

Title:

MAPK PHOSPHORYLATION IN THE ROSTRAL VENTROLATERAL
MEDULLA PLAYS A KEY ROLE IN IMIDAZOLINE (I₁) RECEPTOR
MEDIATED HYPOTENSION

Jian Zhang and Abdel A. Abdel-Rahman

Department of Pharmacology and Toxicology

Brody School of Medicine

East Carolina University

Greenville, NC 27834 (ARA and JJ)

Tel: (252) 744-3470, FAX: (252) 744-3203

E-mail: abdelrahmana@mail.ecu.edu

Running title:

RVLM MAPK_{p42/44} contributes to I₁-receptor mediated hypotension

Abdel A. Abdel-Rahman

Department of Pharmacology and Toxicology, Brody School of Medicine

East Carolina University, Greenville, NC 27834

Tel: (252) 744-3470, FAX: (252) 744-3203

E-mail: abdelrahmana@mail.ecu.edu

Document statistics:

Text pages: 27

Number of figures: 7

Number of tables: 1

Number of references: 26

Abstract: 201 words

Introduction: 490 words

Discussion: 1389 words

Abbreviations:

α -methylnorepinephrine (α -MNE)

Artificial cerebrospinal fluid (ACSF)

Mitogen-activated protein kinase (MAPK)

Nucleus tractus solitarius (NTS)

Phosphatidylcholine-specific phospholipase C (PC-PLC)

Rostral ventrolateral medulla (RVLM)

Abstract

Our previous study showed that rilmenidine, a selective I₁-imidazoline receptor agonist, enhanced the phosphorylation of mitogen-activated protein kinase (MAPK_{p42/44}) via the phosphatidylcholine-specific phospholipase C (PC-PLC) pathway in pheochromocytoma cell line (PC12). In the present study we tested the hypothesis that enhancement of MAPK phosphorylation in the rostral ventrolateral medulla (RVLM) contributes to the hypotensive response elicited by I₁ receptor activation in vivo. Systemic rilmenidine (600 µg/kg, i.v.) elicited hypotension and bradycardia along with significant elevation in MAPK_{p42/44}, detected by immunohistochemistry, in RVLM neurons. To obtain conclusive evidence that the latter response was I₁-receptor mediated, similar hypotensive responses were elicited by intracisternal (i.c.) rilmenidine (25 µg/rat) or the highly selective α₂-agonist α-methylnorepinephrine (α-MNE, 4 µg/rat). An increase in RVLM MAPK_{p42/44} occurred only following rilmenidine. Further, pretreatment with efaroxan (0.15 µg/rat, i.c.), a selective I₁-imidazoline receptor antagonist, or with PD 98059 (5 µg/rat, i.c.), a selective ERK1/2 inhibitor, significantly attenuated the hypotensive response and the elevation in RVLM MAPK_{p42/44} elicited by i.c. rilmenidine. The findings suggest that MAPK phosphorylation in the RVLM contributes to the hypotensive response induced by I₁ receptor activation and presents in vivo evidence that distinguishes the neuronal responses triggered by the I₁-receptor from those triggered by the α₂-adrenergic receptor.

Introduction

The imidazoline (I_1) receptor is considered a novel receptor distinct from the α_2 -adrenergic receptor (α_2 -AR) based on the findings: (i) the mixed I_1/α_2 agonist clonidine and the selective I_1 agonists rilmenidine and moxonidine elicit greater hypotensive response when microinjected into the RVLM as compared with the nucleus tractus solitarius (NTS), (ii) the selective α_2 agonist α -methylnorepinephrine (α -MNE) elicits appreciable hypotensive response when microinjected into the NTS (Ernsberger et al., 1995; Ernsberger and Haxhiu, 1997), (iii) imidazolinergic and adrenergic receptor signal transduction pathways seem to differ in rabbits (Chan et al., 2005), and (iv) a newly selective I_1 receptor agonist LNP509 reduced the blood pressure even in genetically engineered mice lacking functional α_2 -AR (Bruban et al., 2002; Bousquet et al., 2003).

Evidence has shown that the activation of phosphatidylcholine-sensitive phospholipase C (PC-PLC) is triggered by I_1 -receptor in PC12 cells (Separovic et al., 1997; Zhang et al., 2001). The finding that microinjection of D609, a selective PC-PLC inhibitor, into the RVLM attenuated the hypotension caused by systemic moxonidine (Separovic et al., 1997) supports the existence of I_1 -receptor coupled PC-PLC pathway in vivo. Furthermore, our previous findings in PC12 cells demonstrated that activation of the I_1 -receptor, which is coupled to PC-PLC, results in downstream activation of mitogen-activated protein kinase (MAPK_{p42/44}) (Zhang et al., 2001). Notably, the PC12 cells, which lack α_2 -AR (Ernsberger et al., 1995; Separovic et al., 1996), exhibit plasma membrane imidazoline I_1 binding sites (Ernsberger et al., 1995). The enhanced phosphorylation of MAPK by the selective I_1 -receptor agonist rilmenidine and the counteraction of such response by the selective I_1 -receptor antagonist efaroxan established a clear link between

the I₁-receptor and the MAPK signal pathway in vitro (Zhang et al., 2001). Whether enhanced phosphorylation of MAPK in vivo is essential for the mediation of the pharmacological action of selective I₁ agonists has not been investigated.

The objective of the present study was to test the hypothesis that an enhanced neuronal expression of phosphorylated MAPK in the RVLM is functionally linked to the hypotension elicited by I₁-receptor activation. To this end, we measured brainstem neuronal MAPK_{p42/44} (immunohistochemistry) and blood pressure following I₁-receptor activation. We focused on the RVLM, the major site of action for the selective I₁-receptor agonists such as rilmenidine and moxonidine (Gomez et al., 1991; Haxhiu et al., 1994). However, since rilmenidine exhibits α_2 -AR agonist activity (Szabo et al., 1993; Regunathan et al., 1995; Zhu et al., 1999) and the RVLM contains both I₁- and α_2 -ARs (Reis, 1996), we investigated the effects of the pure α_2 -AR agonist α -MNE on MAPK phosphorylation in the RVLM. As a control, we investigated whether rilmenidine enhanced the phosphorylation of MAPK in the NTS, which is devoid of functional I₁-receptor (Gomez et al., 1991). To confirm the involvement of the I₁-receptor and the ERK-MAPK pathway in the observed responses, we investigated the effects of the selective I₁-receptor antagonist efaroxan and the ERK1/2 inhibitor PD98059 on the hypotensive response and the enhanced RVLM MAPK_{p42/44} elicited by rilmenidine.

Methods

A total 56 Sprague-Dawley (SD) male rats, weighing 320-360 g (Harlan, Indianapolis, IN, USA) were used. All rats were housed in a room with controlled environment at a constant temperature of $23 \pm 1^\circ\text{C}$, humidity of $50 \pm 10\%$ and a 12:12 hours light dark cycle. Food and water were available *ad libitum*. Surgical procedures and postoperative care were performed in accordance with the Institutional Animal Care and Use Guidelines.

Intracisternal cannulation. Four to six days before starting the experiment, we implanted a stainless steel guide cannula into the cisterna magna under pentobarbital anesthesia (50mg/kg, i.p.) as in our previous studies (El-Mas et al., 1994a; El-Mas and Abdel-Rahman, 1998). Briefly, the steel cannula (23 G; Small Parts, FL, USA) was passed between the occipital bone and the cerebellum so that its tip protruded into the cisterna magna. The cannula was secured by dental acrylic cement (Duralon: Thomas Dental Supply, Raleigh, NC, USA) as described in our previous studies. The guide cannula was considered patent when spontaneous outflow of cerebrospinal fluid was observed and by gross postmortem histological verification after perfusion with fixation solution. Each rat received a subcutaneous injection of the analgesic buprenorphine hydrochloride (Buprenex; 0.3 $\mu\text{g}/\text{rat}$) and an intramuscular injection of 60,000 U of penicillin G benzathine and penicillin G procaine in aqueous suspension (Durapen). After intracisternal cannulation, the rats were housed individually.

Intravascular cannulation. For measurement of blood pressure (BP) and the heart rate (HR), the method described in our previous studies (El-Mas et al., 1994b; El-Mas and Abdel-Rahman, 1998) was adopted. In brief, the rats were anesthetized with pentobarbital

sodium (50 mg/kg, i.p.). Catheters (polyethylene 50) were placed in the abdominal aorta and vena cava via the femoral artery and vein for measurement of BP and i.v. administration of drugs, respectively. The catheters were inserted ~ 5 cm into the femoral vessels and secured in place with sutures. The catheters were flushed with heparin (200U/ml). The arterial catheter was connected to a Gould-Statham pressure transducer (Oxnard, CA, USA), and BP was displayed on Grass polygraph (model 7D; Grass Instrument Co., Quincy, MA, USA). Heart rate (HR) was computed from BP waveforms by a Grass tachograph and was displayed on another channel of the polygraph.

Immunohistochemistry. The procedure reported in *Current Protocols in Neuroscience* for immunohistochemistry for light microscopy (Gerfen, 1997; Ince and Levey, 1997) was followed. Briefly, brain fixation was achieved by trans-cardiac perfusion of the fixation solution (4% paraformaldehyde) after a lethal dose of pentobarbital. Then, the brain was transferred into 30% sucrose in Tris-buffered saline (TBS) for infiltration until the brain sinks. The brain was cut serially at -22 °C with a microtome cryostat (HM 505 E, Microm International GmbH). Six to eight sections (16 µm thick) of the brain were collected in each well of a cell culture plate (12 wells, Becton Dickinson Inc.) containing cold TBS. Prior to the immunostaining procedure, a section from each well was stained with thionin (Gerfen, 1997) for the segment mapping according to the *Paxinos and Watson Atlas* (Paxinos and Watson, 1982). The sections from the coordinated segments (-12.8 ~ -11.80 mm) were used for immunostaining (Fig.2). ABC method was used following the manufacturer's instruction (Vectastain ABC kits, Vector Laboratories Inc. Burlingame, CA) with minor modification (Ince and Levey, 1997). The sections from treatment and control groups were simultaneously incubated with the primary antibody,

anti-phospho-MAPK (p42/44) monoclonal antibody (Cell Signaling Technology, Inc., Beverly, MA) (1:400 dilutions). Following rinsing with TBS, H₂O₂/DAB (3.3'-Diaminobenzidine) solution was added and the sections were examined under a microscope (Nikon Diaphot 300, Tokyo, Japan) for the appearance of reddish brown staining. After dehydrating and clearing, the sections were sealed with Permount (Fisher Scientific, Pittsburgh, PA) and observed under the microscope. The images were processed by Micropublisher (QImaging, Burnaby, B.C., Canada). For collecting the data of the phosphorylated MAPK, the cell body with dark brown immunoreactive product was considered a positive cell. The positive cells in the defined RVLM or NTS of the segments from -12.8 to -11.8 mm according to the rat brain atlas (Paxinos and Watson, 1982) were counted on each side of the RVLM or NTS from the 6 sections of similar segment of each rat. The data of the positive cell count from the RVLM represented the 'numbers/side/rat'. The average of the positive cell number from each rat was used for the statistical analysis.

Protocols and experimental groups

After the arterial catheter was connected to a pressure transducer for measurement of BP and HR, at least 30 min stabilization period was allowed at the beginning of each experiment. Ten groups of rats were used (Table 1). In the first experiment, two groups of rats received i.v. rilmenidine (600 µg/kg, gift from Technologie Servier, Neuilly Sur Seine, France), or equal volume of saline. In the second experiment, 3 groups were used for the intracisternal administration of: rilmenidine (25 µg/rat), α-MNE (4 µg/rat, Sigma-Aldrich, St Louis, MO), or equal volume (4 µl) of artificial cerebrospinal fluid (ACSF in mM: NaCl 123, CaCl₂ 0.86, KCl 3, MgCl₂ 0.89, NaHCO₃ 25, NaH₂PO₄ 0.5, Na₂HPO₄ 0.25, pH 7.4). In

the third experiment, efaroxan (0.15 $\mu\text{g}/\text{rat}$, i.c., Sigma-Aldrich, St Louis, MO) or equal volume of ACSF was administered and followed 10 min later with i.c. rilmenidine (25 $\mu\text{g}/\text{rat}$). In the fourth experiment, the rats received the ERK1/2 inhibitor PD 98059 (5 $\mu\text{g}/\text{rat}$, Sigma-Aldrich, St Louis, MO) or equal volume of vehicle (DMSO) i.c. 10 min before rilmenidine (25 $\mu\text{g}/\text{rat}$, i.c.). In all experiments, the blood pressure and heart rate were recorded for additional 15 min after rilmenidine or its vehicle (ACSF) following which each rat was prepared for trans-cardiac perfusion and brain preparation for immunohistochemical detection of phosphorylated MAPK as detailed earlier.

Statistical Analysis.

Values are presented as mean \pm S.E.M. Mean arterial pressure (MAP) was calculated as “diastolic + [(systolic - diastolic)/3]”. Statistical comparison was made by analysis of variance (ANOVA) followed by post-hoc multiple comparisons of the mean with Tukey’s test. Student’s t-test (unpaired, two-tailed) was used for comparing the mean of the baseline and positive cell count data. The probability levels less than 0.05 were considered as statistically significant.

Results

The baseline values for blood pressure and heart rate were not significantly different between the experimental and control groups in each experiment (Table 1). The specificity of the anti-phosphorylated MAPK antibody was examined in our previous western blot studies (Zhang et al., 2001). Also, in the immunohistochemical studies, a negative control (omitting the primary antibody) was run, which resulted in a clean background (data not shown). The specific immunostaining of cells was mostly distributed in the RVLM (Fig. 1). Both the cell body and the processes of the neuron were stained. Some of the cerebral blood vessels were stained nonspecifically, but were not included in the data analysis. For comparison of the level of expression of phosphorylated MAPK, all brain sections were processed similarly and the number of cells expressing the phosphorylated MAPK in the RVLM was counted in similar segments of the sections obtained from treatment and control rats. Possible immunostaining for MAPK_{P42/44} in the neurons of the NTS in the same section was also evaluated. The sections from the desired segment of the RVLM were verified by thionin staining before performing the immunohistochemical studies. Fig. 1 (A, B) shows a representative example of phosphorylated MAPK immunostaining (B) and the verification of the site by thionin staining (A) following i.v. rilmenidine

Effect of the I₁ imidazoline receptor agonist rilmenidine on blood pressure, heart rate and the phosphorylation of MAPK in the brainstem. Compared with the vehicle (saline), systemic rilmenidine (600 µg/kg, i.v.) elicited a brief pressor response followed by hypotensive and bradycardic responses. The maximal reductions in the blood pressure and heart rate caused by rilmenidine during the 15 min observation period were -21.9 ± 7.1 mmHg and -65 ± 21 beats/min, respectively (Fig.1). In the same rats, rilmenidine produced

significant increase in the phosphorylation of MAPK in the RVLM, but not in the NTS. The number of the positive cells expressing phosphorylated MAPK in the RVLM was markedly ($p < 0.05$) increased by rilmenidine (25 ± 3) vs saline (7 ± 2) (Fig. 1).

Effect of central (i.c.) administration of rilmenidine or α -MNE on blood pressure and MAPK phosphorylation in the RVLM. To verify whether the phosphorylation of MAPK in the RVLM was elicited by the I_1 -imidazoline receptor or the α_2 -adrenergic receptor, rilmenidine ($25 \mu\text{g}/\text{rat}$) or the pure α_2 -adrenergic receptor agonist α -MNE ($4 \mu\text{g}/\text{rat}$), in doses selected in anticipation of causing equal hypotensive responses, was injected into the cisterna magna. Rilmenidine and α -MNE elicited similar hypotensive responses while ACSF had no effect on blood pressure or heart rate (Fig. 2). However, only rilmenidine caused a significant increase in the expression of phosphorylated MAPK in RVLM (Fig. 3). The number of the positive cells expressing phosphorylated MAPK in RVLM was markedly increased ($p < 0.05$) by rilmenidine (19 ± 1) compared with ACSF (7 ± 1). Compared with ACSF, α -MNE had no effect on neuronal expression of MAPK_{P42/44} in the RVLM (Fig. 3). Neither rilmenidine nor α -MNE increased MAPK phosphorylation in the NTS (Fig. 3).

Effect of efaroxan pretreatment on the hypotensive response and the enhanced phosphorylation of MAPK in RVLM elicited by rilmenidine. To further verify the involvement of the I_1 -receptor in rilmenidine-induced enhancement of the phosphorylation of MAPK in RVLM and its involvement in the hypotensive response, efaroxan, a selective I_1 -imidazoline receptor antagonist (Haxhiu et al., 1994) was used in this experiment. Intracisternal efaroxan ($0.15 \mu\text{g}/\text{rat}$), which had no effect on blood pressure or heart rate, attenuated the hypotensive and bradycardic responses elicited by subsequent i.c. rilmenidine ($25 \mu\text{g}/\text{rat}$, Fig. 4). Compared with ACSF, efaroxan significantly ($p < 0.05$) attenuated the

hypotensive (-10.0 ± 1.9 vs. -20.6 ± 4.5 mmHg) and bradycardic (-49 ± 9 vs. -113 ± 15 beats/min) responses and substantially ($p < 0.05$) reduced the number of positive cells, which expressed phosphorylated MAPK (P42/44) in the RVLM (7 ± 1 vs. 16 ± 2) elicited by i.c rilmenidine (Fig. 5).

Effect of ERK1/2 inhibition on the hypotensive response and the enhanced

phosphorylation of MAPK in RVLM elicited by rilmenidine. To support a causal

relationship between the rilmenidine-induced enhancement of the phosphorylation of MAPK (P42/44) in RVLM and the hypotensive response, PD 98059, a specific inhibitor of the phosphorylation of MAPK kinase (Alessi et al., 1995) was used in this experiment.

Intracisternal PD 98059 had no significant effect on blood pressure (-2.1 ± 3.3 mmHg) or heart rate (-14 ± 17 beats/min) during the observation period (Fig.6). Compared with the vehicle (DMSO), pretreatment with PD 98059 virtually abolished the hypotensive response (Fig. 6) and the enhancement of MAPK phosphorylation (MAPK_{P42/44}) in the RVLM neurons (Fig. 7) caused by i.c rilmenidine.

Discussion

In the present study, we tested the hypothesis that the I₁-imidazoline receptor lowers blood pressure by a unique neuronal signaling process. The most important findings of the present study are: (i) we demonstrate, for the first time, an association between enhanced phosphorylation of MAPK (p42/44) in the RVLM neurons and the hypotensive response elicited by the selective I₁-receptor agonist rilmenidine, (ii) no change in the level of MAPK (p42/44) occurred in the RVLM or the NTS when a similar hypotensive response was elicited by the highly selective α_2 -agonist α -MNE and (iii) the selective I₁-receptor antagonist efaroxan or the ERK1/2 inhibitor PD 98059 significantly attenuated the hypotensive response and virtually abolished the enhancement of neuronal MAPK phosphorylation elicited by rilmenidine. Together, these findings suggest a functional role for RVLM neuronal MAPK_{p42/44} in the hypotension elicited by the I₁-receptor activation and presents evidence that highlights a neuronal mechanism for the I₁ receptor, which is distinct from the signal transduction pathway triggered by the α_2 -AR.

Results of the present study demonstrated that rilmenidine-evoked hypotension was associated with significant enhancement of MAPK phosphorylation in the RVLM neurons. This finding extends our previous findings in differentiated PC12 cells, which demonstrated concentration-dependent enhancement of MAPK phosphorylation by rilmenidine (Zhang et al., 2001). The finding was later confirmed by Edwards et al (Edwards et al., 2001). More importantly, the current findings highlight the interesting possibility that the enhanced phosphorylation of MAPK in the RVLM, triggered by the I₁-receptor activation, represents a novel neuronal mechanism for the hypotensive action of the second generation centrally acting drugs such as rilmenidine and moxonidine. The present findings may explain, at least

partly, the very recently reported difference in the neural pathways that mediate the hypotensive actions of clonidine and moxonidine (Chan et al., 2005).

We measured phosphorylated MAPK as intracellular signal that reflects the interaction between the I₁-receptor and PC-PLC. Reported studies including ours have delineated this signal transduction pathway in differentiated (NGF treated) PC12 cells (Separovic et al., 1997; Zhang et al., 2001). Notably, the present study is the first to replicate such a pathway in vivo and circumvents some of the limitations of the cell culture studies such as the required addition of NGF to the culture medium (Separovic et al., 1997; Zhang et al., 2001). It was imperative that we observe enhanced phosphorylation of MAPK in the RVLM neurons if such neuronal signaling bears relevance to the pharmacological (hypotensive) action produced by I₁-receptor activation. This is because the RVLM is the major site for the I₁-mediated hypotension (Haxhiu et al., 1994; Ernsberger et al., 1995; Ernsberger and Haxhiu, 1997; Head et al., 1998). Notably, the findings of the first set of experiments in the present study provided correlative association between the enhanced neuronal MAPK phosphorylation in the RVLM and the hypotensive response elicited by systemic rilmenidine. Further, it may be argued that the neuronal signal and/or the hypotensive response resulted, at least in part, from the activation of the α_2 -AR because: (i) the RVLM contains I₁ and α_2 -ARs (Reis, 1996), (ii) rilmenidine, even though a selective I₁-agonist, also activates the α_2 -AR (Szabo, 2002). In effect, some investigators believe that the hypotensive action of the second generation centrally acting drugs depends, directly or indirectly, on the α_2 -AR (Szabo et al., 1993; Regunathan et al., 1995; Zhu et al., 1999).

To rule out a possible involvement of the α_2 -AR in the enhanced MAPK phosphorylation in the RVLM caused by rilmenidine, we administered intracisternally α -

MNE, which is considered pure α_2 -receptor agonist (Szabo, 2002). Because α -MNE failed to enhance the phosphorylation of MAPK in the RVLM, while producing a hypotensive response similar to that elicited by rilmenidine, our findings rule out the involvement of α_2 -AR in the modulation MAPK phosphorylation in the RVLM. It is also important to note that the following findings rule out the possibility that the enhanced phosphorylation of MAPK in the RVLM was caused by changes in blood pressure. First, the enhancement of RVLM MAPK phosphorylation after i.c rilmenidine rules out the possibility that the initial pressor response or other peripheral effects caused by i.v. rilmenidine in our preliminary study (Fig. 1) contributed to this response. Second, a reduction in blood pressure elicited by α -MNE, similar to that elicited by rilmenidine, was not associated with enhanced phosphorylation of MAPK in the RVLM. Together, these findings suggest that the enhanced phosphorylation of MAPK in the RVLM is mediated via the activation of central I_1 -receptor.

It must be remembered that we report significant increase in RVLM neuronal MAPK phosphorylation (P42/44) at one time point, 15 min after drug administration. This time was chosen because our previous findings in PC12 cells showed MAPK phosphorylation starts at 2-5 min and reaches a maximal response 15 min after I_1 -receptor activation (Zhang et al., 2001). Nonetheless, the possibility must be considered that the enhanced phosphorylation of MAPK in the RVLM and the hypotensive response caused by rilmenidine may be unrelated events. Notably, earlier attempts to link the PC-PLC signal transduction pathway to the hypotensive response elicited by another I_1 -receptor agonist, moxonidine, were confounded by some experimental conditions. Ernsberger et al. (Separovic et al., 1997) showed that pretreatment with the PC-PLC inhibitor D609 abolished the hypotensive effect of moxonidine. However, the significant pressor response caused by intra-RVLM D609 in the

reported study may have confounded the data interpretation. Further, the specificity of D609 to PC-PLC, which has been questioned in cell culture studies including ours (Zhang et al., 2001) has not been evaluated in vivo. No neuronal marker that reflects the activation of the PC-PLC in the absence and presence of D609 was investigated in the reported in vivo study (Separovic et al., 1997). In the present study, the selective I₁- antagonist efaroxan, used in a dose that preferentially blocks the I₁-receptor (El-Mas and Abdel-Rahman, 2001) virtually abolished the enhancement in MAPK phosphorylation in RVLM neurons and the hypotensive response elicited by I₁-receptor activation.

Furthermore, we reasoned that if the enhanced phosphorylation of MAPK in the RVLM plays a causal role, at least partly, in the I₁-mediated hypotension, then inhibition of MAPK phosphorylation is expected to attenuate the hypotensive response. To achieve this goal, we used PD98059, a selective inhibitor of the activation of MAPK kinase (MEK), an upstream regulator of MAPK (Alessi et al., 1995), in a dose that exhibits no neurotoxic effect in vivo (Seyedabadi et al., 2001). In support of our hypothesis, PD98059 virtually abolished the enhancement of MAPK phosphorylation (MAPK_{P42/44}) in the RVLM and the hypotensive response caused by rilmenidine. These results support a possible causal role for phosphorylated MAPK_{P42/44} in the RVLM in the hypotensive action of rilmenidine. However, a contradictory role for MAPK_{P42/44} in the RVLM in blood pressure regulation has been reported. In anesthetized SHR and WKY rats, intra-RVLM PD98059 lowered blood pressure, which has been attributed to inhibition of PD98059-sensitive genomic processes (Seyedabadi et al., 2001). The objective of the reported study was to allow time (14 hr) for possible transcriptional and translational consequences to take place after kinase inhibition (Seyedabadi et al., 2001). We focused on the rapid effects of PD98059 because the I₁-

mediated phosphorylation of MAPK starts within minutes and peaks at 15 min (Zhang et al., 2001). Nonetheless, in spite of the differences in experimental conditions between the present and the reported (Seyedabadi et al., 2001) studies, the blood pressure remained virtually unaltered during the first 30-60 min after i.c injection (present study) or intra-RVLM (Seyedabadi et al., 2001) injection of PD 98059. Whether, the delayed hypotensive response observed after intra-RVLM PD98059 was related to changes in RVLM MAPK_{p42/44} is not known because the latter was not measured in the reported study (Seyedabadi et al., 2001).

In conclusion, the present findings suggest a causal relationship between enhanced phosphorylation of MAPK in the RVLM neurons and the hypotensive response elicited by the central I₁-receptor activation. The enhanced phosphorylation of MAPK serves as a reliable marker for the PC-PLC signal transduction pathway triggered by I₁- receptor activation in cell culture as well as in vivo, which establishes relevance for this pathway to the hypotensive response elicited by the second generation centrally acting drugs such as rilmenidine. The present findings provide the first evidence that yields insight into the consequence of the rapid phosphorylation of MAPK_{p42/44} in the RVLM on blood pressure and supports a unique neuronal signaling for the I₁-receptor in vivo.

Acknowledgements

The authors thank Ms. Kui Sun for her excellent technical assistance.

References

- Alessi DR, Cuenda A, Cohen P, Dudley DT and Saltiel AR (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. *J Biol Chem* **270**:27489-27494.
- Bousquet P, Grenay H, Bruban V, Schann S, Ehrhardt JD, Monassier L and Feldman J (2003) I(1) imidazoline receptors involved in cardiovascular regulation: where are we and where are we going? *Ann N Y Acad Sci* **1009**:228-233.
- Bruban V, Estato V, Schann S, Ehrhardt JD, Monassier L, Renard P, Scalbert E, Feldman J and Bousquet P (2002) Evidence for synergy between alpha(2)-adrenergic and nonadrenergic mechanisms in central blood pressure regulation. *Circulation* **105**:1116-1121.
- Chan CK, Burke SL, Zhu H, Piletz JE and Head GA (2005) Imidazoline receptors associated with noradrenergic terminals in the rostral ventrolateral medulla mediate the hypotensive responses of moxonidine but not clonidine. *Neuroscience* **132**:991-1007.
- Edwards L, Fishman D, Horowitz P, Bourbon N, Kester M and Ernsberger P (2001) The I1-imidazoline receptor in PC12 pheochromocytoma cells activates protein kinases C, extracellular signal-regulated kinase (ERK) and c-jun N-terminal kinase (JNK). *J Neurochem* **79**:931-940.
- El-Mas MM and Abdel-Rahman AA (1998) Ethanol selectively counteracts hypotension evoked by central I(1)- imidazoline but not alpha2-adrenergic receptor activation in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **32**:382-389.

- El-Mas MM and Abdel-Rahman AA (2001) Evidence for the involvement of central I1 imidazoline receptor in ethanol counteraction of clonidine hypotension in spontaneously hypertensive rats. *J Cardiovasc Pharmacol* **38**:417-426.
- El-Mas MM, Carroll RG and Abdel-Rahman AA (1994a) Centrally mediated reduction in cardiac output elicits the enhanced hypotensive effect of clonidine in conscious aortic barodenervated rats. *J Cardiovasc Pharmacol* **24**:184-193.
- El-Mas MM, Tao S, Carroll RG and Abdel-Rahman AA (1994b) Ethanol-clonidine hemodynamic interaction in normotensive rats is modified by anesthesia. *Alcohol* **11**:307-314.
- Ernsberger P, Graves ME, Graff LM, Zakieh N, Nguyen P, Collins LA, Westbrook KL and Johnson GG (1995) I1-imidazoline receptors. Definition, characterization, distribution, and transmembrane signaling. *Ann N Y Acad Sci* **763**:22-42.
- Ernsberger P and Haxhiu MA (1997) The I1-imidazoline-binding site is a functional receptor mediating vasodepression via the ventral medulla. *Am J Physiol* **273**:R1572-1579.
- Gerfen C (1997) Basic Neuroanatomical Methods, in *Current Protocols in Neuroscience* (Crawley JN, Gerfen CR, Rogawski MA, Sibley DR, Skolnick P and Wray S eds) pp 1.1.1-1.1.11, John Wiley & Sons, Inc.
- Gomez RE, Ernsberger P, Feinland G and Reis DJ (1991) Rilmenidine lowers arterial pressure via imidazole receptors in brainstem C1 area. *Eur J Pharmacol* **195**:181-191.

- Haxhiu MA, Dreshaj I, Schafer SG and Ernsberger P (1994) Selective antihypertensive action of moxonidine is mediated mainly by I1-imidazoline receptors in the rostral ventrolateral medulla. *J Cardiovasc Pharmacol* **24**:S1-8.
- Head GA, Burke SL and Chan CK (1998) Site and receptors involved in the sympathoinhibitory actions of rilmenidine. *J Hypertens Suppl* **16**:S7-12.
- Ince E and Levey A (1997) Immunohistochemical Localization of Neurochemicals, in *Current Protocols in Neuroscience* (Crawley JN, Gerfen CR, Rogawski MA, Sibley DR, Skolnick P and Wray S eds) pp 1.1.1-1.1.11, John Wiley & Sons, Inc.
- Paxinos G and Watson C (1982) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, Sydney.
- Regunathan S, Bramwell S and Reis DJ (1995) Effects of rilmenidine on signal transduction mechanisms associated with alpha 2-adrenergic and imidazoline receptors in brain. *Ann N Y Acad Sci* **763**:290-294.
- Reis DJ (1996) Neurons and receptors in the rostroventrolateral medulla mediating the antihypertensive actions of drugs acting at imidazoline receptors. *J Cardiovasc Pharmacol* **27**:S11-18.
- Separovic D, Kester M and Ernsberger P (1996) Coupling of I1-imidazoline receptors to diacylglyceride accumulation in PC12 rat pheochromocytoma cells. *Mol Pharmacol* **49**:668-675.
- Separovic D, Kester M, Haxhiu MA and Ernsberger P (1997) Activation of phosphatidylcholine-selective phospholipase C by I1- imidazoline receptors in PC12 cells and rostral ventrolateral medulla. *Brain Res* **749**:335-339.

- Seyedabadi M, Goodchild AK and Pilowsky PM (2001) Differential role of kinases in brain stem of hypertensive and normotensive rats. *Hypertension* **38**:1087-1092.
- Szabo B (2002) Imidazoline antihypertensive drugs: a critical review on their mechanism of action. *Pharmacol Ther* **93**:1-35.
- Szabo B, Urban R and Starke K (1993) Sympathoinhibition by rilmenidine in conscious rabbits: involvement of alpha 2-adrenoceptors. *Naunyn Schmiedebergs Arch Pharmacol* **348**:593-600.
- Zhang J, El-Mas MM and Abdel-Rahman AA (2001) Imidazoline I(1) receptor-induced activation of phosphatidylcholine- specific phospholipase C elicits mitogen-activated protein kinase phosphorylation in PC12 cells. *Eur J Pharmacol* **415**:117-125.
- Zhu QM, Lesnick JD, Jasper JR, MacLennan SJ, Dillon MP, Eglen RM and Blue DR, Jr. (1999) alpha 2A-adrenoceptors, not I1-imidazoline receptors, mediate the hypotensive effects of rilmenidine and moxonidine in conscious mice. In vivo and in vitro studies. *Ann N Y Acad Sci* **881**:287-289.

Footnotes

This work was supported by the grant NIAAA 07839 from the National Institute on Alcohol Abuse and Alcoholism.

Legends for Figures

Fig. 1. Effect of systemic administration of rilmenidine (Ril, 600 $\mu\text{g}/\text{kg}$, i.v.) or equal volume of saline on blood pressure, heart rate and the phosphorylation of MAPK in anesthetized Sprague-Dawley rats. Upper panels show sections of the brain stained with thionin (A) or with antibody against phosphorylated MAPK (B). The positive neuronal cells demonstrated strong dark-brown signal in the cell body and processes in RVLM. (Amb: ambiguous nucleus). (Scale bar is 200 μm). The Lower two panels show that rilmenidine increased the number of cells expressing the phosphorylated MAPK (P42/44) in RVLM. No cells in the NTS were immunostained for phosphorylated MAPK (scale bar is 100 μm). Bar graph represents blood pressure and heart rate 15 min after rilmenidine along with the number of positive cells in the RVLM. Values are mean \pm S.E.M., and the number in parentheses indicates the number of the rats in each group. *, $P < 0.05$, compared with the corresponding control value.

Fig. 2. Intracisternal rilmenidine (Ril, 25 μg) or α -methylnorepinephrine (α -MNE, 4 μg) elicited similar hypotensive (A) and bradycardic (B) responses. Equal volume of ACSF (i.c.) served as control. Values are mean \pm S.E.M., and the number in parentheses indicates the number of the rats used in each group. * or #, $P < 0.05$, compared with the corresponding control value.

Fig. 3. Intracisternal rilmenidine (Ril, 25 μg), but not α -methylnorepinephrine (α -MNE, 4 μg) increased the number of cells expressing phosphorylated MAPK (p42/44) in the RVLM. The photomicrograph shows the immunohistochemical image of

phosphorylated MAPK. No cell in the NTS was immunostained for phosphorylated MAPK (scale bar is 100 μm). Bar graph represents the number of the positive cell in the RVLM. Values are mean \pm S.E.M., and the number in parentheses indicates the number of the rats used in each group. *, $P < 0.05$, compared with the value of ACSF control. #, $P < 0.05$, compared with the mean between rilmenidine and α -methylnorepinephrine groups.

Fig. 4. Effect of intracisternal efaroxan (Efa, 0.15 μg) compared with ACSF on the hypotensive (A) and bradycardic (B) responses elicited by subsequent rilmenidine (Ril, 25 μg ; i.c) administration. Values are mean \pm S.E.M., and the number in parentheses indicates the number of the rats used in each group. * $P < 0.05$, compared with the corresponding control.

Fig. 5. Effect of pretreatment with efaroxan (Efa, 0.15 μg , i.c.) compared with ACSF on the enhanced phosphorylation of MAPK induced by rilmenidine (Ril, 25 μg , i.c.) in the RVLM. The photomicrographs show the immunohistochemical image of phosphorylated MAPK (scale bar is 100 μm). Bar graph represents the number of the positive cells. Values are mean \pm S.E.M., and the number in parentheses indicates the number of the rats used in each group. * $P < 0.05$, compared with the value of ACSF-Ril control group.

Fig. 6. Effect of pretreatment with the ERK1/2 inhibitor PD98059 (PD, 5 μg , i.c.) compared with DMSO (the vehicle of PD98059) on the hypotensive (A), and

bradycardic (B) responses elicited by subsequent rilmenidine (Ril, 25 μ g; i.c) administration. Values are mean \pm S.E.M., and the number in parentheses indicates the number of the rats used in each group. * or #. $P < 0.05$, compared with the corresponding control value.

Fig. 7. Pretreatment with PD98059 (PD, 5 μ g, i.c.) virtually abolished the enhanced phosphorylation of the MAPK (p42/44) induced by rilmenidine (Ril, 25 μ g, i.c.) in the RVLM. The photomicrographs show the immunohistochemical image of phosphorylated MAPK (p42/44) (scale bar is 100 μ m). Bar graph represents the number of the positive cells. Values are mean \pm S.E.M., and the number in parentheses indicates the number of the rats used in each group. *, $P < 0.05$, compared with the PD-ACSF group. #. $P < 0.05$, comparing DMSO-Ril and PD-Ril groups.

Table 1: Baseline values of mean arterial pressure (MAP, mmHg) and heart rate (HR, beats/min). Values are mean \pm S.M.E.; n is the number of the rats in each group.

Groups	MAP (mmHg)	HR (beats/min)	n
Saline	101.0 \pm 5.0	335 \pm 6	4
Rilmenidine (i.v.)	102.2 \pm 6.7	350 \pm 18	6
ACSF	88.3 \pm 2.8	280 \pm 10	4
Rilmenidine (i.c.)	94.4 \pm 6.6	283 \pm 12	4
α -MNE	96.0 \pm 2.7	311 \pm 10	7
ACSF- Rilmenidine	99.0 \pm 5.3	296 \pm 11	5
Efaroxan - Rilmenidine	88.3 \pm 5.4	294 \pm 14	7
PD 98059 - ACSF	97.1 \pm 14.8	304 \pm 20	5
DMSO - Rilmenidine	103.1 \pm 5.9	355 \pm 15	6
PD 98059 - Rilmenidine	101.1 \pm 8.5	330 \pm 22	8

Fig. 1

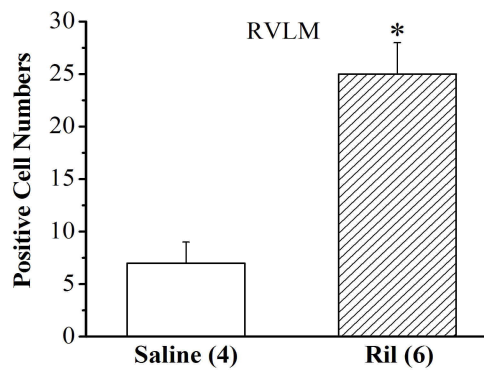
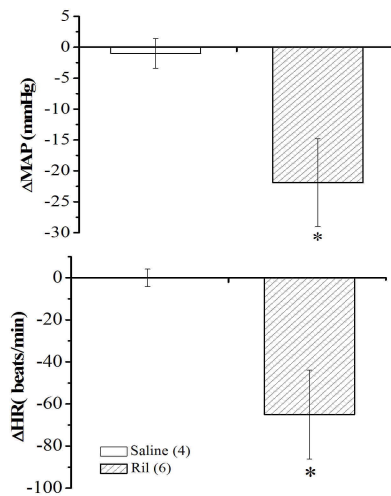
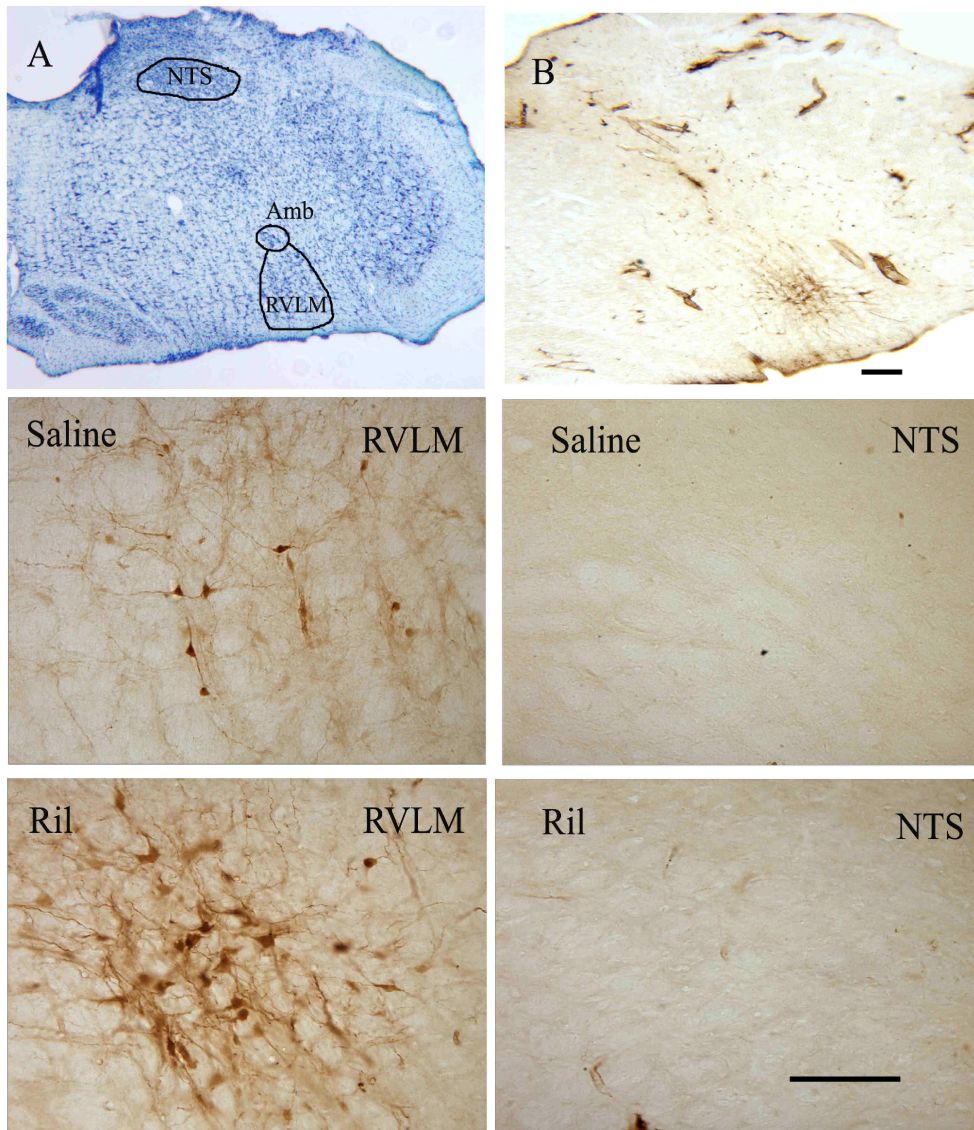


Fig. 2

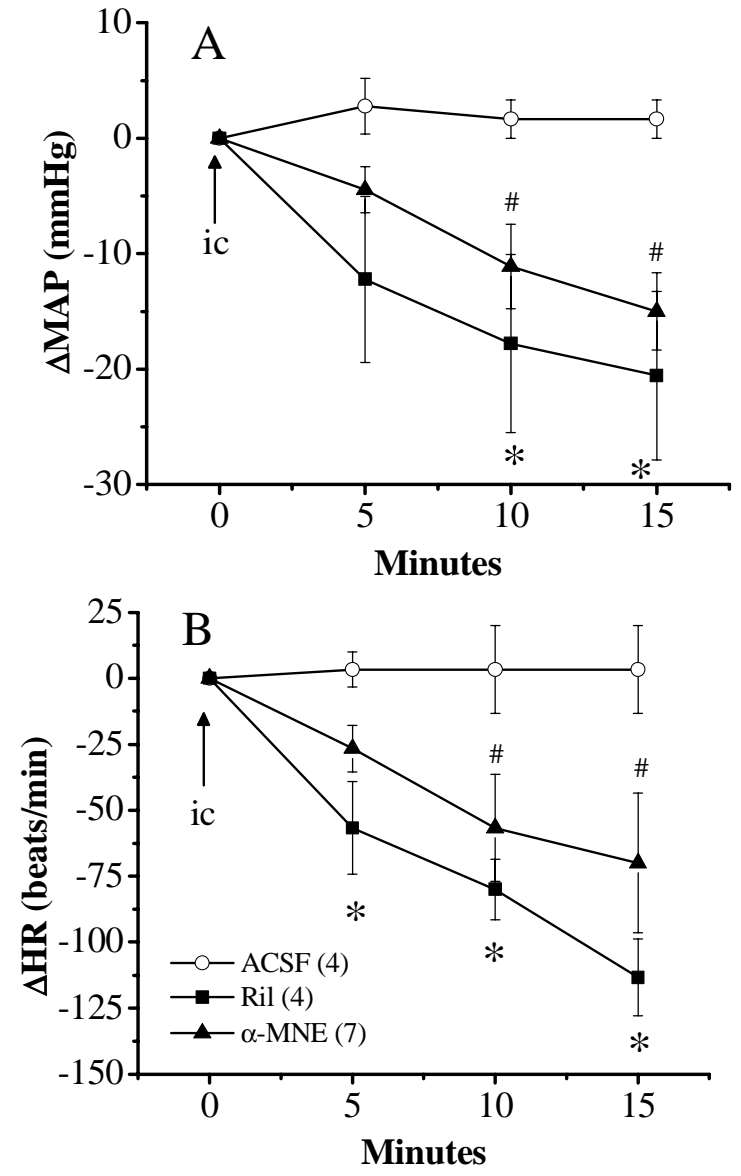


Fig. 3

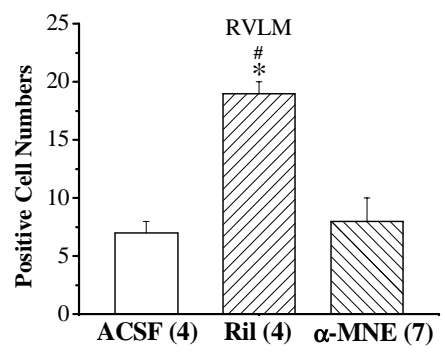
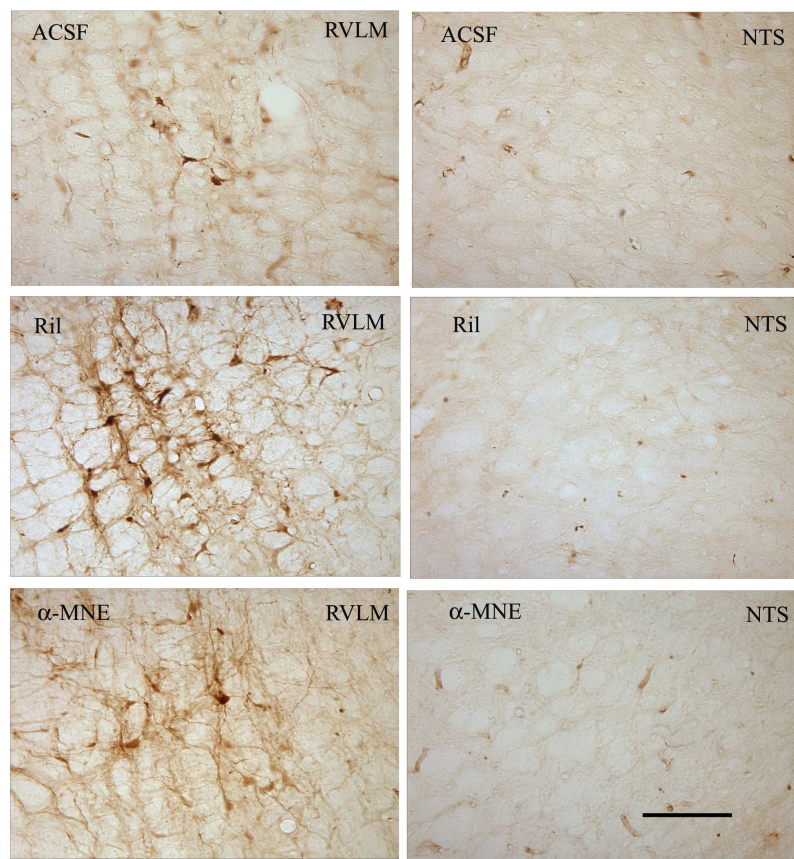


Fig. 4

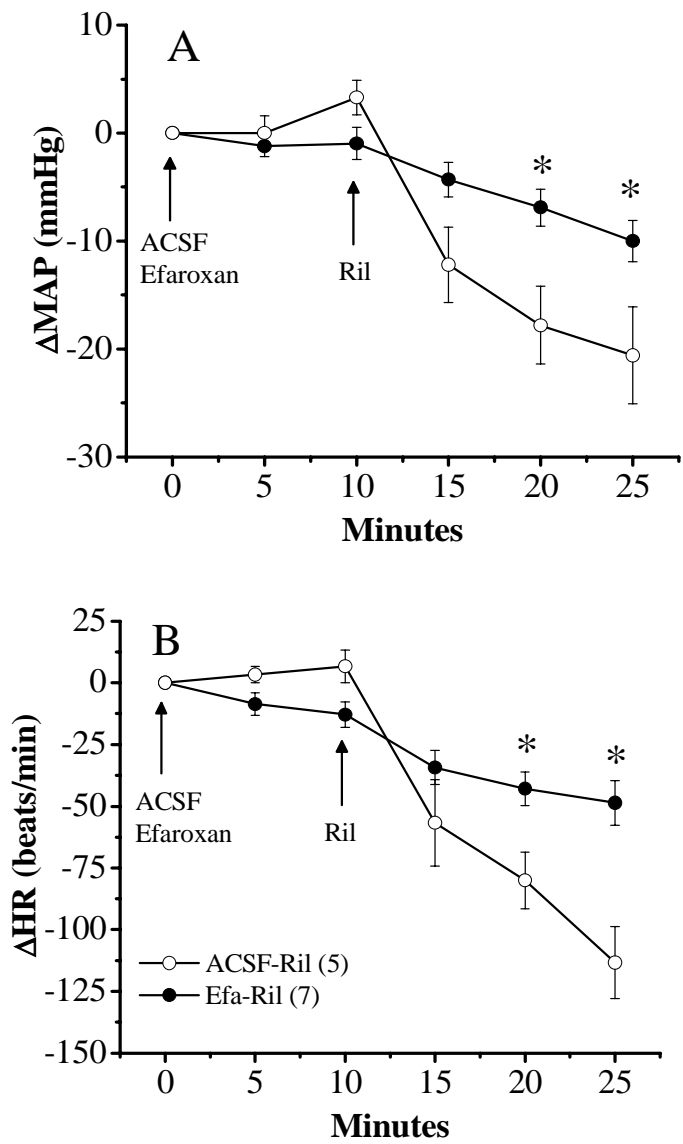


Fig. 5

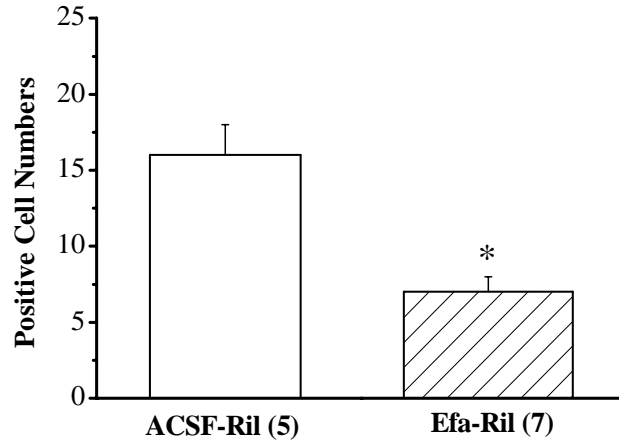
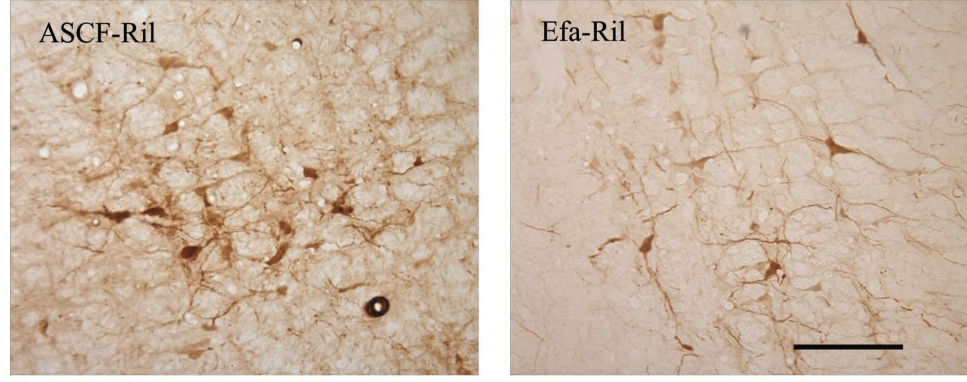


Fig. 6

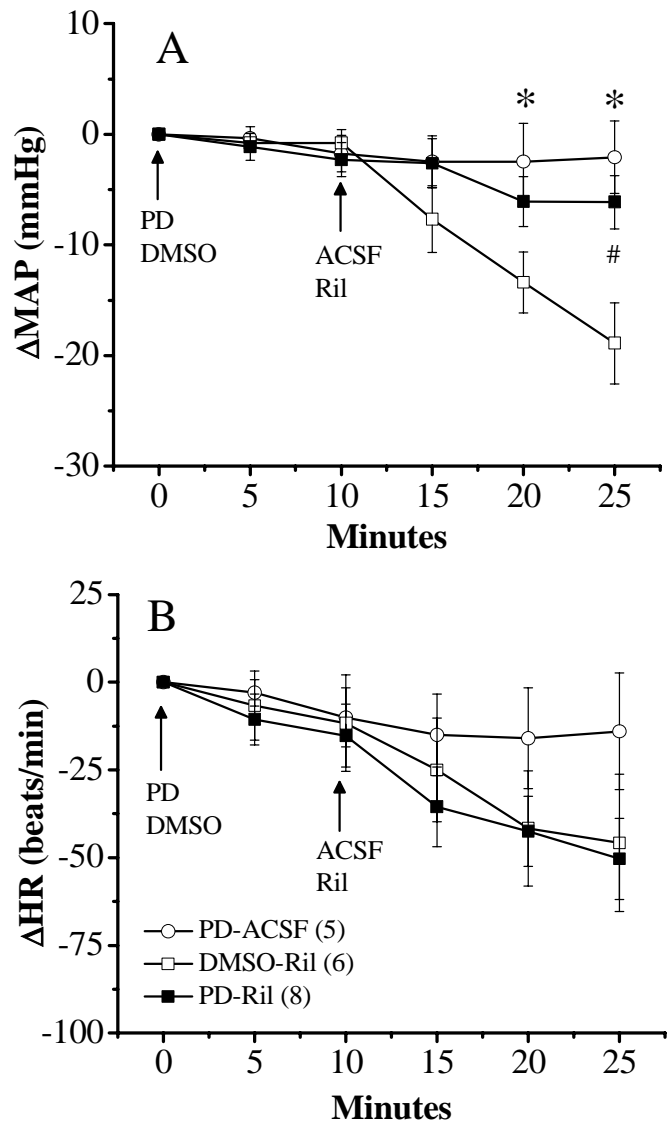


Fig. 7

