline binding sites that are localized to plasma membrane fractions (Ernsberger et al., 1995). The lack of α_2-adrenoceptor receptor in PC12 cells needs to be highlighted because the available selective imidazoline I_1 receptor agonists such as rilmenidine used in the present study and moxonidine used elsewhere (Separovic et al., 1996), exhibit weak α_2-adrenoceptor agonist activity.

The present finding that imidazoline I_1 receptor activation by rilmenidine increased the accumulation of diacylglycerol in PC12 cells is consistent with reported findings on moxonidine (Separovic et al., 1996, 1997). These findings suggest that the imidazoline I_1 receptor is coupled to phospholipase C to generate diacylglycerol as a second messenger. However, the possibility must be considered that other signaling pathways, e.g. phospholipase D or phosphatidylinositol-specific phospholipase C, may be involved in the generation of diacylglycerol following imidazoline I_1 receptor activation. Notably, the imidazoline I_1 receptor-evoked increase in diacylglycerol production cannot be attributed to receptor-mediated activation of phospholipase D in our model system because the increase in diacylglycerol is not associated with increased phosphatidic acid levels in PC12 cells (Separovic et al., 1996). Further, previous studies have ruled out a role for the phosphatidylinositol-specific phospholipase C signaling system in diacylglycerol accumulation evoked by imidazoline I_1 receptor activation (Liedtke et al., 1993; Regunathan and Reis, 1994; Regunathan et al., 1991). This notion is supported by the findings that imidazoline I_1 receptor activation has no effect on the basal or carbachol-mediated increases in phosphoinositide turnover in adrenal chromaffin cells (Regunathan et al., 1990, 1991). Similarly, cAMP and cGMP levels are not affected by imidazoline I_1 receptor activation (Liedtke et al., 1993; Regunathan and Reis, 1994; Regunathan et al., 1990, 1991). Taken together the present findings supported by reported findings (Separovic et al., 1996, 1997) implicate the phosphatidylcholine-specific phospholipase C in the accumulation of diacylglycerol in response to imidazoline I_1 receptor activation.

The present study tested the hypothesis that the accumulation of diacylglycerol, triggered by the imidazoline I_1 receptor activation, elicits downstream mitogen-activated protein kinase phosphorylation. The findings that rilmenidine produced concentration- and time-dependent increases in the phosphorylation of both mitogen-activated protein kinase isoforms suggest the involvement of the latter in the signal transduction mechanism coupled to imidazoline I_1 receptor activation. In order to further support the imidazoline I_1 receptor-mitogen-activated protein kinase interaction, experiments were undertaken to evaluate the effects of imidazoline I_1 receptor blockade or phospholipase C inhibition, by efaroxan and D609, respectively, on mitogen-activated protein kinase phosphorylation. Pretreatment of PC12 cells with efaroxan attenuated the rilmenidine-induced $p42^{mapk}$ and $p44^{mapk}$ phosphorylation. Further, the rilmenidine evoked mitogen-activated protein kinase phosphorylation was significantly reduced after inhibition of phospholipase C by D609. It is notable that the use of efaroxan or D609 attenuated the increase in diacylglycerol accumulation in response to imidazoline I_1 receptor activation by moxonidine, another imidazoline I_1 receptor agonist, in the same model system (Separovic et al., 1996). Taken together, the findings that imidazoline I_1 receptor activation increased diacylglycerol accumulation and mitogen-activated protein kinase phosphorylation and that both responses were attenuated after imidazoline I_1 receptor and phospholipase C inhibition suggest that the increase in mitogen-activated protein kinase phosphorylation by rilmenidine is an imidazoline I_1 receptor-mediated event and follows diacylglycerol accumulation. Further studies are needed, however, to determine the signalling mechanisms, e.g. protein kinase C (van Dijk et al., 1997b), involved in the imidazoline I_1 receptor modulation of phospholipase C-mitogen-activated protein kinase pathway.

Notably, the present study showed that the rilmenidine-induced mitogen-activated protein kinase phosphorylation was demonstrated in cells differentiated with nerve growth

factor but not in the undifferentiated PC12 cells, probably because of the reduced imidazoline I_1 binding sites in the undifferentiated cells (Ernsberger, 1992). Whether rilmenidine affects diacylglycerol accumulation in the undifferentiated cells was not investigated in the current study. Other investigators (Separovic et al., 1996), however, have shown elevated diacylglycerol contents in the undifferentiated PC12 cells in response to imidazoline I_1 receptor activation. The findings that imidazoline I_1 receptor activation increased diacylglycerol accumulation (Separovic et al., 1996) but had no effect on mitogen-activated protein kinase phosphorylation (present study) in the undifferentiated PC12 cells should not be interpreted to suggest that the two events are not related. Notably, in the study by Separovic et al. (1996), moxonidine was used to activate imidazoline I_1 receptors versus rilmenidine in this study. More importantly, the increase in diacylglycerol accumulation peaked as early as 1 min after exposure of the undifferentiated cells to moxonidine and disappeared thereafter (Separovic et al., 1996) whereas in the present study, a minimum of 5 min of rilmenidine exposure was allowed before evaluating the phosphorylation of mitogen-activated protein kinase. Therefore, it is possible that the brief accumulation of diacylglycerol might be associated with a similarly short-lived (< 5 min) phosphorylation of mitogen-activated protein kinase.

It is imperative to comment on the notion that D609 may inhibit other phospholipases, e.g. phospholipase D, in addition to phospholipase C. As discussed above, the signaling triggered by imidazoline I_1 receptor activation does not seem to involve phospholipase D (Separovic et al., 1996). In support of our hypothesis, previous studies with D609 have established a correlation between diacylglycerol accumulation through the phospholipase C route and mitogen-activated protein kinase phosphorylation in the absence of any change in phospholipase D (van Dijk et al., 1997a). Finally, the present finding that D609 had no effect on nerve growth factor-induced mitogen-activated protein kinase activation lends further support to the notion that the phosphorylation of mitogen-activated protein kinase by imidazoline I_1 receptor activation is a specific event. The present findings that nerve growth factor enhanced the phosphorylation of both mitogen-activated protein kinase isoforms ($p42^{mapk}$ and $p44^{mapk}$) and that such a response was not influenced by D609 agree with previous reports (Hunter, 1995; Widmann et al., 1999).

In summary, our recent study employed Western blotting and immunofluorescence techniques to investigate the role of mitogen-activated protein kinase in transmembrane signaling pathway coupled to imidazoline I_1 imidazoline receptors in PC12 cells. The incubation of PC12 cells with the imidazoline I_1 receptor agonist rilmenidine produced increases in the intracellular diacylglycerol accumulation and mitogen-activated protein kinase phosphorylation. The ability of efaroxan or D609 to attenuate rilmenidine but not nerve growth factor induced mitogen-activated protein kinase phosphorylation suggests that this effect of rilmenidine is a receptor-mediated event, which is dependent on phosphatidylcholine-specific phospholipase C. It is concluded that the increase in diacylglycerol accumulation in response to the activation of imidazoline I_1 receptors causes downstream phosphorylation of $p42^{mapk}$ and $p44^{mapk}$.

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