Dexmedetomidine Increases Hippocampal Phosphorylated Extracellular Signal–regulated Protein Kinase 1 and 2 Content by an α2-Adrenoceptor–Independent Mechanism

Evidence for the Involvement of Imidazoline II Receptors

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Background: Dexmedetomidine is a potent and selective α2-adrenoceptor (α2AR) agonist that exhibits a broad pattern of actions, including sedation, analgesia, and neuroprotection. Recent studies have emphasized the role of phosphorylated intracellular signal–regulated protein kinases (pERK1 and 2) in coupling rapid events such as neurotransmitter release and receptor stimulation long-lasting changes in synaptic plasticity and cell survival. Here, the authors hypothesized that dexmedetomidine increases pERK1 and 2 content and examined the mechanisms involved in this effect.

Methods: The effects of dexmedetomidine and their sensitivity to various pharmacologic agents on expression of pERK1 and 2 were studied by Western blots in hippocampal slices obtained from rats, wild-type mice, and mice carrying targeted deletions of the α2AR subtypes.

Results: Dexmedetomidine induced a concentration-related increase in the expression of pERK1 and 2 in rat hippocampal slices (EC50 [95% confidence interval] for pERK1, 0.97 [0.68–1.37] μM; for pERK2, 1.15 [0.62–2.14] μM). This effect was insensitive to the inhibitors of the α2AR-mediated signaling pathway, to prazosin, and to PP2, an inhibitor of the focal adhesion kinase–Src kinases. In contrast, it was still present in mice deleted for each of the α2AR subtypes and was markedly decreased by the antagonist of the II-imidazoline receptor eserine, by phospholipase C and protein kinase C inhibitors, and by PD 98059, a direct inhibitor of ERK1 and 2 phosphorylation.

Conclusion: Dexmedetomidine increases the expression of pERK1 and 2 via mechanisms independent of α2AR activation. The II-imidazoline receptors likely contribute to these effects. The results may be relevant to some long-term effects (e.g., neuroprotective) of dexmedetomidine in the brain.

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Received from INSERM U 676, Hopital Robert Debré, Paris, France; the Department of Anesthesia and Intensive Care, Beaujon University Hospital, Assistance Publique des Hôpitaux de Paris, Clichy, France; the Institute of Experimental and Clinical Pharmacology and Toxicology, University of Freiburg, Freiburg im Breisgau, Germany; and the Department of Anesthesia and Intensive Care, Schleswig Holstein University Hospital, Campus Kiel, Kiel, Germany. Submitted for publication June 22, 2007. Accepted for publication October 24, 2007. Supported by grants from INSERM, Paris, France, and the Société Française d’Anesthésie et de Réanimation, Paris France. Outi Mäki-Ikkola, Ph.D. (Senior Researcher, Orionpharma, Turku, Finland), provided dexmedetomidine.

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Dexmedetomidine is a potent and highly selective agonist of the α2-adrenergic receptors with a broad spectrum of actions on the human brain, including clinical sedation, anesthetic-sparing effects, and analgesia.1–5 Besides its action at α2-adrenergic receptors, dexmedetomidine also exhibits some affinity for imidazoline binding sites.6–8 Recent experimental work indicates that dexmedetomidine also exhibits more long-term effects on the brain, such as neuroprotection against ischemic injury.9–14 We have shown that dexmedetomidine reduces the severity of excitotoxic brain injury in the perinatal period in mice.9 Well-designed experiments performed in knockout animals have shown that this effect is mediated by the activation of the α2A-adrenoceptor subtype.10,11 These neuroprotective properties of dexmedetomidine most likely proceed via activation of intracellular signaling cascades, while an indirect effect through a reduction in the release of glutamate or catecholamines cannot be ruled out.12,13 We have shown that dexmedetomidine preconditions brain tissue against ischemic cell death and caspase-3 expression in hippocampal slices subjected to oxygen glucose deprivation.14 This effect is mediated in part by an increase in phosphorylation of focal adhesion kinase, a nonreceptor tyrosine kinase coupling rapid events such as ligand-receptor interactions, or action potential propagation, to long-lasting changes in synaptic plasticity.15 Also, dexmedetomidine exerts synergistic neuroprotective effects with xenon that have been observed 30 days after ischemic injury.16

Extracellular signal–regulated kinases 1 and 2 (ERK1 and 2) are two members of the mitogen-activated protein kinases that play a pivotal role in cell signaling that control many forms of cellular activities, synaptic plasticity, long-term potentiation, and cell survival.17,18 Activation of ERK1 and 2 results from phosphorylation on threonine(202) and tyrosine(204) via various stimuli, including activation of N-methyl-D-aspartate receptors, neurotrophic factors, and G proteins.19 Once phosphorylated, ERK1 and 2 translocate from the cytosol to the nucleus to activate specific transcription factors, leading to inducible gene expression.20 Therefore, ERK1 and 2 may represent putative cellular targets that may mediate some long-lasting effects of dexmedetomidine on the brain. The aim of the current study was to examine the effect of dexmedetomidine on the content of phosphor-
yalted (active) ERK1 and 2 (pERK1 and 2) in the hippocampus, and whether the α2-adrenergic and/or imidazole 11 receptors played a role in mediating this effect.

Materials and Methods

Animals

Handling procedures according to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD) were followed throughout. Approval was obtained from the Institutional Animal Care and Use Committee at Paris VII University (Paris, France). This study, including care of the animals involved, was conducted according to the official edict presented by the French Ministry of Agriculture (Paris, France) and the recommendations of the Declaration of Helsinki. Therefore, these experiments were conducted in an authorized laboratory and under the supervision of an authorized researcher (Gressens Pierre, M.D., Ph.D., Institut National de la Santé et de la Recherche Médicale U 676, Paris, France). Experiments were performed on adult male Sprague-Dawley rats weighing 250 g (range, 220–275 g; Ifla-Credo, L’Arbresle, France) and C57BL6 mice. In house adult wild-type and transgenic mice lacking α2A, α2B, or α2C adrenoceptor subtypes were used for the experiments (weight, 18–27 g), as previously reported. Because initial reports used mice generated on a 75% C57BL6/J/25% 129Sv background to do partial embryonic lethality of congenic α2B-deficient mice. They were maintained on a C57BL6/129Sv background. In contrast, α2B-deficient mice were maintained on a 75% C57BL6/J/25% 129Sv background to do partial embryonic lethality of congenic α2B-deficient mice. They were maintained on a C57BL6/129Sv background.

Experimental Protocol

The procedures for hippocampal slice preparation have been extensively described elsewhere. Briefly, the hippocampus of each hemisphere was carefully dissected and incubated in Ca2+-free artificial cerebrospinal fluid (4°C, 126.5 mM NaCl, 27.5 mM NaHCO3, 2.4 mM KCl, 0.5 mM KH2PO4, 1.93 mM MgCl2, 0.5 mM Na2SO4, 4 mM glucose, and 11 mM HEPES, adjusted to pH 7.4 with 95% O2–5% CO2 (vol/vol) mixture. Hippocampal slices (each 300 µm thick) prepared with a MacIlwain tissue chopper were transferred to polypropylene tubes (three slices per tube) containing 1 ml artificial cerebrospinal fluid (60 min, 37°C). Slices were incubated for 60 min at 37°C with moderate agitation under a humidified atmosphere of 95% O2–5% CO2 (vol/vol) until pharmacologic treatments were added together with CaCl2. Dexmedetomidine was applied at various concentrations (10−5 M to 10−3 M) for 5 min. This duration of incubation was determined according to preliminary experiments in which the increase in pERK1 and 2 induced by 0.2 µm dexmedetomidine was plotted against duration of incubation (1, 2, 5, 10, 20, and 30 min) in the presence of the phosphatase inhibitors orthovanadate (an inhibitor of tyrosine phosphatases, 1 mM) and okadaic acid (an inhibitor of serine–threonine phosphatases, 10−5 M). It corresponded to the point for which the ceiling was reached after a linear increase in phosphorylation between 1 and 5 min. Agents supposed to increase the expression of pERK1 and 2 were applied for 5 min as well. Agents supposed to block dexmedetomidine (or any other activator)–induced changes in phosphorylated ERK1 and 2 expression were administered 1 h before dexmedetomidine (or any other activator). At the end of the experiments, cerebrospinal fluid was aspirated, and slices were frozen in liquid nitrogen, homogenized by sonication in 200 µl of a solution of 1% (wt/vol) sodium dodecyl sulfate, 1 mM sodium orthovanadate, and antiproteases (50 µg/ml leupeptin, 10 µg/ml aprotinin, and 5 µg/ml pepstatin) in water at 100°C, and then placed in a boiling bath for 5 min. Homogenates were stored at −80°C until processing.

Immunoblot Analysis

Protein concentration in the homogenates was determined with a bicinechonic acid–based method, using bovine serum albumin as the standard. Equal amounts of protein (30 µg) were subjected to 6% (wt/vol) polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and transferred electrophoretically to nitrocellulose. Blots were incubated in a primary rabbit polyclonal antibody against pERK1 and 2 (threonine 202/tyrosine 204; Cell Signaling, Beverly, MA: 1:1,000), ERK1 and 2 (Cell Signaling; 1:1,000), and β-actin (specific monoclonal antiactin A5316 antibody; Sigma, St-Quentin Fallavier, France) overnight at 4°C and then labeled with goat peroxidase–coupled secondary antibodies against rabbit immunoglobulin G (Jackson Immuno Research Laboratory, West Grove, PA; 1:5,000). These were detected by exposure of molecular probe autoradiographic films in the presence of a chemiluminescent reagent (ECL; Amersham, Little Chalfont, United Kingdom). Immunoreactive bands were digitalized and analyzed using a Cohu High Performance charge-coupled device camera (Gel Analyst 3.01 pci; Paris, France). The same primary antibodies were used in mice and rats because they worked well in both species. Variations between gels were controlled by expressing the results as a percentage of increase or decrease from control of ERK1 and 2 or pERK1 and 2 normalized to β-actin (quan-
Chemicals
The following agents were studied alone or in combination: dexmedetomidine (10⁻⁷ m to 10⁻³ m), tetrodotoxin (1 μm; Sigma), N-methyl-D-aspartate (10⁻³ m; Sigma), MK801 (10⁻⁴ m; Sigma), pertussis toxin (10⁻⁴ m; Sigma), PD 098059 (a direct inhibitor of ERK1 and 2 phosphorylation, 50 μm; Sigma), chelerythrine and bisindolylinmaleimide I (RO318220, two structurally distinct inhibitors of protein kinase C, 10⁻³ M; Merck Biosciences, Fontenay-sous-Bois, France), phorbol 12-myristate 13-acetate (an activator of protein kinase C, 10⁻⁷ M; Sigma), carbachol (an activator of phospholipase C, 10⁻⁴ m; Sigma), neomycin (an inhibitor of phospholipase C, 5 × 10⁻⁶ m; Sigma), the analog of cyclic adenosine monophosphate (cAMP) 8 bromo cAMP (4 × 10⁻³ m; Sigma), the inhibitor of phosphodiesterases 3-isobutyl-1-methylxanthine (10⁻⁴ m; Sigma), methoxamine (an agonist of the α₁ adrenoceptors, 10⁻⁵ m; Sigma), the inhibitor of cAMP-dependent protein kinase H89 (10⁻⁵ m; Sigma), prazosin (an antagonist of the α₁ adrenoceptors, 10⁻⁴ m; Sigma), yohimbine (an antagonist of the α₂ adrenoceptors, 10⁻⁻⁷ m; Sigma), 4-amino-5-(4-chlorophenyl)-7-(4-t-butyl)pyrazolo[3,4 days]pyrimidine (PP2, an inhibitor of Src [Rous sarcoma virus tyrosine kinase] tyrosine kinase, 10⁻⁵ m; Calbiochem, Nottingham, United Kingdom), 2-aminoethyl diphenyl borate (2APB, an inhibitor of the inositol 3 phosphate receptors, 10⁻⁴ m; Sigma), rilmenidine (a preferential agonist of the imidazoline I1 receptors and agonist of the α₂ adrenoceptors, 10⁻⁴ m; Sigma), efaroxan (an antagonist of the I1-imidazoline receptors and agonist of the α₂ adrenoceptors, 10⁻⁻⁷, 10⁻⁶, 10⁻⁵ m; Sigma), orthovanadate (an inhibitor of the tyrosine phosphatases, 1 μm; Sigma), and okadaic acid (an inhibitor of the serine-threonine phosphatases, 1 μm; Sigma).

Results
Selection of the Antibodies Used for Immunoblots and Behavior of Phosphorylated versus Nonphosphorylated ERK1 and 2
Omitting the primary anti-ERK1 and 2 or anti-phospho-ERK1 and 2 antibodies in blots with protein extracts from both mice and rat hippocampal slices produced no detectable immunoreactivity (typical example obtained in rats shown fig. 1). A single band was detected when using the primary antibody in the molecular weight

Statistical Analysis
The data presented were collected from at least 10 independent experiments (one rat per experiment) run in triplicate for Western blot analysis. Therefore, the sample size included in the analysis was never lower than 30, and data were considered normally distributed. Statistical analysis used analysis of variance with post hoc analysis using the Bonferroni correction. Factorial analysis of the effects of the interacting variables: control, PD 098059, and chelerythrine on dexmedetomidine-induced ERK1 and 2 activation in knockout and wild-type mice were also performed.

Concentration–response curves and statistical analysis were generated using the (Intuitive Software for Science, San Diego, CA). The functions used to fit the curves to the data were the following four-parameter logistic equation: Y = A + (B − A)/(1 + (X/EC50)⁴). Analysis of variance was generated using GraphPad 4.0 software (GraphPad Software, San Diego, CA). Factorial analysis was generated using SPSS 15.0 (SPSS Inc., Chicago, IL). Results (mean ± SD) are expressed as a percentage of control expression of phosphorylated ERK1 and 2. A P value less than 0.05 was considered the threshold for significance.
predicted for total ERK1 and 2 and biphosphorylated ERK1 and 2 (pERK1 and 2; fig. 1). N-methyl-d-aspartate produced an increase in pERK1 and 2 that was totally antagonized by MK 801 as previously described28 (figure 1). This emphasizes the selectivity of the ERK1 and 2 and pERK1 and 2 antibodies in this assay. The use of tetrodotoxin (1 μm) did not significantly modify the expression of ERK1 and 2 and pERK1 and 2 (data not shown). This indicates that neuronal firing did not have significant influence on the expression of pERK1 and 2. In addition, in all experiments performed in the current study leading to increased pERK1 and 2, total ERK1 and 2 immunoreactivity was not found to be different from control (data not shown). Expression of pERK1 also paralleled that of pERK2 in all experiments. Therefore, to simplify the presentation of the results, all figures display pERK2 content only, except from the dose–response curve displayed in figure 2.

Effects of Dexmedetomidine on ERK1 and 2 Phosphorylation in Rat Hippocampal Slices

Dexmedetomidine produced a significant, concentration-related increase in pERK1 and 2 expression, the EC50 values (95% confidence interval) being 0.97 (0.68 – 1.37) and 1.15 (0.62–2.14) μM for pERK1 and pERK2, respectively (fig. 2A). In contrast, dexmedetomidine did not affect basal levels of total ERK1 and 2 (fig. 2B). The magnitudes of the increase in phosphorylation at the EC50 values (mean ± SD) were 334 ± 30% and 316 ± 28% for pERK1 and pERK2, respectively. Dexmedetomidine (1 μM)–induced increase in pERK1 and 2 expression was completely blocked by PD 098059, an inhibitor of ERK1 and 2 phosphorylation (10–5 M; fig. 3). In contrast, it was insensitive to the α2-adrenergic antagonist yohimbine (10–4 M) or pertussis toxin (an activator of adenylyl cycles, 1 μM) (fig. 3). H89 (10–5 M), a CAMP-dependent inhibitor of protein kinase A or the association of the pem cant cAMP analog 8 bromo CAM (4 × 10–5 M) and the phosphodiesterase inhibitor 5-isobutyl-1-methylxanthine (10–4 M) did not modify pERK1 and 2 expression (fig. 4). PP2 (10–5 M), an Src tyrosine kinase inhibitor, failed to block dexmedetomidine-induced increase in pERK1 and 2 phosphorylation (fig. 4). The α1-adrenergic antagonist prazosin (10–4 M) also failed to affect dexmedetomidine-induced increase in pERK1 and 2 expression, whereas it completely blocked the increase induced by methoxamine (an agonist of the α1 adrenergic receptors, 10–5 M; fig. 5).

Dexmedetomidine-induced increase in pERK1 and 2 expression was blocked by efaroxan (an antagonist of the 11-imidazoline receptors and α2 adrenergic receptors, 10–5, 10–6, 10–7 M) in a concentration-related way. This agent (10–5 M) also significantly attenuated the rilmenidine (a preferential agonist of the imidazoline I1 receptors and agonist of the α2 adrenergic receptors, 10–4 M)–induced increase in pERK1 and 2 (fig. 6). The phospholipase C inhibitor neomycin (5 × 10–6 M for pERK1 and 2 (fig. 6).

Fig. 2. (B) Upper: Western blots of the effects of dexmedetomidine (Dex, 10–8 to 10–3 M) on total extracellular signal–regulated protein kinase 1 and 2 immunoreactivity (tERK1 and tERK2), and biphosphorylated extracellular signal–regulated protein kinase 1 and 2 immunoreactivity (pERK1 and pERK2). Lower: Concentration–response curves of the effects of dexmedetomidine (10–8 to 10–3 M) on biphospho-extracellular signal–regulated protein kinase (ERK) 2 immunoreactivity. Data (mean ± SD) are expressed as a fractional tERK2 phosphorylation on actin ratio increase from basal phosphorylation (control: 100%). Analyses of variance for tERK1 and 2 were F = 172.5, P < 0.0001 and F = 172.5, P < 0.0001, respectively. Post hoc analysis (Bonferroni correction): *** P < 0.0001 versus control, both pERK1 and pERK2. (A) Upper: Western blots of the effects of dexmedetomidine (10–8 to 10–3 M) on total extracellular signal–regulated protein kinase 1 and 2 immunoreactivity (tERK1 and tERK2). Lower: Effects of dexmedetomidine (10–8 to 10–3 M) on total extracellular signal–regulated protein kinase 1 and 2 immunoreactivity (tERK1 and tERK2). Data (mean ± SD) are expressed as a fractional tERK1 and tERK2 on actin ratio increase from basal phosphorylation. Analyses of variance for tERK1 and tERK2 were F = 1.105, P = 0.35 and F = 1.56, P = 0.32, respectively. NS = not significant.
or the protein kinase C inhibitors chelerythrine or RO318220 (10^{-5} M) also blocked or decrease dexmedetomidine-induced increase in pERK1 and 2 expression (fig. 7). This was observed while both carbachol (10^{-4} M) and 12-myristate 13-acetate (10^{-7} M)–induced increase in pERK1 and 2 was blocked by neomycin (chelerythrine and RO318220, respectively; fig. 7). Incubating slices with a Ca^{2+}-free medium or with 2APB (2-aminoethyl diphenyl

Fig. 3. Effects of PD 098059 (PD, 50 \mu M), yohimbine (Yoh, 10^{-4} \mu M), and pertussis toxin (PT, 10^{-4} \mu M) on dexmedetomidine (Dex, 10^{-6} \mu M)–induced extracellular signal–regulated protein kinase 2 biphosphorylation (pERK2). Data (mean ± SD) are expressed as a fractional pERK2 phosphorylation on actin ratio increase from basal phosphorylation (control: 100%). Analysis of variance for pERK2 was F = 1.002, P < 0.0001. Post hoc analysis used Bonferroni correction. * P < 0.01, *** P < 0.0001 versus control. NS = not significant.

Fig. 4. Effects of 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo[3,4-d]pyrimidine (PP2, 10^{-5} M), 8 bromo cAMP (8B, 4 \times 10^{-3} \mu M), 3-isobutyl-1-methylxanthine (IBMX, 10^{-5} \mu M), and H89 (10^{-5} \mu M) on dexmedetomidine (Dex, 10^{-6} \mu M)–induced extracellular signal–regulated protein kinase 2 biphosphorylation (pERK2). Data (mean ± SD) are expressed as a fractional pERK2 on actin ratio increase from basal phosphorylation (control: 100%). Analysis of variance for pERK2 was F = 901.9, P < 0.0001. Post hoc analysis used Bonferroni correction. *** P < 0.0001 versus control, # P < 0.0001 versus PP2. NS = not significant.
bortate, $10^{-4}$ M) failed to affect dexmedetomidine-induced increase in pERK1 and 2 expression (fig. 8).

**Effects of Dexmedetomidine on ERK1 and 2 Phosphorylation in Mouse Hippocampal Slices**

In wild-type mice, dexmedetomidine (1 μM) induced a similar increase in pERK1 and 2 expression as was observed in the rat. This response was insensitive to yohimbine but was blocked by chelerythrine and PD 098059 as was observed in the rat. In mice lacking α2A, α2B, or α2C adrenoceptors, dexmedetomidine-induced increase in pERK1 and 2 expression and sensitivity to PD 098059 and chelerythrine was not different from that of the wild-type animals. Factorial analysis found no effects of the interacting variables: control, ERK1 and 2 inhibition (PD 098059), and protein kinase C inhibition (chel-
crythrine) on dexmedetomidine-induced ERK1 and 2 activation in knockout and wild-type mice.

Discussion

In the current study, we have shown that dexmedetomidine increases the expression of pERK1 and 2, a key enzyme in signal transduction. This phenomenon is independent from the α2 adrenoceptors, and the I1-imidazoline receptors are a good candidate to account for this effect. These results may contribute to a better understanding of some long-term effects of dexmedetomidine, such as preconditioning and/or neuroprotection.

Methodologic Considerations and Limitations

Both rats and mice were used to examine the effects of dexmedetomidine on ERK1 and 2 phosphorylation. The use of rats allowed comparison with previous studies performed by our group and others on hippocampal slices and/or cultures.12,14,25–27,29 We have previously shown in rat hippocampal slices that dexmedetomidine increases phosphorylation of focal adhesion kinase via an α2-adrenoceptor-mediated mechanism.14 For comparative purposes, the signaling cascade involved in the effects reported in the present study (increase in pERK1 and 2 expression) was performed in the same experimental conditions. However, the use of genetically modified animals was required to definitely address the role of the α2 adrenoceptors in dexmedetomidine actions. This led us to use mice carrying targeted deletions of the α2-adrenoceptor subtypes. A common criticism and limitation of studies using transgenic mice is the possible induction of compensatory mechanisms that are not operational in wild-type animals and may mask the functional results of a targeted mutation. This possibility seems unlikely because yohimbine, an antagonist of the α2 adrenoceptors, was ineffective in blocking dexmedetomidine- and 2-adrenoceptor–mediated mechanism.14 For comparative purposes, the signaling cascade involved in the effects reported in the present study (increase in pERK1 and 2 expression) was performed in the same experimental conditions. However, the use of genetically modified animals was required to definitely address the role of the α2 adrenoceptors in dexmedetomidine actions. This led us to use mice carrying targeted deletions of the α2-adrenoceptor subtypes. A common criticism and limitation of studies using transgenic mice is the possible induction of compensatory mechanisms that are not operational in wild-type animals and may mask the functional results of a targeted mutation. This possibility seems unlikely because yohimbine, an antagonist of the α2 adrenoceptors, was ineffective in blocking dexmedetomidine-induced increase in pERK1 and 2 in rats, wild-type mice, and transgenic mice as well. The efficacy of 10−4 m yohimbine in blocking α2-adrenoceptor–mediated effects in similar experimental conditions has been extensively characterized in our laboratory.14 Our strategy consisted of reproducing in mice the cornerstone effects reported in the present study (increase in pERK1 and 2 expression) was performed in the same experimental conditions. However, the use of genetically modified animals was required to definitely address the role of the α2 adrenoceptors in dexmedetomidine actions. This led us to use mice carrying targeted deletions of the α2-adrenoceptor subtypes. A common criticism and limitation of studies using transgenic mice is the possible induction of compensatory mechanisms that are not operational in wild-type animals and may mask the functional results of a targeted mutation. This possibility seems unlikely because yohimbine, an antagonist of the α2 adrenoceptors, was ineffective in blocking dexmedetomidine-induced increase in pERK1 and 2 in rats, wild-type mice, and transgenic mice as well. The efficacy of 10−4 m yohimbine in blocking α2-adrenoceptor–mediated effects in similar experimental conditions has been extensively characterized in our laboratory.14 Our strategy consisted of reproducing in mice the cornerstone effects reported in the present study (increase in pERK1 and 2 expression) was performed in the same experimental conditions. However, the use of genetically modified animals was required to definitely address the role of the α2 adrenoceptors in dexmedetomidine actions. This led us to use mice carrying targeted deletions of the α2-adrenoceptor subtypes. A common criticism and limitation of studies using transgenic mice is the possible induction of compensatory mechanisms that are not operational in wild-type animals and may mask the functional results of a targeted mutation. This possibility seems unlikely because yohimbine, an antagonist of the α2 adrenoceptors, was ineffective in blocking dexmedetomidine-induced increase in pERK1 and 2 in rats, wild-type mice, and transgenic mice as well. The efficacy of 10−4 m yohimbine in blocking α2-adrenoceptor–mediated effects in similar experimental conditions has been extensively characterized in our laboratory.14 Our strategy consisted of reproducing in mice the cornerstone
pharmacologic results obtained in rats, i.e., insensitivity to yohimbine and sensitivity to protein kinase C inhibitors and PD 098059 to demonstrate the coherence of the data obtained in both species. Inhibition of pERK1 and 2 expression by PD 098059 indicates the selectivity of the cellular signaling pathway examined.

Our results also indicate that dexmedetomidine increases the content of phosphorylated ERK1 and 2 and not simply the total amount of ERK1 and 2. This indicates that the increase in pERK1 and 2 content was related to changes in the phosphorylation status, not in the amount of total ERK protein content. We observed a parallelism in the expression of pERK1 and pERK2 induced by dexmedetomidine and rilmenidine. This suggests that phosphorylation of both protein kinases is equally sensitive to $\alpha_2$-adrenoceptor and imidazoline I1 receptor agonists. The primary anti-pERK1 and 2 antibody was used in mice and rats because it exhibited excellent cross-reactivity between these two species.

It can be argued that nonspecific activation of ERK1/2 by phosphorylation may have occurred in our study. However, both the dose-dependent effect of dexmedetomidine and rilmenidine on ERK1/2 phosphorylation and the dose-dependent reversal of dexmedetomidine effects by efaroaxan strongly argue against nonspecificity of ERK1/2 phosphorylation by these agents. Phosphorylation is the most common posttranslational modification of proteins regulating their properties by numerous extracellular signals. ERK1 and 2 activation consists of its phosphorylation on both tyrosine and threonine residues located at the threonine-glutamine-tyrosine residue of its catalytic domain. This phenomenon is dependent on both kinases and phosphatas activities. More generally, phosphatas are also involved in the spatiotemporal regulation of pERK1 and 2 and their subsequent effects on cellular functions. Therefore, we cannot exclude that the increase in pERK1 and 2 content induced by dexmedetomidine involves phosphatas activities.

**Dexmedetomidine Increases pERK1 and 2 Content via $\alpha_2$-Adrenoceptor-independent Mechanisms**

A major original finding of the current study is the demonstration that dexmedetomidine produces changes on a major cellular signaling pathway via mechanisms independent from the $\alpha_2$-adrenoceptors. This hypothesis is strongly supported by the inability of the $\alpha_2$-adrenoceptor antagonist yohimbine, the Gi protein blocker pertussis toxin, and the permeant adenylyl cyclase activator 8-BrcAMP coadministered with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine to block the increase in pERK1 and 2 expression induced by dexmedetomidine. Further, the cAMP-dependent protein kinase inhibitor H89 was unable to mimic the effects of dexmedetomidine. Finally, the preservation of dexmedetomidine effects on pERK1 and 2 expression in mice that did not express the different $\alpha_2$-adrenoceptor subtypes clearly shows that these effects proceed via mechanisms independent from the $\alpha_2$-adrenoceptors.

Dexmedetomidine-induced increase in pERK1 and 2 content was blocked by chelerythrine and bisindolylmaleimide I, two structurally distinct inhibitors of protein kinase C that blocked phorbol 12-myristate 13-acetate-induced activation of pERK1 and 2 expression. Further, it was blocked by neomycin, an inhibitor of phospholipase C. Neomycin also blocked carbachol-induced increase in pERK1 and 2 expression. Altogether, these results support that dexmedetomidine-induced increase in pERK1 and 2 content proceeds via activation of the phospholipase C-protein kinase C pathway. Methoxamine, an agonist of the $\alpha_1$-adrenoceptors, increased pERK1 and 2 phosphorylation, and this effect was sensitive to prazosin. It is likely that methoxamine effect proceeds via an increase in cytosolic calcium, as this mechanism has been reported to activate ERK phosphorylation. The $\alpha_1$-$\alpha_2$ selectivity ratio of dexmedetomidine is remarkably low (1:1,620). The lack of antagonism of dexmedetomidine effects by prazosin and 2APB, an inhibitor of the inositol 3 phosphate receptor, does not support the involvement of $\alpha_1$-adrenoceptors in dexmedetomidine effects on ERK phosphorylation.

**Evidence for the Involvement of Imidazoline I1 Receptors in Dexmedetomidine Effects on ERK1 and 2 Phosphorylation**

In our study, we investigated the role of imidazoline I1 receptors in dexmedetomidine effects on ERK phosphorylation. Different imidazoline receptor subtypes, including I1, I2, and non-I1/I2 (or I3) receptors, have been described. Subtype I1 comprises sites that can be labeled with clonidine or idazoxan, whereas subtype I2 comprises those that do not recognize clonidine but are labeled by idazoxan. The I1-imidazoline receptors are widely expressed in the central nervous system, particularly in the brainstem, where they are thought to participate in the central control of blood pressure, but also the striatum, and the hippocampus. They are coupled to phospholipase C and protein kinase C. Animals have been successfully transfected with a cloned complementary DNA that encodes a protein with I1 receptor–like binding properties, designated as IRAS (imidazoline receptor antisera-selected). However, knockout animals engineered for targeted deletion of imidazoline I1 receptors are not available yet. Therefore, investigating the role of the imidazoline I1 receptors in ERK phosphorylation was restricted to a pharmacologic approach with few (if any) totally selective agonists or antagonists available. Nevertheless, the following arguments support the role of imidazoline I1 receptors in dexmedetomidine-induced increase in ERK1 and 2 phosphorylation: (1) Dexmedetomidine affinity for imidazoline receptors has been reported to be 0.72 $\mu$M, which is consistent with the EC$_{50}$ values reported in our study.
mechanisms may be relevant to the neuroprotective effects of dexmedetomidine against ischemic injury. Interestingly, a functional relation has been proposed for α2 adrenoceptors and imidazoline I1 receptors in some brain areas. It can be speculated that this may apply to dexmedetomidine neuroprotective effects, because IRAS has been reported to exhibit antiapoptotic properties. We have previously shown that dexmedetomidine preconditions brain tissue against ischemic injury, and that this effect is mediated in part by the activation of focal adhesion kinase phosphorylation via an α2 adrenoceptor-mediated mechanism. Here, we provide a possible additional mechanism for the preconditioning effect of dexmedetomidine against brain ischemic injury, which does not depend on the α2 adrenoceptors. This hypothesis remains to be verified in vivo or in experimental models such as organotypic slice cultures, in which long-term actions of pharmacologic/anesthetic agents can be investigated.

In summary, we have shown that dexmedetomidine increases the expression of pERK1 and 2, a key enzyme involved in coupling cellular signaling to long-term phenomena such as neuroprotection and cell survival. This effect is independent from the α2 adrenoceptors and is likely to be mediated via the I1-imidazoline receptors. These results may contribute to a better understanding of dexmedetomidine-induced neuroprotective effects.

**Physiologic Relevance**

Dexmedetomidine has been shown to decrease the expression of proapoptotic and favors that of antiapoptotic factors. On the other hand, phosphorylated ERK activates transcriptional factors responsible for long-term adaptive changes. For example, activated ERK translocates to the cytosol and activates Rsk proteins, which then phosphorylate the transcription factor CREB (cAMP response element binding protein), subsequently enhancing CREB response element-mediated gene expression (fig. 9). CREB expression has been shown to be enhanced by clonidine in dorsal horn neurons, which could contribute to mediate inhibition of pain signaling by this agent. CREB also plays a role in promoting cell survival by regulating the synthesis of protective factors such as the brain-derived neurotrophic factor. These reactions are the major mechanisms by which dexmedetomidine acts to protect the brain against ischemic injury.

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Anesthesiology, V 108, No 3, Mar 2008

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