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Research report

α-Adrenergic stimulation of ERK phosphorylation in astrocytes is α_2-specific and may be mediated by transactivation

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Abstract

The highly specific α_2-adrenergic agonist, dexmedetomidine, has hypnotic-sedative, anesthetic-sparing and analgesic effects, and it protects neurons against ischemia. The α_1-adrenergic agonist, phenylephrine, does not share dexmedetomidine's pharmacological properties, although both dexmedetomidine and phenylephrine increase free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in astrocytes, and most of dexmedetomidine's actions in the brain are exerted on postjunctional receptors. α_2-Adrenergic receptors are abundant on astrocytes. Dexmedetomidine-mediated 'down-streamn' signal transduction was therefore investigated in primary cultures of mouse astrocytes and contrasted with that of phenylephrine. The cultures were incubated with dexmedetomidine concentrations known to be pharmacologically active and to act specifically on α_2-adrenergic receptors (25–100 nM). $ERK_{1/2}$ phosphorylation was measured using specific antibodies. Peak increases of $ERK_{1/2}$ phosphorylation occurred at 50 nM dexmedetomidine, with less effect at higher concentrations. Phenylephrine caused ERK phosphorylation only at a concentration high enough to exert non subtype-specific effects (10 μM), and this effect was counteracted by the α_2-adrenergic antagonist atipamezole. The phosphorylation of ERK was reduced by tyrphostin AG1478, an inhibitor of phosphorylation of the epidermal growth factor receptor (EGFR), and by heparin, which neutralizes heparin-binding epithelial growth factor (HB-EGF), suggesting the involvement of a transactivation process, in which α_2-adrenergic stimulation leads to proteolytic shedding of HB-EGF (and perhaps other EGFR agonists) from transmembrane-spanning precursors.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Second messengers and phosphorylation

Keywords: Astrocyte; α_2-Adrenergic agonist; Dexmedetomidine; Signal transduction

1. Introduction

The best known effect of α_2-adrenergic agonists in brain is presynaptic inhibition of noradrenaline release in noradrenergic neurons, but only a minor fraction of α_2-adrenergic receptors appear to be presynaptic [13]. It is therefore important to investigate the direct effect of α_2-adrenergic agonists on their target cells. One such agonist is dexmedetomidine, a potent and highly specific α_2-adrenergic agonist, which in receptor binding experiments has an α_2/α_1 selectivity ratio of 1600 or several times higher than clonidine [39]. Dexmedetomidine is utilized clinically for its hypnotic/sedative and analgesic effects [3,7,38], and low concentrations of dexmedetomidine also have neuroprotective properties [21]. Most, if not all, of these effects are independent of inhibition of noradrenaline release [9,12,15,36]. Although the α_1-adrenergic agonist, phenylephrine also increases $[Ca^{2+}]_i$ in astrocytes [4,25,28,29,37], it does not share the pharmacological profile of dexmedetomidine, and there are no indications that it has neuroprotective properties.

Dexmedetomidine is an agonist at each of the three subtypes of the α_2-adrenergic receptor (the $\alpha_{2A/D}$, the α_{2B},

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and the α_{2C} receptor), which all are coupled to pertussis toxin-sensitive $G_{i/o}$-coupled receptors [1]. In addition, dexmedetomidine activates an imidazoline-preferring site [18,20]. The target cells in the CNS displaying α_2-adrenergic receptors include astrocytes [2,11,22,27], which mainly express the $\alpha_{2A/D}$-adrenergic receptor subtype [10,16]. Stimulation of cultured astrocytes by dexmedetomidine increases free cytosolic calcium concentration, $[Ca^{2+}]_i$ [10,40], but it has no similar effect in cultured cerebrocortical interneurons [40]. The concentration dependence of the dexmedetomidine-induced Ca^{2+} surge in astrocytes is biphasic, with one peak at 70 nM, a reduction of the magnitude of the increase at 300 nM, and a second peak in the low micromolar range [5]. The increase in $[Ca^{2+}]_i$ is caused by release of intracellularly bound Ca^{2+}, which is in agreement with the observation by Enkvist et al. [10] that dexmedetomidine elevates the content of inositol trisphosphate (IP_3), i.e. activates the phosphatidylinositide second messenger system. The increase in $[Ca^{2+}]_i$ at a dexmedetomidine concentration of 100 nM is completely inhibited by the α_2-adrenergic antagonist, yohimbine [40], whereas the effect at micromolar dexmedetomidine concentrations is partly inhibited by yohimbine and partly by idazoxan, an inhibitor of the imidazoline-preferring site [5].

In a monkey kidney cell line, COS-7, stimulation of the α_2-adrenergic receptor activates a two-stage transactivation process [24,33,34]. The first, α_2-mediated, stage leads to a metalloproteinase-activated shedding of heparin-binding epithelial growth factor (HB-EGF) from its transmembrane-spanning precursor, leading to extracellular release of HB-EGF. In the second stage the released HB-EGF stimulates the EGF receptor (EGFR) in the same and adjacent cells, activating conventional growth factor-stimulated transduction pathways, which eventually lead to phosphorylation of the mitogen-activated protein (MAP) kinases ERK_1 and ERK_2 ($ERK_{1/2}$) via the Ras–Raf–MEK cascade in cells expressing EGFR. The involvement of such a transactivation pathway has been demonstrated by inhibition of the effect of α_2-adrenergic stimulation on ERK phosphorylation by tyrphostin AG 1478, an inhibitor of EGFR tyrosine kinase [33] and by heparin, which binds and thereby inactivates HB-EGF [19]. Transactivation and its potential importance in astrocytes have recently been reviewed by Peng [31].

In the present study we have demonstrated (i) that dexmedetomidine stimulates phosphorylation of $ERK_{1/2}$ in astrocytes and (ii) that the α_1-adrenergic agonist phenylephrine only has a similar effect at excessively high concentrations, when its subtype-specificity is lost. Preliminary evidence was obtained that the stimulatory effect of dexmedetomidine may be exerted by transactivation, since ERK phosphorylation by medium obtained from cultures treated with dexmedetomidine was inhibited by the EGFR tyrosine kinase inhibitor, tyrphostin AG 1478 and by heparin.

2. Materials and methods

2.1. Materials

Chemicals for preparation of media and most other chemicals, including phenylephrine HCl, were purchased from Sigma (St. Louis, MO, USA); dexmedetomidine and its highly specific antagonist, atipamezole, were donated by Orion (Orion Pharma, Turku, Finland). Tyrphostin AG 1478 was purchased from Calbiochem and heparin from Sigma. Santa Cruz Biotechnology supplied first antibodies, raised against ERK (K-23):sc-94 and against phosphorylated ERK (E-4):sc-7383. K-23 is monoclonal antibody, which binds to both ERK_1 (44 kDa) and ERK_2 (42 kDa), and E-4 is a polyclonal antibody reacting with both phosphorylated ERK_1 (P-ERK_1) and phosphorylated ERK_2 (P-ERK_2). The second antibodies goat anti-mouse IgG (H+L) HRP conjugate and goat anti-rabbit IgG HRP conjugate were purchased from Zymed and Santa Cruz Biotechnology, respectively.

2.2. Cell culturing

Primary cultures of mouse astrocytes were prepared as previously described [14,17]. The neopallia, i.e. the parts of the cerebral hemispheres above the lateral ventricles, were dissected out of the brains from newborn Swiss mice and mechanically dissociated. The resulting cell suspension, in tissue culture medium (a slightly modified Dulbecco's medium [17] with horse serum), was seeded in 100-mm Falcon Primaria culture dishes, and grown under a CO_2/air (5:95%) atmosphere at 37 °C. After the first 2 weeks, the culturing was continued in the presence of 0.25 mM dibutyryl cyclic AMP, a procedure known to promote morphological and functional differentiation of the cells [26,35], including an enhanced $\alpha_{2A/D}$receptor expression [10]. The cultures were utilized after 3–4 weeks in culture. Such cultures constitute an excellent model for many functional characteristics of astrocytes in situ; astrocytes constitute >95% of the cell population, and neurons are absent [14,17].

2.3. Drug treatment

For determination of $ERK_{1/2}$ phosphorylation the cells were washed once with phosphate-buffered saline (PBS), containing 137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 7.5 mM glucose, pH 7.4, and subsequently incubated in culturing medium without serum at 37 °C with or without drugs. The reaction was stopped by washing with ice-cold PBS containing 7.5 mM glucose, and the cells were scraped off the dishes and harvested in 0.5 ml of ice-cold buffer A (0.25 M sucrose, 10 mM HEPES, the phosphatase inhibitors α-mercaptoethanol [10 mM] and phenylmethyl-

sulfonyl fluoride [1 mM], and 1 mM sodium orthovanadate, pH 7.4).

2.4. Western blotting

A whole cell lysate was prepared by vortexing for 2 min. Protein content was determined by the Lowry method [23], using bovine serum albumin as the standard. Samples containing fifty μg protein were applied on slab gels of 5 and 12% polyacrylamide and Laemmli buffer (Invitrogen) and electrophoresed. After transfer to nitrocellulose membranes, the samples were blocked by 5% skim milk powder in TBS-T (30 mM Tris–HCl, 125 mM NaCl, 0.1% Tween 20) for 2 h. The nitrocellulose membranes were incubated with the first antibody, specific to P-ERK or ERK for 1.5 h at room temperature or overnight at 4 °C. After washing, specific binding was detected by goat-anti-mouse (P-ERK) or goat-anti-rabbit (ERK) horseradish peroxidase-conjugated secondary antibody. Staining was visualized by luminol reagents (Amersham Pharmacia, Sweden), followed by exposure to Amersham hyper film for a suitable length of time, which could vary between different sets of experiments. The results were collected by Eagle Sight software version 3.2. Band density was measured with Eagle Eye II. This procedure does not allow determination of absolute amounts of either P-ERK or ERK, but the ratio between them provides a measurement of ERK phosphorylation.

3. Results

ERK_1 and P-ERK_1 were visible as bands of 44 kDa, and ERK_2 and P-ERK_2 as bands of 42 kDa. Twenty minutes of exposure to 25 and 50 nM dexmedetomidine caused a distinct increase of phosphorylation of both ERK_1 and ERK_2, with smaller effects at 75 and 100 nM (Fig. 1, upper rows). The increase in phosphorylated $ERK_{1/2}$ could be seen already after 5 min of exposure to dexmedetomidine, but the effect was smaller after 60 min (Fig. 2, upper rows).

Within the same experiment, non-phosphorylated ERK_1 and ERK_2 (Figs. 1 and 2, lower rows) remained relatively constant (the 5-, 20-, and 60-min values in Fig. 2 are from different experiments). The ratios between P-ERK_1 and ERK_1 and P-ERK_2 and ERK_2 are, therefore, mainly determined by the absolute values of P-ERK_1 and P-ERK_2. The average increases in ratios between P-ERK_1 and ERK_1 and P-ERK_2 and ERK_2 (ΔP-ERK/ERK ratios) after exposure to 50 nM dexmedetomidine for either 5 or 20 min ($n=3$) are shown in the left part of Fig. 3 (fully open and fully filled-in column). The increase in the P-ERK_1/ERK_1 ratio amounted to close to 0.5 (open column) and that in the P-ERK_2/ERK_2 ratio to about 0.3 (filled-in column); the effect on ERK_1 is statistically significantly different from zero ($P<0.05$ in ANOVA followed by Fisher's PLSD test), whereas that on ERK_2 does not reach statistical significance in ANOVA, although P equaled 0.05 in a Student's t-test. The effects of dexmedetomidine on phosphorylation of ERK_1 and on ERK_2 are not significantly different from each other.

Ten μM phenylephrine also caused an increase in P-ERK, whereas 0.2 and 1 μM caused no increase in $ERK_{1/2}$ phosphorylation (Fig. 4). Averaging of results for 10 μM phenylephrine after either 5 or 20 min (right part of Fig. 3; horizontally striped columns; $n=3$) showed that the increase in phosphorylation of ERK_1 (expressed as changes in P-ERK/ERK ratios) was statistically significant ($P<$

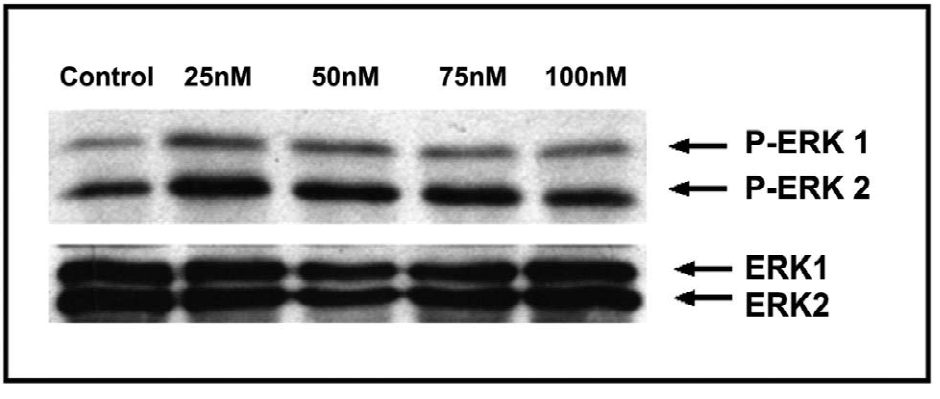

Fig. 1. Bands of 44 and 42 kDa representing P-ERK_1 (phosphorylated ERK_1) and P-ERK_2 (phosphorylated ERK_2), respectively (upper rows), and similar bands representing non-phosphorylated ERK_1 and ERK_2 (lower rows) in primary cultures of mouse astrocytes incubated for 20 min in the absence of any drug (control) or in the presence of the indicated concentrations of dexmedetomidine. Following the incubation solubilized cell samples containing 50 μg protein of whole cell lysate were electrophoresed on polyacrylamide slab gels and transferred to nitrocellulose membranes, which were incubated with either the monoclonal antibody E4 or the polyclonal antibody K-23, recognizing P-$ERK_{1/2}$, and $ERK_{1/2}$, respectively. Specific binding of E-4 was detected by goat-anti-mouse horseradish peroxidase-conjugated secondary antibody, and specific binding of K-23 was detected by goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody. ERK phosphorylation was quantitated as ratios between P-ERK_1 and ERK_1 and between P-ERK_2 and ERK_2. All results in this figure originate from the same experiment, and the 50 nM value is included in the average dexmedetomidine-induced increase in phosphorylation of $ERK_{1/2}$ shown in Fig. 3.

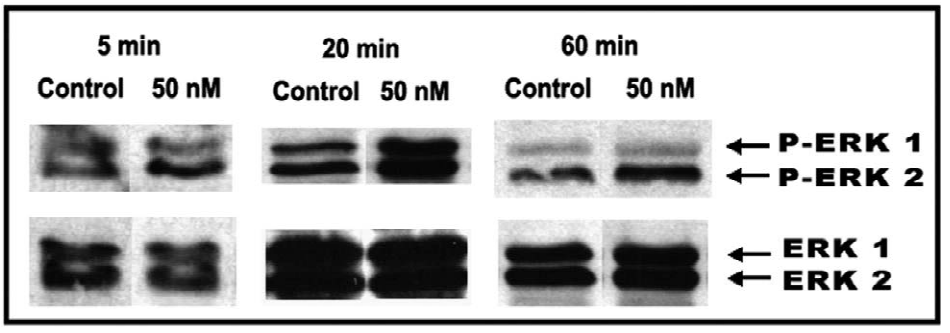

Fig. 2. Bands of 44 and 42 kDa representing P-ERK$_1$ (phosphorylated ERK$_1$) and P-ERK$_2$ (phosphorylated ERK$_2$), respectively (upper rows), or non-phosphorylated ERK$_1$ and ERK$_2$ (lower rows) in primary cultures of mouse astrocytes incubated for 5, 20, or 60 min in the absence of any drug (control) or in the presence of 50 nM dexmedetomidine. Following the incubation the cultures were treated as described in the legend of Fig. 1. Results for different time periods originate from three separate experiments, but control values and values in the presence of dexmedetomidine were always from the same experiment. ERK phosphorylation was quantitated as ratios between P-ERK$_1$ and ERK$_1$ and between P-ERK$_2$ and ERK$_2$ and results for 5 and 20 min are included in the average dexmedetomidine-induced increase in phosphorylation of ERK$_{1/2}$ shown in Fig. 3.

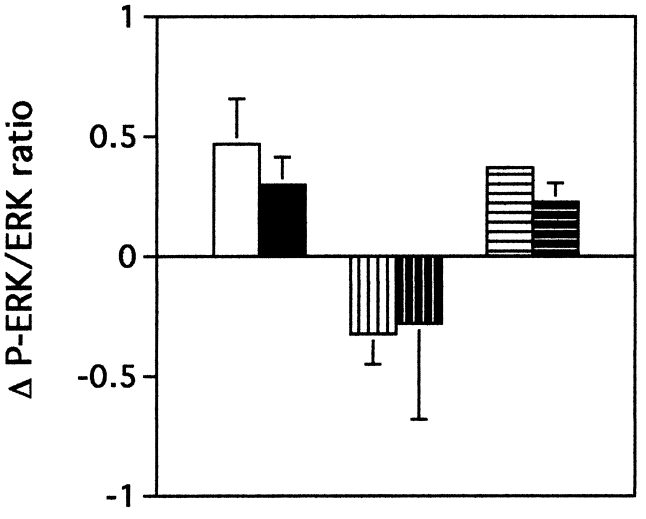

Fig. 3. Changes in phosphorylation of ERK$_1$ and ERK$_2$, measured as alterations of ratios between P-ERK$_1$ and ERK$_1$ and between P-ERK$_2$ and ERK$_2$ (ΔP-ERK/ERK ratios). The left one-third of the figure indicates the average increase in phosphorylation of ERK$_1$ (open column) and ERK$_2$ (filled-in column) induced by exposure to 50 nM dexmedetomidine for 5 or 20 min. The middle one-third of the figure indicates the average decreases in phosphorylation of ERK$_1$ (column with vertical black stripes on white background) and ERK$_2$ (column with vertical white stripes on black background) induced by exposure to 1.0 μM phenylephrine for 5 or 20 min. The right one-third indicates the average increases in phosphorylation of ERK$_1$ (column with horizontal black stripes on white background) and ERK$_2$ (column with horizontal white stripes on black background) induced by exposure to 10 μM phenylephrine for 5 or 20 min. Number of independent experiments (n)=3, except for 1 μM phenylephrine, where $n=2$; S.E.M. values are indicated by vertical bars. The following statistically significant ($P<0.05$) effects were identified by ANOVA followed by Fisher's PLSD test: effect of dexmedetomidine on ERK$_1$ vs. zero; effect of 10 μM phenylephrine on ERK$_1$ vs. zero; effect of dexmedetomidine on ERK$_1$ vs. effect of 1 μM phenylephrine on ERK$_1$; and effect of dexmedetomidine on ERK$_2$ vs. effect of 1 μM phenylephrine on ERK$_2$.

0.05 in ANOVA followed by Fisher's PLSD test), whereas that on ERK$_2$ was not statistically significant in ANOVA, although it was statistically significant ($P<0.05$) in a Student's *t*-test. In contrast, 1 μM phenylephrine ($n=2$) decreased ERK phosphorylation (Fig. 3, middle part; vertically striped columns), and the effect of 1 μM phenylephrine on ERK phosphorylation was significantly different ($P<0.05$ in ANOVA followed by Fisher's PLSD test) from that of 50 nM dexmedetomidine both in the case of ERK$_1$ and of ERK$_2$. Moreover the difference between the effects of 1 and of 10 μM phenylephrine was statistically significantly different for phosphorylation of ERK$_1$ ($P<0.05$ in ANOVA followed by Fisher's PLSD test) and on the borderline of statistical significance for ERK$_2$. This concentration–response relationship may suggest that α_1-adrenergic stimulation by itself decreases ERK phosphorylation, but that a subtype-nonspecific effect overrides the inhibitory effect at a very high concentration of phenylephrine. That the stimulatory effect of 10 μM phenylephrine is caused by activation of α_2-adrenergic receptors is supported by the observation that it was reduced by simultaneous administration of 300 nM of the specific α_2-adrenergic antagonist atipamezole (Fig. 5).

In order to obtain preliminary evidence whether shedding of HB-EGF and subsequent transactivation of EGFR is involved in the phosphorylation of ERK$_{1/2}$ by dexmedetomidine, astrocyte cultures were exposed to 50 nM dexmedetomidine for 1 h (allowing the release of potential EGFR agonist(s) from the membrane-spanning precursors), and the media from these cultures were harvested. Three different cultures of astrocytes were incubated for 20 min in this medium after atipamezole (300 nM) had been added to prevent a direct effect of dexmedetomidine. In addition, tyrphostin AG 1478 (1 μM), a specific inhibitor of EGFR tyrosine kinase, was added to one culture, heparin (100 μg/ml) to another, and no additional drug to the third (control). ERK phosphorylation was pronounced in the presence of the conditioned medium, and this effect was

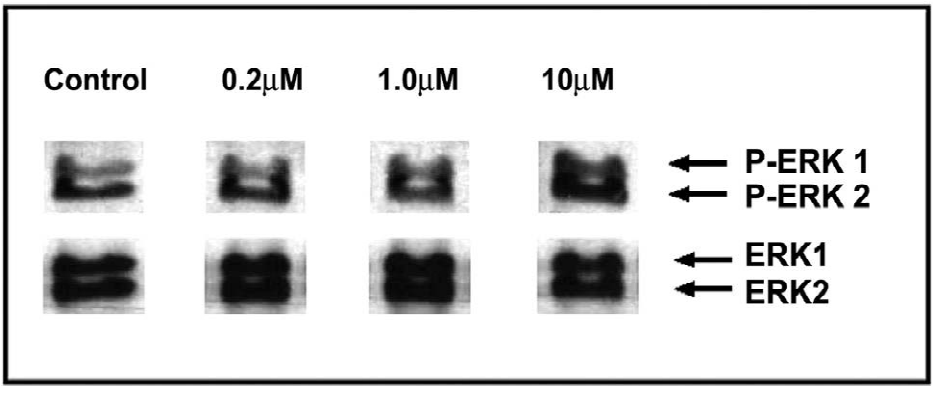

Fig. 4. Bands of 44 and 42 kDa representing P-ERK$_1$ (phosphorylated ERK$_1$) and P-ERK$_2$ (phosphorylated ERK$_2$), respectively (upper rows), and similar bands representing non-phosphorylated ERK$_1$ and ERK$_2$ (lower rows) in primary cultures of mouse astrocytes incubated for 20 min in the absence of any drug (control) or in the presence of the indicated concentrations of phenylephrine, an α_1-adrenergic agonist. Following the incubation the cultures were treated as described in the legend of Fig. 1. All results shown originate from the same experiment. ERK phosphorylation was quantitated as ratios between P-ERK$_1$ and ERK$_1$ and between P-ERK$_2$ and ERK$_2$ and the results for 1 and 10 μM phenylephrine were combined with results from a different experiment using an incubation period of 5 min and used for calculation of the values shown in Fig. 3.

distinctly reduced in the presence of 1 μM tyrphostin AG 1478 and less so in the presence of 100 μg/ml of heparin (Fig. 6).

4. Discussion

Cultured or freshly dissociated astrocytes react to the α_2-adrenergic agonists clonidine [4,25,28] and dexmedetomidine [5,40] with increases in $[Ca^{2+}]_i$ and in IP_3 [10] as well as with translocation of PKC from cytosol to membrane (L. Peng and L. Hertz, unpublished experiments). The increase in $[Ca^{2+}]_i$ and PKC translocation reach their maximum around 50 nM and show a decline at higher concentrations, i.e. a similar concentration dependence as that observed in the present study for phosphorylation of ERK$_{1/2}$. Phenylephrine is known to increase $[Ca^{2+}]_i$ in astrocytes [4,25,28,29,37] across the concentration interval tested (0.2–10 μM). Nevertheless, 0.2 μM phenylephrine had no effect on ERK phosphorylation, 1 μM caused a reduction, which was significantly different from both the effect of 50 nM dexmedetomidine and that of 10 μM phenylephrine, which increased phosphorylation significantly. These observations together with the inhibition of phenylephrine-induced ERK phosphorylation by atipamezole imply that α_1-adrenergic activation does not stimulate ERK phosphorylation. On the contrary, the inhibition of ERK phosphorylation by 1 μM phenylephrine may suggest that activation of α_1-adrenergic receptors rather counteracts a stimulation of ERK phosphorylation in control cultures by unknown EGFR agonists, possibly

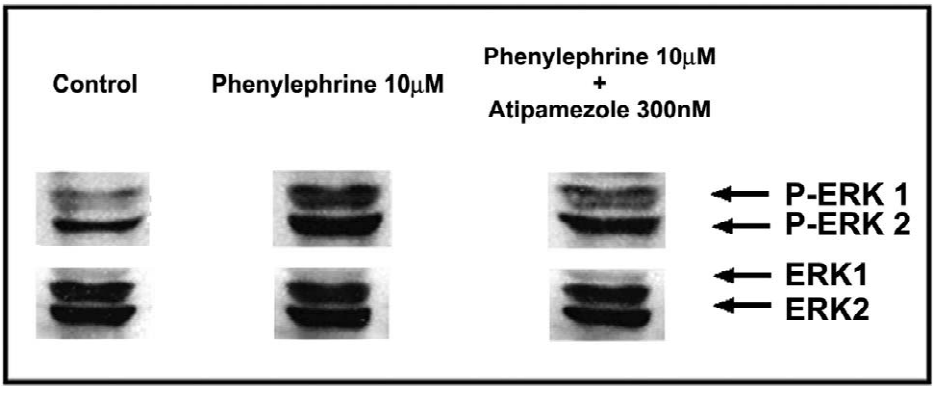

Fig. 5. Bands of 44 and 42 kDa representing P-ERK$_1$ (phosphorylated ERK$_1$) and P-ERK$_2$ (phosphorylated ERK$_2$), respectively (upper rows), and similar bands representing non-phosphorylated ERK$_1$ and ERK$_2$ (lower rows) in primary cultures of mouse astrocytes incubated for 5 min in the absence of any drug (control) or in the presence of the α_1-adrenergic agonist phenylephrine (10 μM) with or without the α_2-adrenergic antagonist atipamezole (300 nM). Following the incubation the cultures were treated as described in the legend of Fig. 1. All results shown originate from the same experiment, which was different from that shown in Fig. 4. ERK phosphorylation was quantitated as ratios between P-ERK$_1$ and ERK$_1$ and between P-ERK$_2$ and ERK$_2$; the result in the absence of atipamezole was included in the value for 10 μM phenylephrine shown in Fig. 5.

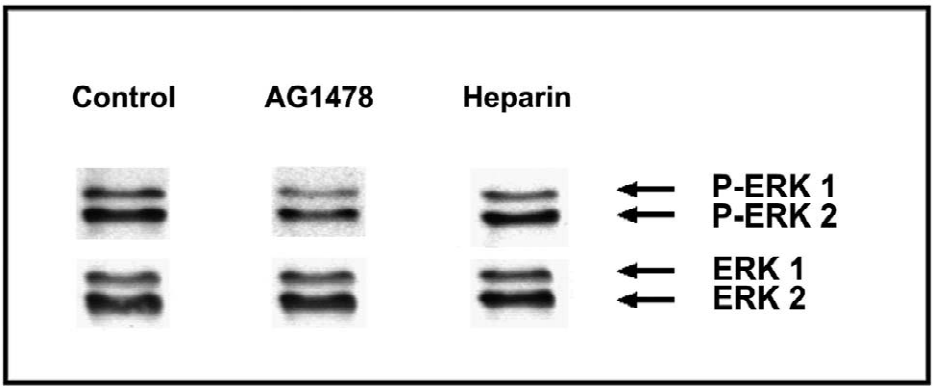

Fig. 6. Bands of 44 and 42 kDa representing P-ERK$_1$ (phosphorylated ERK$_1$) and P-ERK$_2$ (phosphorylated ERK$_2$), respectively (upper rows), and similar bands representing non-phosphorylated ERK$_1$ and ERK$_2$ (lower rows) in primary cultures of mouse astrocytes incubated for 10 min in medium, which had previously been conditioned by a 60-min incubation of cultures treated with 50 nM dexmedetomidine. Dexmedetomidine remaining in the conditioned media was inhibited by the α_2-adrenergic antagonist atipamezole (300 nM), before the cultures represented by the gels shown in this figure were exposed to these media. No other drug was added to the culture shown on the left side (control, potentially stimulated by epithelial growth factor receptor (EGFR) agonist(s) released to the medium), but 1 μM tyrphostin AG1478, a specific inhibitor of EGFR phosphorylation, was added to the culture shown in the middle, and heparin (100 μg/ml) an inhibitor of heparin-binding EGF (HB-EGF) due to its binding to the growth factor, was added to the culture shown on the right side. Following the incubation the cultures were treated as described in the legend of Fig. 1.

growth factors released spontaneously from the cultured astrocytes.

It is well established that stimulation of $G_{i/o}$- or G_q-protein-coupled transmitters can lead to phosphorylation of ERK$_{1/2}$ by transactivation of EGFR in different cell types [24,33,34], including astrocytes [6,8,30,32]. Since neurons also express EGFR and the stimulation of these receptors can cause neuroprotection, such a mechanism might in the intact brain lead to neuroprotection by a paracrine effect [31]. The inhibition of dexmedetomidine-induced phosphorylation of ERK by the specific EGFR antagonist tyrphostin AG 1478 and to some extent also by heparin suggests the involvement of such a mechanism. However, additional experiments are necessary to confirm this hypothesis and experiments are underway to establish whether phosphorylation of EGF receptors, known to exist on astrocytes in primary cultures [8] (T. Nakagawa and J.P. Schwartz, personal communication) is affected by dexmedetomidine and to identify possible EGF receptor agonists released by dexmedetomidine.

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