



Research report

The I₁-imidazoline receptor in PC12 pheochromocytoma cells reverses NGF-induced ERK activation and induces MKP-2 phosphataseLincoln Edwards^{a,b}, Paul Ernsberger^{a,*}^aDepartments of Nutrition, Medicine, Pharmacology, and Neuroscience, Case Western Reserve University School of Medicine, Cleveland, OH 44106-4906, USA^bDepartment of Pharmacology, Loma Linda University School of Medicine, Loma Linda, CA, USA

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Abstract

We sought to further elucidate signal transduction pathways for the I₁-imidazoline receptor in PC12 cells and their interaction with the well-characterized signaling events triggered by nerve growth factor (NGF) in these cells. Stimulation of the I₁-imidazoline receptor with moxonidine, a centrally acting antihypertensive, increased by greater than two-fold the proportion of ERK-1 and ERK-2 in the phosphorylated active form. Similarly, NGF elicited a five-fold increase in activated ERKs. Surprisingly, treatment of NGF-treated cells with moxonidine completely reversed activation of ERK. Moxonidine-induced inhibition of ERK activation in NGF-treated cells was dose-dependent, followed a limited time course and could be blocked by the I₁-antagonist efaroxan. These data suggested possible deactivation of ERK by specific phosphatases. Therefore, we assayed levels of MKP-2, a dual specificity phosphatase whose substrates include ERK. Moxonidine and NGF both increased levels of MKP-2 by three-fold. These effects were additive, as both agents together increased MKP-2 by a total of six-fold. Moxonidine-induced induction of MKP-2 was time- and dose-dependent and could be blocked by the I₁-antagonist efaroxan or by D609, an inhibitor of phosphatidylcholine-selective phospholipase C known to block downstream signaling events coupled to I₁-receptors. Thus, I₁-receptors can abrogate the primary signaling cascade activated by NGF, most likely by increasing levels of a specific phosphatase to return dually phosphorylated ERK to its unphosphorylated state.

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Keywords: Receptor, imidazoline; Cell, cultured; Pheochromocytoma; PC12 cell; ERK; MAPK; Phospholipase C**1. Introduction**

The existence of a novel imidazoline receptor was first proposed to account for differential responses to imidazoline and phenylethylamine α_2 -adrenergic agonists [3]. Subsequently, non-adrenergic binding sites specific for imidazolines were characterized [14]. It is now accepted that there are at least two subtypes of imidazoline receptors, the I₁- and I₂-subtypes, and possibly a third I₃-subtype present in the endocrine pancreas [7]. The

I₁-subtype is characterized by a high affinity for a group of agents which act in the brainstem to lower blood pressure, including clonidine, rilmenidine and moxonidine [11,12,27]. The I₂-subtype shows lower affinity for these antihypertensives with a central nervous system site of action but higher affinity for other imidazolines and guanidines, and represents a novel recognition site on mitochondrial monoamine oxidase [22].

A gene encoding an imidazoline binding protein has been cloned from a human brain cDNA library [26]. The encoded protein contains motifs commonly associated with cytokine receptors, including leucine-rich repeats and serine-rich regions. When the gene is expressed in Chinese hamster ovary (CHO) cells, high-affinity binding sites for imidazolines are induced that show nanomolar affinity for clonidine and moxonidine. Functional I₁-imidazoline receptors have been identified in neural cells, including the rostral ventrolateral medulla oblongata (RVLM) region which mediates sympatholytic actions of imidazoline

Abbreviations: DAG, diacylglycerides; DMSO, dimethylsulfoxide; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinases; NGF, nerve growth factor; PC12 cells, PC12 pheochromocytoma cell line; PC-PLC, phosphatidylcholine-selective phospholipase C; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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agonists [11,13]. Many ligands active at imidazoline receptors also bind to α_2 -adrenergic receptors. Therefore, functional studies are typically carried out with prior blockade of α_2 -adrenergic receptors or in mice with inactive α_{2A} -adrenergic receptors. These mice fail to show falls in blood pressure in response to various α_{2A} -adrenergic agonists, or to moxonidine administered peripherally [17,34], but are equivalent to wild type mice in their response to centrally administered moxonidine [31]. Thus, the central nervous system action of this agent does not depend upon α_{2A} -adrenergic receptors.

The predominant cellular model for investigation of I_1 -imidazoline receptor signaling pathways has been PC12 pheochromocytoma cells. These adrenal tumor cells express I_1 -imidazoline receptors but lack α_2 -adrenergic receptors, as shown by radioligand binding as well as molecular approaches. Stimulation of the I_1 -imidazoline receptor in PC12 cells with the agonist moxonidine leads to activation of phosphatidylcholine-selective phospholipase C (PC-PLC) [9,29,30]. Activation of PC-PLC is characteristic of the signaling pathways coupled to certain cytokine receptors, including some of the interleukin receptors [5,19], and also mediates some of the actions of thromboxanes in astrocytes [21]. Activation of PC-PLC by imidazoline agonists results in increased formation of the second messenger diacylglyceride (DAG) from phosphatidylcholine, and the release of phosphocholine. These effects can be blocked by both efaroxan, an I_1 -imidazoline receptor antagonist, and by D609, an inhibitor of PC-PLC. Cell signaling steps subsequent to the accumulation of DAG have recently been characterized for I_1 -imidazoline receptor signaling, and include activation of the β_{II} and zeta isoforms of PKC, ERK and JNK, and a modest increase in cell proliferation [6].

The best known and strongest stimulus for the activation of ERK in PC12 cells is nerve growth factor (NGF) [32]. Activation of ERK in response to NGF is dramatic in magnitude and prolonged in duration. ERK activation is both necessary and sufficient for the differentiation of PC12 cells from a chromaffin-like phenotype into a neuron-like phenotype with long processes extending from the cell body [1]. Given that imidazoline agonists induce ERK activation in PC12 cells, we postulated that imidazoline agonists might modulate the response to NGF, which may give further clues regarding the cellular function of imidazoline receptors.

MAPKs are unique among protein kinases in that in order to be activated they must be phosphorylated on both tyrosine and threonine residues in their activation loop by MAP kinase kinase [2]. MAP kinase inactivation occurs by dephosphorylation of either or both phosphoamino residue. Consequently, three types of phosphatases may play a role in inhibiting MAP kinase: serine/threonine-specific protein phosphatase by dephosphorylating only the phosphothreonine; protein tyrosine phosphatases (PTPase) by de-

phosphorylating only the phosphotyrosine residue; or dual-specificity phosphatases that can remove both phosphates [28]. The latter appear to play a predominant role. In PC12 cells and in the central nervous system, a major dual-specificity phosphatase is MAP kinase phosphatase (MKP-2) [24]. Dual-specificity phosphatases are transcriptionally regulated very rapidly and can be induced by treating cells with growth factors or other activators of MAPK [18]. Thus, in the present study we sought to determine whether imidazoline receptors, like other receptors which activate ERK, might induce inactivation of MAPK through induction of MKP-2. We further sought to determine whether induction of MKP-2 by imidazoline receptor stimulation might contribute to interactions with NGF receptor signaling.

2. Materials and methods

2.1. Materials

RPMI medium and horse serum were obtained from GIBCO (Gaithersburg, MD, USA). Fetal bovine serum, rat tail collagen and anti-ERK affinity purified antibodies were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Moxonidine was kindly provided by Kali-Chemie (Hannover, Germany). Efaroxan and clonidine were purchased from Research Biochemicals International (Natick, MA, USA). The enzyme inhibitors D609 and H-7 were purchased from Biomol (Plymouth Meeting, PA, USA). Anti-active ERK antibody and donkey anti-rabbit horseradish peroxidase antibody were purchased from Promega (Madison, WI, USA). Anti-MKP-2 antibodies were obtained from Transduction Laboratories (Lexington, KY, USA). Nerve growth factor (NGF) was obtained from Austral Biologicals (San Ramon, CA, USA). Protein assay reagents were obtained from Pierce (Rockford, IL, USA). All other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA) or Fisher (Pittsburgh, PA, USA) and were of analytical grade.

2.2. PC12 cell culture

PC12 cells were cultured as previously reported [29]. Briefly, PC12 cells were grown on 75 cm² flasks coated with rat tail collagen at 5% CO₂ in RPMI 1640 supplemented with 10% (v/v) heat-inactivated horse serum, 5% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin (complete medium). Cells were subcultured at a plating density of 1:6 once per week and medium was refreshed every 2 days. On the day before the experiments, the growth medium was switched to RPMI 1640 medium supplemented with 1% FBS to decrease basal levels of ERK activation.

2.3. Assay of ERK activation

PC12 cells in 75 cm² culture flasks were treated for 90 min with moxonidine (100 nM), NGF (50 ng/ml), efaroxan (10 μM), D609 (10 μM), or vehicle (0.1 mM acetic acid) alone, or in combination. After treatment, cells were washed with cold PBS, and then removed from the flask by scraping into lysis buffer (1% Triton X-100, 0.5% NP-40, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, and protease inhibitor cocktail; Boehringer Mannheim GmbH, Germany). Cells were subsequently homogenized with a polytron (Tecmar Tissuemizer, 15 s at setting 60) followed by centrifugation (16,000×*g* at 4 °C for 10 min). Equal amounts of protein (20 μg) from the resulting supernatants were subjected to SDS–PAGE on a 10% gel and proteins were electrophoretically transferred to a nitrocellulose membrane for immunodetection with anti-active MAPK and anti-MAPK antibodies. The anti-active MAPK recognizes the dually phosphorylated activated forms of ERK-1 and ERK-2 species of MAPK, whereas anti-MAPK recognizes all forms of ERK-1 and ERK-2. A donkey anti-rabbit secondary antibody coupled to horseradish peroxidase was utilized to visualize protein bands by chemiluminescence. Film images were quantified by using a scanning densitometer (United States Biochemical, Cleveland, OH, USA). Results were expressed as a ratio of arbitrary density*area units between anti-active and anti-MAPK blots and then normalized to the vehicle-treated control run on the same gel.

2.4. Assay of MKP-2 expression

PC12 in 75 cm² culture flasks were treated with 100 nM moxonidine for various times (0 min to 4 h) or with varying doses of moxonidine for 90 min (0.1 nM to 1 μM). In some experiments, cells were treated for 90 min with moxonidine (100 nM), NGF (50 ng/ml), efaroxan (10 μM), D609 (10 μM), vehicle (0.1 mM, acetic acid) alone or in combination. After treatment, cells were processed for Western blotting as described previously. MKP-2 expression was detected with anti-MKP-2 monoclonal antibody. An anti-mouse secondary antibody coupled to horseradish peroxidase was utilized to visualize protein bands by chemiluminescence, and quantitation was by scanning densitometry. Data were expressed as a ratio to vehicle-treated control flasks of cells run in parallel.

2.5. Data analysis

Statistical comparisons were performed by *t*-test for two groups or analysis of variance for multiple comparisons, with Newman–Keuls post-hoc tests. Dose–response data were fitted to logistic equations [25] using the Prism data

analysis package (GraphPAD software, San Diego, CA, USA) to obtain EC₅₀ values.

3. Results

3.1. Effect of moxonidine with and without NGF

We first determined the interaction between the I₁-imidazoline receptor agonist moxonidine and NGF, as both substances have been reported to activate ERK. The activation of ERK-1 and ERK-2 was determined as the ratio of the amount of dually phosphorylated active form to total ERK immunoreactivity. Confirming our previous results, 100 nM moxonidine increased by more than three-fold the relative proportion of ERK-1 in the active dually phosphorylated state (Fig. 1). As expected based on prior literature, NGF induced an even greater level of activation. Surprisingly, co-treatment with both moxonidine and NGF produced less activation than with either substance alone. This suggested an interaction between the signaling events triggered by the imidazoline and NGF receptors. To test whether the effect of moxonidine in the presence of NGF was receptor-mediated, we evaluated the effect of efaroxan, an imidazoline receptor antagonist. Efaroxan blocked the inhibition produced by moxonidine and restored ERK activation to the level observed in the presence of NGF alone.

We next sought to determine whether the inhibitory action of moxonidine in the presence of NGF was dose-dependent (Fig. 2). The decrease in ERK activation was significant at 1.0 nM (*P*<0.05, paired *t*-test), and at 1.0 μM the response to NGF was suppressed by more than two-thirds. Actions in the range of 1–1000 nM are consistent with the binding affinity of moxonidine at I₁-imidazoline sites in PC12 cells (*K_i* = 8 nM [29]). Moreover, the dose–response relationship for inhibition of NGF-mediated ERK activation (Fig. 2) is comparable to previously obtained dose–response curves for ERK activation [6], arachidonic acid release [8] and diacylglyceride accumulation [29]. In the presence of the I₁-imidazoline antagonist efaroxan, none of the doses of moxonidine had any significant effect on ERK activation by NGF.

3.2. Effect of moxonidine on MKP-2 expression in PC12 cells

A representative blot is shown in Fig. 3, illustrating the time course of the response to 100 nM moxonidine. An increase in the amount of immunoreactivity to the anti-MKP-2 antibody is apparent at the middle time points. The amount of total ERK-2 immunoreactivity was constant between lanes, indicating equal loading (less than 5% variation in optical density from mean value; not shown). Mean data from four experiments showed that moxonidine

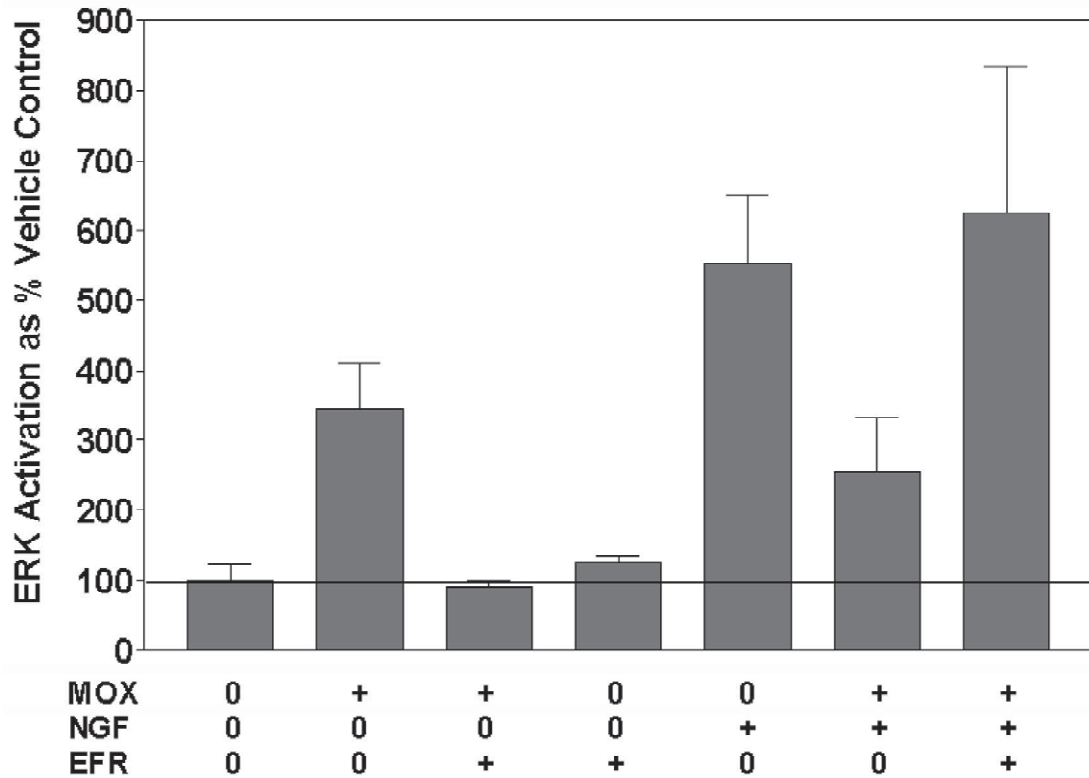


Fig. 1. Effects of moxonidine, NGF and their combination on ERK-2 activation, and the antagonism of moxonidine’s effects by efaroxan. The relative activation of ERK-1 and ERK-2 was defined by the ratio of total immunoreactive protein, in arbitrary absorbance units, to the dually phosphorylated form. The data were expressed relative to vehicle-treated controls run in parallel. Data are presented as mean percent change±standard error from nine to 12 separate experiments run in duplicate.

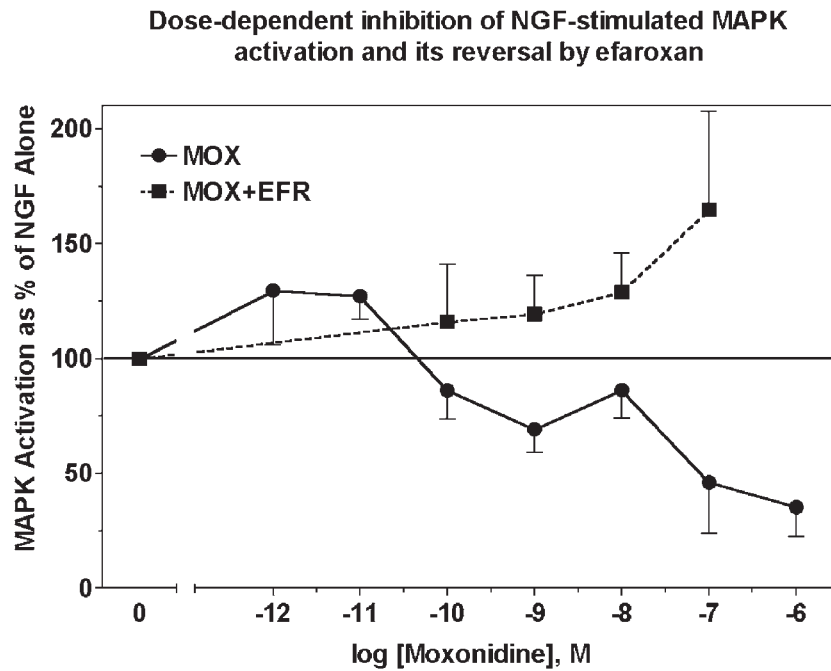


Fig. 2. Dose dependence of the deactivation of ERK by moxonidine in NGF-treated PC12 cells in the presence and absence of efaroxan. PC12 cells were treated with NGF (50 ng/ml) in the presence or absence of increasing concentrations of moxonidine (MOX; 0.1 nM to 1 μM) with and without efaroxan (EFR; 10 μM) for 90 min, prepared for analysis of total and activated ERK as described for Fig. 1. Efaroxan was also present during a 10 min preincubation. Data are presented as mean percent change±standard error from three to five separate experiments run in duplicate. Significant inhibition of ERK activation was detected at 1.0 nM, 100 nM and 1.0 μM ($P < 0.05$, Newman–Keuls) only in the absence of efaroxan.

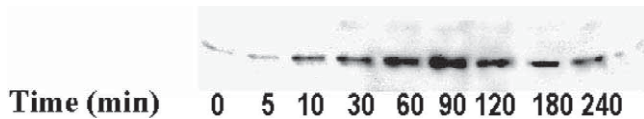


Fig. 3. Western blot illustrating the time course of MKP-2 induction by moxonidine. Each lane was obtained from different flasks of PC12 cells incubated with 100 nM moxonidine for increasing periods of time.

treatment of PC12 cells elicited a rapid increase in the level of MKP-2 expression, which was significant within 10 min ($P < 0.05$, Newman–Keuls test) and peaked at 90 min at about 230% relative to vehicle-treated controls (Fig. 4). The level of MKP-2 rapidly declined after this peak and returned to baseline within 4 h.

The dose-dependence for the action of moxonidine on MKP-2 expression is illustrated in Fig. 5. Concentrations

Timecourse of mkp2 induction by moxonidine

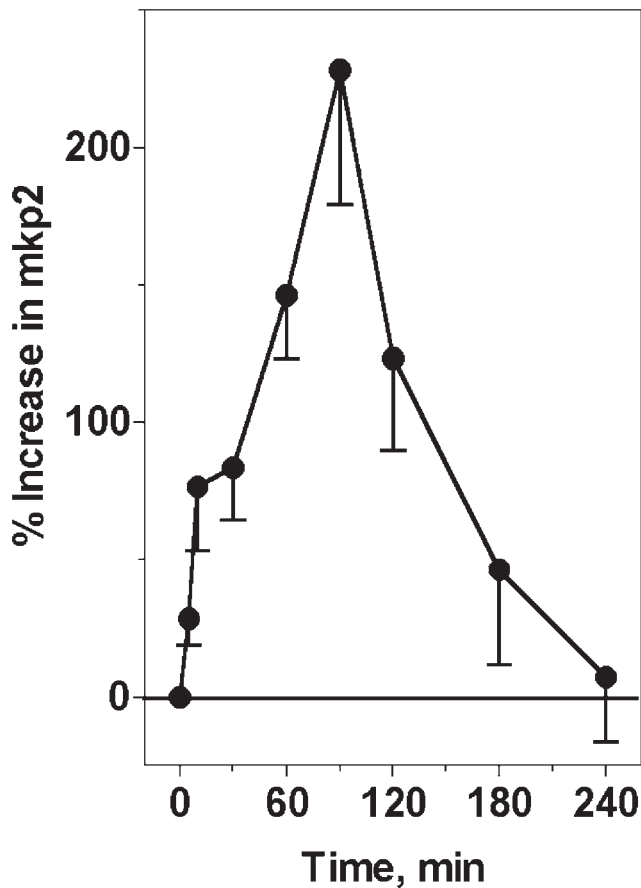


Fig. 4. Time course of MKP-2 expression in PC12 cells following moxonidine treatment. PC12 cells were treated with moxonidine (100 nM) for various times and then analyzed for MKP-2 protein expression by quantitative Western blot. Data are presented as mean percent change relative to vehicle-treated control flasks run in parallel on the same blot \pm standard error from four separate experiments run in duplicate.

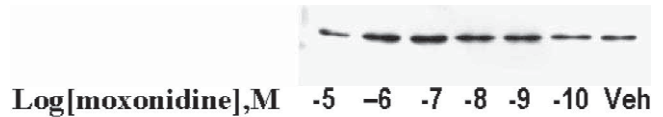


Fig. 5. Western blot illustrating the dose-dependence of MKP-2 induction by moxonidine. The log of the moxonidine concentration is given below each band. Each lane was obtained from a different 75 cm² flask of PC12 cells. Total ERK immunoreactivity was equal between lanes (not shown).

of moxonidine as low as 1 nM significantly increased MKP-2 immunoreactivity, and increased further up through 100 nM. Gel loading was evaluated by the total amount of ERK-2 protein, which was constant (not shown). Summary data from four separate experiments show that moxonidine's effect on ERK was dose-dependent up to 100 nM, with an ED_{50} of 4.2 ± 1.4 nM (Fig. 6). A higher concentration of moxonidine, 10 μ M, induced MKP-2 to a lesser extent than 100 nM. Comparable biphasic dose–response relationships have been reported for DAG accumulation [29] and ERK-2 activation [6].

In order to test whether the effect of moxonidine on ERK stimulation was mediated by the I_1 -imidazoline receptor and through its known transmembrane signaling pathways, we treated the cells with efaroxan, a selective I_1 -imidazoline receptor antagonist, or with D609, an inhibitor of phosphatidylcholine-selective phospholipase C which blocks I_1 -imidazoline receptor signaling in PC12 cells (Figs. 7 and 8). Efaroxan (10 μ M) abolished ERK activation by 100 nM moxonidine treatment, but had no significant effect when given alone. The PC-PLC inhibitor D609 (1.0 μ M) also effectively abolished the effect of moxonidine.

We next sought to test whether the induction of MKP-2 by moxonidine treatment might contribute to the interaction between NGF and imidazoline receptor activation. As shown in the representative blot in Fig. 7 and as summarized in Fig. 8, NGF increased the level of MKP-2 immunoreactivity by three-fold after 90 min of treatment. In flasks of cells run in parallel, 100 nM moxonidine elicited a nearly identical response. Combined treatment with NGF and moxonidine produced an additive effect, resulting in a nearly six-fold increase in MKP-2 immunoreactivity. The effect of moxonidine was abrogated by the I_1 -imidazoline antagonist efaroxan, either in the presence or absence of NGF. The PC-PLC inhibitor D609 also blocked the effect of moxonidine and had no effect when given alone.

4. Discussion

The present study shows that stimulation of I_1 -imidazoline receptors in PC12 rat pheochromocytoma cells can either activate or inactivate ERK-1 and ERK-2 mitogen-activated protein kinases (MAPKs) depending on the presence of other stimuli. In PC12 cells, the activation

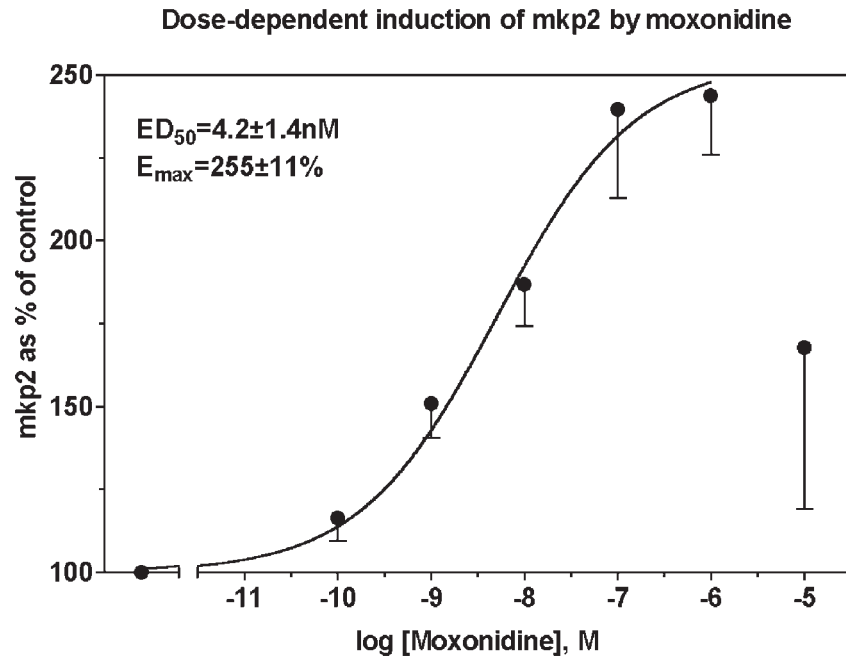


Fig. 6. Dose–response relationship for moxonidine induction of MKP-2 immunoreactivity. PC12 cells were treated with increasing concentrations of moxonidine for 90 min and then analyzed for MKP-2 protein expression by quantitative Western blot. Data are presented as percent change relative to vehicle-treated control flasks of cells run in parallel on the same blot and represent mean \pm S.E. from four separate experiments run in duplicate.

of ERK by NGF is a potent and well-characterized signaling response which is both necessary and sufficient for the neuronal differentiation of these cells [32]. In neuronal cells, EGF stimulation of MAP kinase results in cell proliferation whereas NGF stimulation results in neuronal survival and differentiation [4]. Imidazoline treatment almost completely reverses the activation of ERKs by NGF. This inhibitory effect is dose-dependent and is abrogated by receptor antagonists and by blockade

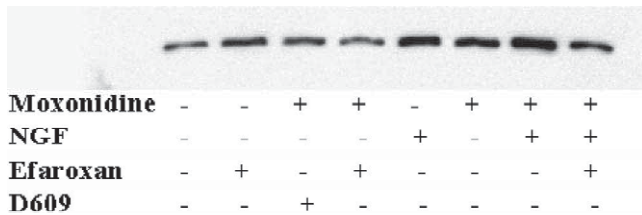


Fig. 7. Effects of NGF, moxonidine and selective inhibitors on MKP-2 immunoreactivity. PC12 cells were incubated with or without moxonidine (100 nM) or NGF (50 ng/ml) in the presence or absence of the I_1 -imidazoline antagonist efaroxan (10 μ M) or the PC-PLC inhibitor D609 (10 μ M) for 90 min. Efaroxan or D609 were also present during a 10 min preincubation. MKP-2 expression was then determined by Western blotting. Both NGF (fifth lane) and moxonidine (sixth lane) increased MKP-2 immunoreactivity relative to controls treated only with vehicle (first lane), and co-treatment with both agents showed additive stimulation (seventh lane). Efaroxan abolished the additional effect of moxonidine (eighth lane) and the effect of moxonidine alone (fourth lane) as did D609 (third lane). Although efaroxan appeared to have a stimulating effect on its own, this effect was not consistent.

of phosphatidylcholine hydrolysis by PC-PLC. We did not test whether imidazolines could block downstream effects of NGF, such as neuronal differentiation.

We have shown previously that moxonidine treatment of PC12 cells causes a dose- and time-dependent activation of ERK-1 and ERK-2, with maximum activation occurring at a 100 nM concentration for 90 min of treatment [6]. This activation of ERK by imidazoline receptors in PC12 cells is associated with a mild cell proliferation response 24 or 48 h later. The present study demonstrates that I_1 -imidazoline receptors participate in either up- or downregulation of the ERK kinase cascade depending on the status of the cell. In agreement with previous reports, both NGF and the I_1 -imidazoline receptor agonist induced a three-fold or greater increase in the proportion of ERK-1 and ERK-2 in the dually phosphorylated active state. Since both moxonidine and NGF separately activated ERK, we anticipated that the combination of moxonidine and NGF would produce an additive or synergistic effect. In contrast, co-stimulation with NGF and moxonidine resulted in a decreased phosphorylation of ERK compared to NGF stimulation alone. This decreased phosphorylation could be reversed by efaroxan, an I_1 -imidazoline receptor blocker. Efaroxan can also act as an α_2 -adrenergic antagonist in some cells in the dose range used in the present study, but these receptors are not present in PC12 cells. Efaroxan has negligible affinity for the mitochondrial I_2 -imidazoline subtype [23] which is present in these cells.

The dual specificity phosphatases such as MKP-1 and MKP-2 are important convergence points for various extra-

MKP-2 Protein Expression is Regulated by I₁-Imidazoline and NGF Receptors

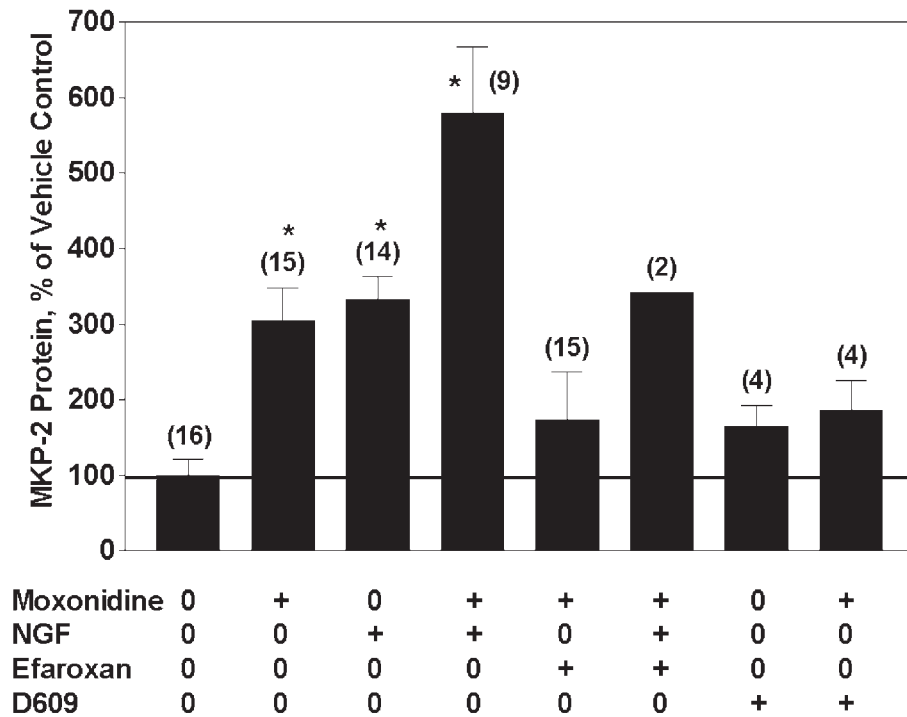


Fig. 8. MKP-2 protein expression is regulated by NGF and I₁-imidazoline receptors. Cells were treated as described for Fig. 7. Values are expressed as a percent of vehicle-treated controls run in parallel on the same blot. Each value represents the mean \pm S.E.M. of separate experiments. The number of experiments is given in parentheses. Asterisks indicate statistically significant effects ($P < 0.05$, Newman–Keuls test). Both efaroxan and D609 abolished induction of MKP-2 expression by moxonidine treatment, but had no significant effect when given alone. Efaroxan reversed the additive effect on MKP-2 expression achieved by combination treatment with NGF and moxonidine.

cellular ligand-mediated signaling pathways, including nerve growth factor (NGF) and imidazoline receptor pathways [20,24]. The decrease in ERK phosphorylation following co-stimulation with moxonidine and NGF could be due to increased expression and activity of MKP isoforms in response to moxonidine and NGF. Others have shown that NGF and EGF caused a substantial increase in MKP-2 mRNA levels but only modest increases in MKP-1 in PC12 cells [24]. A major involvement of MKP-1 induction in the rapid deactivation of MAP kinase in PC12 cells has also been ruled out [33]. Our own data (not shown) did not reveal consistent induction of MKP-1 expression, in contrast to the findings with MKP-2. Hence the present report focused solely on MKP-2, although a contribution from MKP-1 induction cannot be ruled out. Indeed, a number of other phosphatases are active on ERK-1 and ERK-2 [28] and the relative role of MKP-2 cannot be directly determined from the data at hand.

To test the hypothesis that co-stimulation with moxonidine and NGF leads to the induction of the specific MAP kinase attenuator, MKP-2, the expression of this phosphatase was measured in PC12 cells that had been incubated in the absence and presence of moxonidine for various periods of time. NGF stimulation of PC12 cells is

known to cause an induction of MKP-2 within 1 h [24]. The I₁-imidazoline receptor agonist moxonidine caused a time-dependent induction of MKP-2 immunoreactivity with a peak at 90 min. Analysis of the dose-dependence of this induction revealed an ED₅₀ of 4.2 nM, which is consistent with the binding affinity of moxonidine for I₁-imidazoline receptors in PC12 cell plasma membranes (8 nM [29]). Moreover, the previously reported ED₅₀ for the activation of ERK-2 in the absence of NGF treatment was also similar at 1.3 nM [6]. MKP-2 induction by moxonidine is apparently receptor-mediated because it could be blocked by co-treatment with the I₁-imidazoline antagonist efaroxan, and also with D609, an inhibitor of phosphatidylcholine-selective phospholipase C (PC-PLC) which is an intermediate in the I₁-imidazoline receptor signaling pathway in PC12 cells [29,30]. Co-stimulation by moxonidine and NGF produced an additive effect on MKP-2 induction after 90 min of treatment. The additive induction of MKP-2 may provide a mechanism for the decreased phosphorylation of ERK seen at 90 min of co-stimulation with moxonidine and NGF. The additive effect on MKP-2 induction and the reversal of ERK activation is not seen when cells are treated with efaroxan along with NGF and moxonidine, as only the action of

NGF is seen. As expected, efaroxan blocked the contribution by moxonidine.

This state-dependent regulation of ERK by I_1 -imidazoline receptor is not unique to this signaling protein. Stimulation of angiotensin receptors of the AT_2 subtype in PC12W cells by angiotensin II results in enhanced phosphorylation of ERK within 10 min compared to unstimulated cells [20]. Paradoxically, co-stimulation with NGF and angiotensin II resulted not in the expected additive effect on ERK phosphorylation but rather in a decrease compared to NGF stimulation alone. The deactivation of ERK was paralleled by a rise in MKP-1 activity, suggesting that AT_2 induces MKP-1 expression. In the case of I_1 -imidazoline receptors, MKP-2 rather than MKP-1 appears to be more involved in ERK inactivation. A possible mechanism for the differential effects on ERKs by I_1 -imidazoline receptor may involve PC-PLC cleavage of phosphatidylcholine to produce phosphocholine and DAG. PKC activation triggered by DAG ultimately leads to ERK activation. The activation of ERK in response to imidazoline agonists appears to be downstream of PKC activation. Thus, depletion of classical and novel isoforms of PKC by prolonged exposure to a phorbol ester blocked the response to moxonidine. Furthermore, a non-selective PKC inhibitor blocked the response to moxonidine as well. These results implicate the stimulation of PKC in the activation of ERK by imidazoline agonists. Through the same pathway involving PC-PLC hydrolysis, imidazoline receptor activation also leads to induction of MKP-2.

MKP-2 is highly expressed in PC12 cells, and is also widely distributed in the central nervous system where it may participate in neuronal signaling [24,32]. Imidazoline receptors are also found in PC12 cells and in the CNS, suggesting that they may contribute to the regulation of MKP-2 in the CNS and ultimately neuronal signaling. Activation of I_1 -imidazoline receptors in the brain leads to sympathoinhibition [10,30,31] and may also be linked to control of mood [15]. The sympathoinhibition induced by moxonidine has been linked to phosphatidylcholine hydrolysis by PC-PLC [30]. The sympatholytic actions of imidazolines are relatively slow in onset and prolonged in duration compared to the effects of agonists active at conventional G-protein coupled receptors such as α_2 -adrenergic receptors [16]. The ability of moxonidine to induce expression of a specific dual specificity phosphatase, thereby inducing long-term alterations in the responses to neurotrophic factors, is an example of a relatively long lasting effect compared to direct agonists at G-protein coupled receptors. Thus, it is possible that I_1 -imidazoline receptors may influence neuronal activity on a longer timescale than receptors coupled to short-lived second messengers.

A limitation of this study is that only a single I_1 -imidazoline agonist, moxonidine, was tested. The specific antagonism of moxonidine's effects by the known I_1 -imidazoline antagonist efaroxan and by the pathway

inhibitor D609 is consistent with mediation of the response by this receptor. However, to unequivocally link the observed cell signaling events to the I_1 -imidazoline receptor it would be necessary to test a series of agonists and show that the order of potency corresponds to that of I_1 -imidazoline receptors. Future studies should follow such an approach.

In summary, although both NGF and the I_1 -imidazoline agonist moxonidine activated ERK-1 and ERK-2 in PC12 cells, combined treatment with both agents produced no net activation. The inhibitory action of moxonidine appeared to be receptor mediated, as it could be blocked by the appropriate competitive antagonist. We hypothesized that the apparent deactivation of ERK by imidazoline receptor stimulation was mediated by a phosphatase capable of deactivating ERK. Consistent with this hypothesis, both NGF and moxonidine induced expression of the dual specificity phosphatase MKP-2. In this case, the effects of these two agents were additive. The effect of moxonidine could be blocked by a receptor antagonist or by inhibition of the signaling cascade utilized by I_1 -imidazoline receptors. These data are consistent with, but do not conclusively establish, an interaction between NGF and imidazoline receptor signaling pathways mediated by induction of a specific phosphatase.

Acknowledgements

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