Activation of Akt1 by Human 5-Hydroxytryptamine (Serotonin)\textsubscript{1B} Receptors Is Sensitive to Inhibitors of MEK

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ABSTRACT
Akt1/protein kinase B and the mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase 1 (ERK1) and ERK2 have been shown to promote cell survival in a cell-specific manner. Since many receptors activate both pathways, inhibitors are commonly used to study the relative role of each pathway. In the present study, we examined the effects of PD098059 and U0126, two structurally dissimilar inhibitors of MAP kinase kinase (MEK1/2), on the activation of ERK and Akt stimulated by human 5-hydroxytryptamine\textsubscript{1B} (serotonin) (5-HT\textsubscript{1B}) receptors. Surprisingly, pathways for activation of both ERK and Akt were found to be sensitive to the two MEK inhibitors at concentrations commonly used to selectively inhibit the activation of ERK. Both compounds caused complete inhibition of phosphorylation of ERK and a maximal 60% inhibition of 5-HT\textsubscript{1B} receptor-mediated phosphorylation of Akt. Inhibition of Akt activation required almost complete inhibition of ERK. Transfection with cDNA for activated forms of MEK1/2 caused increased phosphorylation of ERK but not of Akt, demonstrating that independent activation of MEK/ERK was insufficient for activation of Akt. Therefore, it is not clear whether inhibition of activation of Akt resulted from selective inhibition of MEK or from additional actions on other unidentified common pathways. Nevertheless, our findings that PD098059 and U0126 inhibit activation of Akt at commonly used concentrations demonstrate that in at least some systems, these compounds inhibit activation of both ERK and Akt, and cannot be used to discern the relative roles of each pathway in mediating cellular responses.

Akt1 (also referred to as protein kinase B) and the mitogen-activated protein (MAP) kinases extracellular signal-regulated kinase 1 (ERK1) and ERK2 have been found to suppress apoptosis (Coffer et al., 1998; Campana et al., 1999; Encinas et al., 1999; Erhardt et al., 1999). The relative contribution of each pathway to cell survival appears to be at least partially cell-dependent. Since agonists for some receptors have been found to activate both ERK and Akt (Coffer et al., 1998; Campana et al., 1999; Encinas et al., 1999; Miikke et al., 1999; Leone et al., 2000), inhibitors are commonly used as tools to discern the relative importance of each pathway in regulating specific cellular responses.

Complete activation of Akt requires phosphorylation at both Thr\textsuperscript{308} and Ser\textsuperscript{473} (see Coffer et al., 1998, for review). Phosphorylation at these two sites appears to be independently regulated. Thr\textsuperscript{308} is phosphorylated by phosphatidylinositol (3,4,5)P\textsubscript{3}-dependent protein kinase 1 (PDK1) and Ser\textsuperscript{473} is thought to be phosphorylated by an unidentified PDK2. Additionally, the phosphatidylinositol 3-kinase (PI3K) product, phosphatidylinositol-3,4,5-bisphosphate, can bind to the pleckstrin homology domain of Akt and cause direct activation (Franke et al., 1997). Although most studies of Akt have examined tyrosine kinase growth factor receptors, it has been found that G protein receptors can also activate Akt. PI3K appears to be required for activation by receptors coupling to G\textsubscript{ai} and G\textsubscript{iq} (Tilton et al., 1997; Murga et al., 1998). β\textsubscript{y} subunits appear to mediate the effects of G\textsubscript{a}, while both G\textsubscript{iq} and β\textsubscript{y} subunits appear to mediate the effects of G\textsubscript{q} (Murga et al., 1998).

Many G protein-coupled receptors have also been reported to activate the MAP kinases ERK1 and ERK2 (Meloche et al., 1992; Koch et al., 1994; Flordellis et al., 1995; Luttrell et al., 1995; Cowen et al., 1996; Garnovskaya et al., 1996; Pullarkat et al., 1998; Leone et al., 2000). The pathways for activation of ERK and Akt, while distinct, share some common components. For example, the activation of ERK by G\textsubscript{a}-coupled receptors has been reported to require G protein β\textsubscript{y} subunits, a tyrosine kinase, PI3K, She, Grb2/SOS, and Ras (Touhara et al., 1995), in addition to Raf and the MAP kinase kinases 1 and 2 (MEK1 and MEK2).

Although the pathways for activation of ERK and Akt share some components, they have been previously reported to be independently regulated. Oncogenic v-p3k, which codes

ABBREVIATIONS: MAP kinase, mitogen-activated protein kinase; 5-HT, 5-hydroxytryptamine (serotonin); ERK, extracellular signal-regulated kinase; MEK, MAP kinase kinase; PDK, phosphatidylinositol (3,4,5)P\textsubscript{3}-dependent protein kinase; PI3K, phosphatidylinositol 3-kinase; JNK, c-Jun NH\textsubscript{2}-terminal kinase; CMV, cytomegalovirus.
for the p110α catalytic subunit of PI3K, for example, has been reported to cause constitutive activation of Akt but not ERK (Aoki et al., 2000). In contrast, overexpression of B-Raf in Rat-1 fibroblast cells has been reported to cause increased phosphorylation of ERK but not of Akt (Erhardt et al., 1999). Additionally, B-cell antigen receptor-mediated activation of Akt, but not ERK, has been found to be sensitive to inhibition of PI3K (Craxton et al., 1999).

The MEK inhibitors PD098059 and U0126 are commonly used to inhibit activation of ERK, with the assumption that they do not affect other signaling pathways. However, the role of MEK and ERK in the activation of Akt1 stimulated by G protein-coupled receptors has not been directly addressed. The aim of the current studies was to examine the effect of MEK inhibitors on the activation of Akt stimulated by G coupled 5-HT1B receptors. Since inhibition of MEK was associated with inhibition of Akt activation, the effect on Akt of constitutively activated forms of MEK was also studied. Our findings suggest a possible role for MEK in the pathway for activation of Akt by agonists for 5-HT1B receptors, and therefore possibly for other G-coupled receptors. However, the lack of effect of activated MEK suggests either that MEK potentiates, but does not directly activate, phosphorylation of Akt or that the MEK inhibitors PD098059 and U0126 have previously unidentified common actions, in addition to inhibiting MEK, that mediate the attenuation of activation of Akt.

### Experimental Procedures

**Materials.** Sumatriptan succinate was kindly provided by GlaxoWellcome (Hertfordshire, UK). U0126, PD098059, anisomycin, and 5-HT were purchased from Sigma (St. Louis, MO).

**Cell Culture.** A previously described (Leone et al., 2000) stable transfected BE(2)-C neuroblastoma cell line expressing human 5-HT1B receptors at a density of 500 fmol/mg membrane was used in these studies. Cells were routinely cultured in Ham's F-12 nutrient mixture with 1-glutamine, Eagle's minimal essential medium with nonessential amino acids (1:1), 10% dialyzed fetal bovine serum, 5-HT1B receptors (Leone et al., 2000) cultured overnight under serum- and geneticin-free conditions. Cells were stimulated with the specified concentrations of agonists and lysed with a 26-gauge needle in 25 mM HEPES (pH 7.4), 50 mM sodium fluoride, 5 mM EDTA, 1 mM sodium orthovanadate, 250 μM 4-(2-aminoethyl)-benzene-sulfonfonyl fluoride hydrochloride, 0.1% aprotinin, and 10 μg/ml leupeptin. Proteins were separated on 12% resolving gels (Bio-Rad Laboratories, Hercules, CA) and transferred to 0.45-μm Immobolin-P polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Membranes were blocked overnight with 3% powdered milk before incubation with primary and secondary antibodies. Bound antibodies were visualized using Enhanced Lumino Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA) and exposure to a Kodak Image Station 440CF with a cooled, full-frame-capture CCD camera (Eastman Kodak, Rochester, NY). Net intensity of bands was calculated using Kodak Digital Science 1D Image Analysis Software (version 3.02) on defined regions of interest.

**Akt Kinase Activity.** The day before use, cells were washed with phosphate-buffered saline and cultured overnight under serum- and genetin-free conditions. Cells were stimulated with the specified concentrations of agonists and lysed with a 26-gauge needle in 25 mM HEPES (pH 7.4), 50 mM sodium fluoride, 5 mM EDTA, 1 mM sodium orthovanadate, 250 μM 4-(2-aminoethyl)-benzene-sulfonfonyl fluoride hydrochloride, 0.1% aprotinin, and 10 μg/ml leupeptin. Proteins were separated on 12% resolving gels (Bio-Rad Laboratories, Hercules, CA) and transferred to 0.45-μm Immobolin-P polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA). Membranes were blocked overnight with 3% powdered milk before incubation with primary and secondary antibodies. Bound antibodies were visualized using Enhanced Lumino Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA) and exposure to a Kodak Image Station 440CF with a cooled, full-frame-capture CCD camera (Eastman Kodak, Rochester, NY). Net intensity of bands was calculated using Kodak Digital Science 1D Image Analysis Software (version 3.02) on defined regions of interest.

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### Results

**Similar Concentration-Response Relationships and Kinetics for Activation of ERK and Akt.** Studies of the pathways required for coupling of human 5-HT1B receptors to Akt1 and the MAP kinases ERK1 and ERK2 were performed in a stable transfected BE(2)-C human neuroblastoma cell line (referred to in this paper as BE2–1B). BE2–1B cells express 5-HT1B receptors at a density of 500 fmol/mg membrane protein (Leone et al., 2000). Activation of ERK requires phosphorylation, by MEK1/2, of both threonine 202 and tyrosine 204 (Cobb and Goldsmith, 1995). Therefore, detection of double phosphorylation was used as a measure of activation of MAP kinase. Similarly, activation of Akt was assayed by measuring phosphorylation of Akt at serine 473. We have previously reported that sumatriptan, an agonist selective for human 5-HT1B receptors, stimulates activation of both ERK and Akt in BE2–1B cells, but not in nontransfected BE(2)-C cells that do not express the receptor (Leone et al., 2000). The EC50 values for phosphorylation (calculated by nonlinear regression analysis of the net intensities of bands) of ERK and Akt were found to be similar, 0.87 and 0.66 μM, respectively (Fig. 1A; Table 1). This difference was not statistically significant. When 5-HT was used as an agonist, the concentration-response relationship for phosphorylation of ERK was also found to be similar to that for Akt. The EC50 values for phosphorylation of ERK and Akt were 2.40 and 2.88 nM, respectively (Table 1). The kinetics for activation of ERK and Akt were also found to be very similar (Fig. 1B). Phosphorylation of both kinases was detected within 2 min of treatment with sumatriptan, and was maximal at 5 min. After treatment for 30 min, the levels of phosphorylated ERK and Akt both approached those seen at baseline, although the level of phosphorylated Akt declined at a slower rate. Therefore, the concentration-response relationship of agonists and the kinetics for stimulation of phosphorylation are very similar for ERK and Akt.
Inhibition of MEK Attenuates Activation of Both ERK and Akt. Pretreatment of cells with U0126, a selective inhibitor of MEK1/2 (Favata et al., 1998), was found to completely block the increase in activated ERK stimulated by 1 μM sumatriptan (Fig. 2A; Table 2). Surprisingly, it also inhibited the activation of Akt. Attenuation of activation of Akt was incomplete and required almost complete inhibition of MEK. For example, pretreatment with 0.1 μM U0126 caused a 62% inhibition of phosphorylation of ERK, but only a 5% inhibition of phosphorylation of Akt (Fig. 2B). In contrast, pretreatment with 10 μM PD098059 caused complete inhibition of ERK and a 62% inhibition of activation of Akt.

The observed attenuation of phosphorylation of Akt was reflected in assays of Akt kinase activity. Pretreatment of cells with 10 μM U0126 caused a 69% inhibition of the increase in activity of Akt immunoprecipitated from cells incubated with 1 μM sumatriptan (Fig. 3A). This inhibition was not the result of a direct effect of U0126 on Akt, but was mediated by one or more other cellular components. When 10 μM U0126 was included in the in vitro kinase reactions for Akt immunoprecipitated from cells treated only with sumatriptan, no inhibition of activity was evident when compared with inclusion of vehicle only (0.2% dimethyl sulfoxide, Fig. 3B).

To further study whether the apparent role of MEK in activating Akt was a nonseselective effect of U0126, we also performed studies with the structurally dissimilar MEK1/2 inhibitor PD098059. Results very similar to those observed for U0126 were obtained. For example, pretreatment with 5 μM PD098059 caused a 51% inhibition of phosphorylation of ERK, but only a 13% inhibition of phosphorylation of Akt (Fig. 4A; Table 2). In contrast, pretreatment with 50 μM PD098059 caused complete inhibition of ERK and a 62% inhibition of activation of Akt.

Since the antibody used to detect phosphorylation of Akt recognizes both Akt1 and Akt2, it was not clear whether our findings were relevant to the better studied Akt1. To directly study the effect on Akt1, cells were transiently transfected with cDNA for a cMyc-tagged Akt1. As shown in Fig. 4B, treatment with 1 μM sumatriptan stimulated phosphorylation of the tagged Akt1, and this phosphorylation was completely inhibited by pretreatment of cells with 25 μM PD098059.

Although PD098059 is commonly used at 25 to 50 μM concentrations, we sought to determine whether lower concentrations could also inhibit activation of Akt. When cells were treated with only 100 nM sumatriptan, ERK was activated to a lesser extent than when stimulated with 1 μM, and this activation was almost completely inhibited by 10 μM PD098059 (Fig. 5). Under these same conditions, phosphorylation of Akt was inhibited by 55%. Therefore, the concentration of MEK inhibitors required to attenuate activation of Akt was consistently that required for almost complete inhibition of ERK.

It should be pointed out that phosphorylation of Akt was routinely detected in our studies with an antibody against Akt phosphorylated at Ser^473. Significantly, when membranes were reprobed with antibody against phosphorylated Thr^308, identical inhibition by PD098059 and U0126 of sumatriptan-stimulated phosphorylation of Akt was seen (not shown).

The Effect of Activated MEK on Phosphorylation of Akt. The inhibition of activation of Akt1 caused by PD098059 and U0126 suggested a role for MEK and/or ERK. Cells were therefore transfected with cDNA for activated forms of MEK1 and MEK2 to determine whether MEK/ERK could independently stimulate phosphorylation of Akt. Activated MEK1 stimulated a greater than 4-fold average increase in the basal level of phosphorylated ERK (Fig. 6). However, no change was observed in the basal level of phosphorylated Akt. Additionally, transfection with activated MEK1 did not increase the level of phosphorylated Akt stimulated by sumatriptan. Similar results were obtained when cells were transfected with an activated form of MEK2. Activated MEK2 stimulated a 4.5-fold increase in the basal level of phosphorylated ERK, but no change in the basal level of activated Akt (Fig. 7). Nor did transfection with activated MEK2 increase the level of phosphorylated Akt stimulated by sumatriptan. Significantly, even when the magnitude of activation of ERK stimulated by MEK2 in individual experiments was similar to that stimulated by sumatriptan in control cells, no increase in Akt was observed (Fig. 8). Therefore, although activation of the MEK/ERK pathway appeared
to be required for maximal phosphorylation of Akt, it was found to be insufficient for directly activating Akt.

5-HT1B Receptors Do Not Couple to Activation of p38 and JNK MAP Kinases. Since activated forms of MEK were not found to directly activate Akt, it could be postulated that the MEK inhibitors might have had effects on other unidentified pathways that regulate Akt. Although it is not clear what pathways these would be, we examined whether two other MAP kinases, p38 and JNK, might be involved. Significantly, sumatriptan was not found to stimulate the activation of either p38 or JNK in BE2–1B cells (Table 3). In contrast, anisomycin, a protein synthesis inhibitor known to activate p38 and JNK, stimulated a 5-fold and 15-fold increase, respectively, in phosphorylation of the kinases. Therefore, the MEK inhibitors could not have inhibited 5-HT1B receptor-stimulated activation of Akt by inhibiting the pathways for activation of p38 or JNK.

**Discussion**

The inhibition by PD098059 and U0126 of sumatriptan-stimulated activation of Akt suggests that MEK1/2 and/or ERK1/2 are required for full activation of Akt. However, while transfection with constitutively activated forms of MEK1 and MEK2 increased activation of ERK, there was no observed increase in activation of Akt. This demonstrates that direct activation of MEK and ERK is not sufficient for activation of Akt. Similarly, it has been reported that overexpression of B-Raf in Rat-1 fibroblast cells causes increased phosphorylation of ERK but not Akt (Erhardt et al., 1999).

There are a number of possible explanations for this apparent discrepancy. 1) Prolonged activation of MEK1/2 and ERK1/2 (as occurs with transfection of activated forms of MEK) is different from acute activation of MEK/ERK (as occurs with stimulation by sumatriptan). Possibly, prolonged activation causes compensatory changes, such as desensitization. 2) ERK/MEK potentiates activation of the pathway for phosphorylation of Akt, but does not directly activate the pathway. This would be analogous to the role of PI3K in the activation of ERK1/2. Inhibitors of PI3K attenuate the activation of ERK stimulated by Gi-coupled receptors (Pace et al., 1995; Touhara et al., 1995; Cowen et al., 1996; Garnovskaya et al., 1996; Pullarkat et al., 1998). However, oncogenic v-p3k, which codes for the p110α catalytic subunit of PI3K, does not cause constitutive activation of ERK (Aoki et al., 2000). 3) Inhibition of activation of Akt by MEK inhibitors is not the result of selective inhibition of MEK, but is mediated by actions on another, unidentified, pathway.

We cannot rule out this last possibility. However, the combination of PD098059 and U0126 has been found to be highly selective (Alessi et al., 1995; Favata et al., 1998; Davies et al., 2000). If the inhibition of activation of Akt was the result of the MEK inhibitors acting on another pathway, the identity of that pathway is unclear. We found no activation by sumatriptan of p38 or JNK, two other kinases within the MAP kinase family. Therefore, nonselective inhibition of either of those two pathways would not be expected to mediate inhibition of Akt activation. Additionally, although PD098059 has been shown to inhibit cyclooxygenase-1 and -2 (Borsch-Haubold et al., 1998), U0126 does not (Davies et al., 2000). Significantly, in vitro
studies have shown no direct inhibition of PDK1 by 10 μM U0126, and only a 14% inhibition of PDK1 by 50 μM PD098059 (Davies et al., 2000). Therefore, inhibition of phosphorylation of Akt must occur at a step before PDK1. Interestingly, both PD098059 and U0126 have been reported to inhibit activation of ERK5 at concentrations similar to that required to inhibit activation of ERK1 and ERK2 (Kamakura et al., 1999). However, it has been reported that G₂-coupled receptors and subunits of G₂ do not stimulate activation of ERK5 (Fukuhara et al.,

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**TABLE 2**

Differential sensitivity of phosphorylation of ERK and Akt to inhibitors of MEK

BE2-1B cells were incubated for 30 min in the presence of the indicated concentrations of PD098059 or U0126 before treatment with 1 μM sumatriptan for 5 min and subsequent lyses. Total lysate was analyzed by immunoblotting with antibody to phospho-ERK1/ERK2 and phospho-Akt. Percentage of inhibition of increases in the net intensities of bands above basal conditions was calculated from three separate experiments performed in duplicate and expressed as the means ± S.E.M.

<table>
<thead>
<tr>
<th>Percentage of Inhibition of Phosphorylation (Mean ± S.E.M.)</th>
<th>5 μM PD098059</th>
<th>50 μM PD098059</th>
<th>0.1 μM U0126</th>
<th>10 μM U0126</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERK</td>
<td>51.0 ± 9.3</td>
<td>99.0 ± 0.6</td>
<td>62.0 ± 7.7</td>
<td>99.8 ± 0.2</td>
</tr>
<tr>
<td>Akt</td>
<td>13.2 ± 8.4**</td>
<td>61.8 ± 8.2**</td>
<td>5.0 ± 1.7***</td>
<td>59.7 ± 8.6***</td>
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**p < 0.01; ***p < 0.001 versus ERK at the same concentration of inhibitor ANOVA, Bonferroni analysis.**

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**Fig. 3.** U0126 inhibits the activation of Akt but does not directly inhibit the activity of Akt. BE2-1B cells were incubated for 5 min with 1 μM sumatriptan (sum) and then lysed. Akt was immunoprecipitated from total lysate, and kinase activity was measured in an in vitro assay using a GSK-3α/β fusion protein as a substrate. Phosphorylation of substrate was analyzed by immunoblotting with antibody to phospho-GSK-3α/β (pGSK-3α/β). U0126 (10 μM) was added 30 min before treatment of cells with sumatriptan (A) or after immunoprecipitation at the initiation of the in vitro kinase assay (B). Net intensities of bands are expressed as the means ± S.E.M. for pGSK-3α/β. *p < 0.05; ***p < 0.001 versus sumatriptan in the absence of U0126, ANOVA.

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**Fig. 4.** Inhibition of activation of both ERK and Akt by PD098059. A, BE2-1B cells were incubated for 30 min in the presence or absence of the indicated concentrations of PD098059 (PD) before treatment with 1 μM sumatriptan (sum) for 5 min and subsequent lyses. Total lysate was analyzed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK) and phospho-Akt (p-Akt). B, BE2-1B cells were transfected 24 h before use with cDNA for cMyc-tagged Akt1. Cells were incubated for 30 min in the presence or absence of 25 μM PD098059 before treatment with 1 μM sumatriptan for 5 min and subsequent lyses. Tagged Akt1 was immunoprecipitated from total lysate with rabbit polyclonal antibody to cMyc and was analyzed by immunoblotting with mouse monoclonal antibody to phospho-Akt. Net intensities of bands were calculated from three (A) or six (B) separate experiments, performed in duplicate and expressed as the means ± S.E.M. for p-ERK, p-Akt and p-Akt1. *p < 0.05; **p < 0.01; ***p < 0.001 versus sumatriptan in the absence of PD098059, repeated measures ANOVA.

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Since 5-HT₁B receptors activate both Akt and ERK through a pertussis toxin-sensitive (and therefore G₁-dependent) pathway (Leone et al., 2000), it is unlikely that inhibition of ERK5 mediates the attenuation of phosphorylation of Akt.

In contrast, our findings are consistent with the hypothesis that the inhibition of activation of Akt by PD098059 and U0126 was the result of selective inhibition of MEK1/2. The concentration-response curves and kinetics for sumatriptan-stimulated activation of ERK were very similar to those for activation of Akt. This is consistent with MEK being required for activation of both ERK and Akt. Also, the concentrations of PD098059 and U0126 required to inhibit activation of Akt were consistently those required to completely inhibit MEK1/2. When cells were treated with submaximally stimulating concentrations of sumatriptan, activation of ERK was stimulated to a lesser extent and inhibition of phosphorylation of Akt was seen at reduced concentrations of MEK inhibitors relative to that seen when cells were treated with maximally stimulating concentrations of sumatriptan. In separate studies, we have attempted to reproduce the complete inhibition of ERK caused by U0126 and PD098059 with dominant negative forms of MEK. However, transient transfection of cells with either phosphorylation-resistant or kinase-resistant dominant negative forms of MEK1 did not result in sufficient inhibition of ERK to enable us to study the effect on Akt. Dominant negative forms of MEK1 caused a maximal 60% inhibition of sumatriptan-stimulated phosphorylation of cotransfected hemagglutinin-tagged ERK1. Under these conditions, no inhibition of activation of Akt was observed. Since only minimal inhibition of phosphorylation of Akt was also found when activation of ERK was inhibited 60% by U0126 and PD098059, no conclusion can be made.

Fig. 5. Under conditions where less ERK is activated, lower concentrations of PD098059 are required to inhibit activation of both ERK and Akt. BE2–1B cells were incubated for 30 min in the presence or absence of the indicated concentrations of PD098059 (PD) before treatment with 100 nM sumatriptan (sum) for 5 min and subsequent lyses. Total lysate was analyzed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK) (A) and phospho-Akt (p-Akt) (B). Increases in the net intensities of bands above basal conditions were calculated from four separate experiments performed in duplicate and expressed as the means ± S.E.M. for p-ERK (A) and p-Akt (B). **p < 0.01; ***p < 0.001 versus sumatriptan in the absence of PD098059, repeated measures ANOVA, Bonferroni analysis.

Fig. 6. Activated MEK1 causes phosphorylation of ERK but not Akt. BE2–1B cells were transfected 24 h before use with cDNA for an activated form of MEK1, or an equal amount of empty control plasmid. Cells were incubated in the presence or absence of 100 nM sumatriptan for 5 min and then lysed. Total lysate (10 μg/lane) was analyzed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK) (A) and phospho-Akt (p-Akt) (B). Net intensities of bands were calculated from three separate experiments performed in duplicate and expressed as the means ± S.E.M. for p-ERK (A) and p-Akt (B). **p < 0.01 versus absence of activated MEK1, two-sided paired Student's t test calculated separately for both the presence and absence of sumatriptan.
Whether the MEK inhibitors acted selectively at MEK1/2 or at some unknown site, the attenuation of phosphorylation of Akt can be concluded to occur at a step before PDK1 and PDK2. Phosphorylation of Akt at Thr308 and Ser473 are known to occur at a step before PDK1 and PDK2. Phosphorylation of Akt at Thr308, identical inhibition of phosphorylation by MEK inhibitors was observed. MEK/ERK might be postulated to play a role in the recruitment of Akt to the membrane, where it is phosphorylated, or in the regulation of PI3K.

Interestingly, B cell antigen receptor-mediated phosphorylation of Akt has been reported to be insensitive to treatment with U0126 (Crxton et al., 1999). Similarly, the activation by insulin-like growth factor-1 receptors of ERK, but not Akt, has been reported to be sensitive to treatment with U0126 (Craxton et al., 1999). It is possible that our findings represent a single experiment included in the analysis shown in Fig. 7.

**Fig. 7.** Activated MEK2 causes phosphorylation of ERK but not Akt. BE2–1B cells were transfected 24 h before use with cDNA for an activated form of MEK2 or an equal amount of empty control plasmid. Cells were incubated in the presence or absence of 100 nM sumatriptan for 5 min and then lysed. Total lysate (10 μg/lane) was analyzed by immunoblotting with antibody to phospho-ERK1/ERK2 (p-ERK) (A) and phospho-Akt (p-Akt) (B). Net intensities of bands were calculated from three separate experiments, performed in duplicate and expressed as the means ± S.E.M. for p-ERK (A) and p-Akt (B). **p < 0.01; ***p < 0.001 versus absence of activated MEK2, two-sided paired Student’s t test calculated separately for both the presence and absence of sumatriptan.

**Table 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Net Intensity p-p38</th>
<th>Net Intensity p-JNK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>967 ± 173</td>
<td>4,639 ± 616</td>
</tr>
<tr>
<td>Sumatriptan</td>
<td>574 ± 213 (N.S.)</td>
<td>4,067 ± 660 (N.S.)</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>5,119 ± 913**</td>
<td>69,963 ± 3,603***</td>
</tr>
</tbody>
</table>

N.S., not statistically significant (p > 0.05).
**p < 0.01 vs. control, repeated measures ANOVA, Bonferroni analysis.

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