ERK Activation by G-protein-coupled Receptors in Mouse Brain Is Receptor Identity-specific*

Amanda M. Vanhoose‡, Megan Emery, Lismary Jimenez, and Danny G. Winder§

From the Department of Molecular Physiology and Biophysics, and the Center for Molecular Neuroscience, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0615

Received for publication, August 28, 2001, and in revised form, January 2, 2002
Published, JBC Papers in Press, January 8, 2002, DOI 10.1074/jbc.M108309200

In transfected cells and non-neuronal tissues many G-protein-coupled receptors activate p44/42 MAP kinase (ERK), a kinase involved in both hippocampal synaptic plasticity and learning and memory. However, it is not clear to what degree these receptors couple to ERK in brain. Gα2-coupled β-adrenergic receptor activation of ERK in neurons is critical in the regulation of synaptic plasticity in area CA1 of the hippocampus. In addition, α1- and α2-adrenergic receptors, present in CA1, could potentially activate ERK. We find that, like the β-adrenergic receptor, the Gα2-coupled α2AR activates ERK in adult mouse CA1. However, activation of the Gαi2-coupled α2AR does not activate ERK, nor does activation of a homologous Gαi2-coupled receptor enriched in adult mouse CA1, the 5HT1A receptor. In contrast, the nonhomologous Gα2-coupled γ-aminobutyric acid type B receptor does activate ERK in adult mouse CA1. Surprisingly, activation of α2ARs in CA1 from immature animals where basal phospho-ERK is low induces ERK phosphorylation. These data suggest that although most G-protein-coupled receptor subtypes activate ERK in non-neuronal cells, the coupling of Gαi2 to ERK is tightly regulated in brain.

Protein phosphorylation plays a critical role in synaptic plasticity and learning and memory in vertebrates. A growing body of evidence suggests that the p44/42 MAP kinase (ERK) cascade in particular plays important roles in the modulation of long-term potentiation in area CA1 of the hippocampus and is required for several forms of learning and memory (1). Given the roles of this kinase cascade in transcriptionally regulated processes, initial studies focused on its roles in long-term forms of both long-term potentiation of synaptic transmission in the hippocampus and hippocampus-dependent long term memory formation (2–7). More recent studies, however, implicate a role for this kinase in more moment-to-moment cellular excitability (8, 9). Thus, the ERK signaling cascade regulates several aspects of synaptic transmission.

Because of the multiple roles ERK may play in neuronal function it is critical to understand how the activation of this kinase is regulated in neurons. A number of receptor signaling pathways critical to synaptic plasticity recruit ERK activation in the hippocampus. As in many cell types, ligands for receptor tyrosine kinases, such as neurotrophins, recruit ERK activation in neurons, as does N-methyl D-aspartate receptor activation (10, 11). In addition, neuromodulators such as norepinephrine (NE) that activate GPCRs play critical roles in learning and memory and synaptic plasticity, at least in part through the regulation of ERK activity. For example, the activation of one adrenergic receptor, the Gαs-coupled β-adrenergic receptor, results in increased ERK activity and facilitates ERK-dependent forms of long-term potentiation in CA1 (8, 9, 12, 13). However, this receptor is not likely to be activated alone in vivo but rather in concert with other NE receptors, including the Gα2-coupled α2A-adrenergic receptor (α2AR) and the Gαi2-coupled α2A-adrenergic receptor (α2AR), both present in CA1 (14–16). Both of these classes of adrenergic receptors have been demonstrated to strongly couple to activation of ERK in non-neuronal cell lines (17–19); however, their regulation of the ERK cascade in neurons remains undefined. Because ERK plays predominantly excitatory roles in neuronal function (1), it seems counterintuitive that Gαi2-coupled receptors thought to play inhibitory roles in neuronal function would activate this cascade. Consistent with this idea, recent studies have demonstrated that the α2AR does not activate ERK in stably transfected PC12 cells, even though functional expression of the receptor was demonstrated. However, as in the non-neuronal cell lines, the α1- and β-adrenergic receptors stimulate ERK activation in differentiated PC12 cells (20).

We have begun to examine the role of GPCR receptors in coupling to ERK activation in area CA1 of the mouse hippocampus with an emphasis on the role of Gαi2-coupled receptors. We began with adrenergic receptor subtypes and found that, as in the case of PC12 cells, the β and α1Rs robustly couple to ERK phosphorylation in adult mouse CA1, whereas the α2AR subtype does not. Moreover, we found that the 5HT1A receptor (a Gαi2-coupled receptor enriched in area CA1 and homologous to the α2AR) also fails to couple to ERK activation in this region even though it has been shown to couple to ERK activation in non-neuronal cell types (21). Because these receptors play predominantly inhibitory roles in regulating neuronal function, the lack of coupling of these receptors to ERK in situ is likely through a physiologically important inhibitory constraint. However, in contrast we find that the nonhomologous Gαi2-coupled GABA-B receptor does activate ERK in mouse CA1. This receptor has recently been demonstrated to associate with CREB and ATF4 in hippocampal neurons (22, 23), and receptor activation initiates ERK-dependent CREB2 transcription.
(23), suggesting the activation of ERK by the GABAB receptor may have important physiological consequences. Finally, although the a2AR fails to activate ERK in CA1 from adult mice, we do observe ERK activation in response to a2AR activation in CA1 from immature mice where basal levels of phospho-ERK are dramatically reduced. Taken together these data support the idea that activation of ERK is differentially regulated in neuronal versus non-neuronal cells, that ERK signaling via GPCRs is receptor identity-specific, and that the ERK cascade is developmentally regulated.

EXPERIMENTAL PROCEDURES

Brain Slice Preparation—Preparation of hippocampal slices was performed as previously described (9). Briefly, hippocampi were dissected from C57Bl/6j mice (Jackson), either 6–11-week-old male adults or 17–21-day-old male and female adolescents (no difference was observed between sexes), and 400-μm thick slices were prepared using a McIwain chopper. Slices were then placed in multiwell submerged chambers with oxygenated artificial cerebrospinal fluid (in mM: NaCl, 124; KCl, 4.4; CaCl2, 2; MgSO4, 1.2; NaH2PO4, 1; glucose, 10; and NaHCO3, 26) at 26–28 °C unless otherwise specified. Slices were equilibrated for 2–3 h before drug application was performed. After drug application, slices were transferred directly to a metal surface in dry ice for rapid freezing and microdissection of area CA1. Slices were then stored at −80 °C until homogenization.

Western Blotting—CA1 minislices were added to ice-cold homogenization buffer (TBS, 20 mM; Triton X-100, 0.5%; sodium orthovanadate, 2 mM; and NaF, 2 mM), homogenized, and centrifuged at 4 °C. Protein levels in supernatant were determined by the Bradford method using a Bio-Rad protein assay kit. Samples were then diluted to equal concentrations, mixed with an equal volume of sample buffer (Tris-Cl, pH 6.8, 62.5 mM; glycerol; SDS, 2%; bromphenol blue, 0.5%; and β-mercaptoethanol, 5%), heated at 95 °C for 4 min, and run on a 10% polyacrylamide-resolving gel using a Bio-Rad mini trans-blot apparatus. Protein was then transferred to two immobilon polyvinyldene difluoride membranes in series. The first blot was probed with anti-phospho-p44/42 ERK primary antibody (1:5000; Cell Signaling, Beverly, MA), whereas the second blot was stained for total protein using colloidal gold (Bio-Rad) to verify equal lane loading. In some cases the first blot was stripped and re-probed for total ERK with anti-p44/42 ERK (1:5000; Cell Signaling). To strip, blots were incubated in stripping buffer (62.5 mM; glycerol; SDS, 2%; bromphenol blue, 0.5%; and β-mercaptoethanol, 5%), heated at 95 °C for 4 min, and run on a 10% polyacrylamide-resolving gel using a Bio-Rad mini trans-blot apparatus. Protein was then transferred to two immobilon polyvinyldene difluoride membranes in series. The first blot was probed with anti-phospho-p44/42 ERK primary antibody (1:5000; Cell Signaling, Beverly, MA), whereas the second blot was stained for total protein using colloidal gold (Bio-Rad) to verify equal lane loading. In some cases the first blot was stripped and re-probed for total ERK with anti-p44/42 ERK (1:5000; Cell Signaling). To strip, blots were incubated in stripping buffer (62.5 mM Tris-Cl, pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol) for 30 min at 50 °C with occasional agitation and then washed with TBS-Tween.

To avoid the loss of phosphate groups on targeted proteins during processing all subsequent incubations included 2 mM sodium orthovanadate and 2 mM NaF. Blots were blocked with 5% nonfat dry milk in TBS-Tween for 1 h at room temperature. After rinsing, blots were incubated in the appropriate primary antibody either overnight (4 °C) without agitation or for 1 h (room temperature) on a shaker. After a further rinse, blots were incubated in a horseradish peroxidase-coupled anti-rabbit IgG secondary antibody (4 °C) or for 1 h (room temperature) on a shaker. After a further rinse, blots were incubated in ECL (Amer sham Biosciences, Inc.), exposed to film, and developed. Relative amounts of signal on blots were determined by scanning the films using a Hewlett Packard ScanJet 5200C into a Pentium III-based computer and utilizing an adaptation of NIH Image software (Scion Image) to measure optical density. By running a standard curve of protein concentrations on each blot above and below the concentration utilized in our sample lanes we verified the linearity of this measurement. Results from repeated experiments were then averaged together and differences from basal were tested by Mann-Whitney analysis unless specified otherwise.

cAMP Assay—CA1 minislices were homogenized in 6% trichloroacetic acid and centrifuged at 4 °C. The supernatant was washed with tert-butylmethyl ether to remove residual trichloroacetic acid and then lyophilized. The cAMP residue was dissolved in the buffer supplied with the PerkinElmer Life Sciences cAMP RIA kit. The cAMP assay was performed per manufacturer’s suggestions (PerkinElmer Life Sciences, Boston, MA). Radioactivity was counted on a Cobra II Auto-Gamma Count (Packard). Sample counts were normalized to an average protein amount determined by the Bradford assay with CA1 minislices collected in parallel with the CA1 minislices used in the cAMP assay. Results from repeated experiments were then averaged together and differences were tested by unpaired student’s t test.

RESULTS

Comparison of Adrenergic Receptor Coupling to ERK Phosphorylation in Area CA1 of Mouse Hippocampus—As shown in Fig. 1a, the application of norepinephrine (100 μM, 15 min) elicited robust phosphorylation of ERK2 (273 ± 44%, p = 0.009) in area CA1 of the mouse hippocampus. To determine which classes of adrenergic receptors mediate this action we assessed the ability of various agonists to mimic the action of NE. Consistent with previous studies (9, 12), we found that activation of the G2-coupled β-adrenergic receptor by isoproterenol potently elicited ERK phosphorylation in CA1, with an EC50 of 3.5 mM (Fig. 1a and b). Further, an a-adrenergic receptor agonist, 6-fluoronorepinephrine (100 μM), also elicited ERK2 phosphorylation (241 ± 38%, p = 0.009; Fig. 1a). This agonist was added in the presence of the β-adrenergic receptor antagonist, timolol (10 μM), because cross-activity with the β-adrenergic receptor has been observed (24). Although the general a-adrenergic receptor agonist evoked ERK phosphorylation, activation of the G10-coupled a2ARs by the more specific agonists UK14,304 (1 μM) or Clonidine (10 μM) did not elicit detectable rises in ERK phosphorylation (Figs. 1a and 2a) even though saturating concentrations of agonists were applied (25, 26). Taken together, these data indicate that the NE-evoked rise in ERK activation in adult mouse CA1 is due to β-adrenergic receptor/G2 and a2AR/G10 activation but not a2AR/G10 activation.

G10-coupled Receptors Do Not Detectably Activate ERK in Adult Mouse CA1—The apparent failure of a2AR activation to elicit ERK phosphorylation is surprising given the repeated findings that these receptors couple to ERK phosphorylation in non-neuronal cell lines (27–30). However, these data are reminiscent of recent reports in PC12 cells in which a similar lack of coupling was reported even though functional coupling to other cascades was demonstrated (20). In HEK293 cells, activation of ERK by a2ARs is rapid, peaking within 5 min of drug addition (29). Thus we investigated the possibility that coupling of a2ARs to ERK activation is time-dependent by assessing ERK phosphorylation as a result of Clonidine (10 μM) application for time intervals ranging from 2 to 15 min. However, at none of these time points was a statistically significant increase in ERK phosphorylation observed (Fig. 2a). Another possibility is that the regulation of ERK phosphorylation by a2ARs is temperature-dependent. To test this possibility, we repeated experiments with the full a2AR agonist UK14,304 (1 μM) with slices incubated in 37 °C artificial cerebrospinal fluid. Again, a2AR activation did not increase ERK phosphorylation, whereas NE did (227 ± 51%, p = 0.055). Interestingly, after a
Raf. In many cell types, Ras/Raf signaling is inhibited by actin in the hippocampus, the administration of KT5720 does the idea that basal PKA activity dampens basal phospho-ERK dependent long-term potentiation (9, 39, 40). Consistent with et al., the tissue was analyzed 2 h postinjection.

**Fig. 2.** Do G\(_{\text{oi}}\)-coupled receptors activate ERK in adult mouse CA1? ERK2 phosphorylation in adult (6–11 weeks) mouse CA1 by 10 \(\mu\)M Clonidine applied for various time intervals (a), 1 \(\mu\)M UK14,304 or 100 \(\mu\)M norepinephrine applied for 10 or 15 min at 37 \(^\circ\)C (b), 1 \(\mu\)M UK14,304 applied for 15 min in the presence of 1 \(\mu\)M KT5720, pre-treated for 1 h (c), or 100 \(\mu\)M buspironone, 1 \(\mu\)M 8-hydroxy-DPAT, or 30 \(\mu\)M baclofen applied for 15 min (d). The number in parenthesis indicates the \(n\) for the condition. * and **, significant increases in ERK2 phosphorylation over basal (\(p < 0.05\) and \(p < 0.01\), respectively). For illustrative purposes agonist-elicited ERK2 responses over basal are plotted together in d, although agonists were applied in separate experiments.

10-min application of UK14,304 at 37 \(^\circ\)C, there is a decrease in phospho-ERK2 (70 ± 13%, \(p = 0.047\); Fig. 2b). Thus, at physiological temperatures, G\(_{\text{oi}}\)-coupled receptors may not only fail to increase ERK phosphorylation but also decrease phospho-ERK concentrations. In addition, in vivo experiments by Peng et al. (31), an \(\alpha_2\)AR agonist administered intramuscularly decreased phospho-ERK levels in the rat hippocampus, though the tissue was analyzed 2 h postinjection.

Numerous studies have shown that G\(_{\text{oi}}\)-elicited ERK activation can be achieved by \(\beta\)y signaling through p21\(^{\text{ras}}\) (Ras) and Raf. In many cell types, Ras/Raf signaling is inhibited by activation of PKA (32–35), suggesting the possibility that \(\alpha_2\)ARs can signal ERK activation through Raf, but that tonic inhibition of the cascade may prevent significant coupling in adult mouse CA1. Although the cAMP/PKA cascade has been demonstrated to activate rather than inhibit ERK in neurons, this activation is thought to occur through the activation of a second signaling cascade involving Rap1 and B-ras (36–38). Thus, we tested the possibility that the cAMP/PKA cascade tonically inhibits \(\alpha_2\)AR coupling to ERK through the Ras/Raf cascade by assessing the effects of preincubation of hippocampal slices with 1 \(\mu\)M KT5720, a concentration sufficient to inhibit PKA-dependent long-term potentiation (9, 39, 40). Consistent with the idea that basal PKA activity dampens basal phospho-ERK in the hippocampus, the administration of KT5720 does slightly increase basal phospho-ERK2 (133 ± 13%, \(p = 0.004\)). However, PKA inhibition in adult mouse CA1 had no significant effect on \(\alpha_2\)AR-elicited ERK activity (Fig. 2c).

Another potential explanation for the lack of coupling of the \(\alpha_2\)AR to ERK phosphorylation is that the receptor is not expressed at a threshold level required for ERK phosphorylation. The \(\alpha_2\)AR is functionally expressed in this brain region as assessed by cAMP assays and electrophysiological analysis in rat (41, 42) and in mouse,\(^2\) but to further address this issue we assessed the ability of other G\(_{\text{oi}}\)-coupled receptors to couple to ERK phosphorylation in CA1. Both the 5HT1A and GABA\(_B\) receptors are G\(_{\text{oi}}\)-coupled and enriched in area CA1 (43, 44). However, even at saturating concentrations (45, 46), agonists of the 5HT1A receptor do not significantly increase ERK phosphorylation in adult mouse CA1 (Fig. 2d). In fact, as with \(\alpha_2\)AR activation at 37 \(^\circ\)C, the application of 8-hydroxy-DPAT appeared to decrease ERK phosphorylation (68 ± 8%, \(n = 3, p = 0.049\)). Interestingly, we observed that activation of the GABA\(_B\) receptor, a member of a distinct GPCR family from the adrenergic and serotonin receptors (47), does yield increased ERK activation (148 ± 24% of basal, \(p = 0.003\); Fig. 2d). However, the degree of ERK activation is low relative to that elicited by the G\(_{\text{i}}\) and G\(_{\text{oi}}\)-coupled receptors.

\(\alpha_2\)AR Activation Does Not Regulate ERK Activation by \(\beta\)-adrenergic receptors in Mouse CA1—Although \(\alpha_2\)AR activation does not by itself significantly regulate ERK phosphorylation in mouse CA1, it is important to remember that these receptors are not likely activated alone in vivo but rather in concert with other adrenergic receptors. Thus, it is conceivable that the role of \(\alpha_2\)ARs in ERK activation could be to regulate another receptor’s ability to signal ERK activation. For example, the G\(_{\text{oi}}\)-coupled \(\alpha_2\)AR could indirectly regulate \(\beta\)-adrenergic receptor activity through modulating adenylyl cyclase activity and cAMP production. However, co-application of the \(\alpha_2\)AR agonist with either subsaturating or saturating concentrations of isoproterenol does not affect ERK activity elicited by \(\beta\)-adrenergic receptor activation in adult mouse CA1 (Fig. 3). These data are consistent with the data showing NE activation of ERK that resembles isoproterenol-induced ERK activation, because NE should activate both the \(\beta\)-adrenergic receptor and the \(\alpha_2\)AR.

Do G\(_{\text{oi}}\)-coupled Receptors Activate ERK in Area CA1 of Immature Mice?—In the hippocampus a number of effector systems regulated by GPCRs are developmentally controlled. The ERK cascade proves to be among these as we found that basal levels of phospho-ERK2 were dramatically lower in area CA1 of slices taken from 17–21-day-old adolescent mice compared with 7–8-week-old adult mice (Fig. 4a). This was not due to an alteration in total ERK levels as ERK expression was similar across these two age groups. In addition, the difference observed in basal phospho-ERK levels is not due to a difference in basal cAMP levels as we observed no change in cAMP levels between the two age groups (Fig. 4b). Forskolin treatment, a positive control, elicited robust rises in cAMP levels in both groups (data not shown). Further, in addition to GABA\(_B\) receptor activation of ERK, which is observed in both age groups, we found that in area CA1 of hippocampal slices from young mice \(\alpha_2\)AR activation elicits a significant increase in ERK phosphorylation over basal (Fig. 4, c and d). Because ERK is activated by the \(\alpha_2\)AR in CA1 from the young animal, the question arises whether norepinephrine responses are altered in CA1 from the

---

\(^2\) D. G. Winder, unpublished observations.
using an unpaired student’s t test. 

The principal findings of the present study are: 1) that the degree of coupling of GPCRs to ERK activation in mouse CA1 is highly subtype-specific, 2) that two G_{i/o}-coupled receptors (α_2AR and 5HT_{1A}R) that robustly activate ERK in mitotic non-neuronal cells do not activate ERK in adult mouse CA1 whereas the nonhomologous GABA_B receptor does, and 3) that basal phospho-ERK levels, at least in the adult mouse CA1, as well as each of the other adrenergic agonists and antagonist (Fig. 4d). The number in parenthesis indicates the n for the condition. ** and *** significant increases in ERK2 phosphorylation over basal (p < 0.01 and p < 0.001, respectively). Basal ERK2 phosphorylation between young and adult slices was compared using an unpaired student’s t test. 

Consistent with previous work, we show that G_{s} and G_{i/o}-coupled adrenergic receptors (β and α_2Rs) robustly couple to ERK phosphorylation in adult mouse CA1 whereas the G_{i/o}-coupled α_2AR does not. In addition, we observed a similar lack of ERK coupling with the homologous 5HT_{1A} receptor. This is surprising given that these receptors so readily couple to ERK activation in mitotic non-neuronal cell types (21, 29). There is the possibility that these receptors couple to ERK in a very discrete location and that the coupling is not detected with an immunoblot of the tissue homogenate. However, β-adrenergic receptor-induced ERK phosphorylation, which is readily detectable via immunoblot, has been localized to CA1 pyramidal cell somas and dendritic shafts by immunocytochemical techniques (9). The apparent lack of coupling of the α_2AR to ERK phosphorylation and the inhibition of basal ERK phosphorylation at physiological temperatures by the α_2AR is in concert with the classically described inhibitory actions of the Gi/o-ergic receptor (43). Indeed, similar results were reported when adrenergic receptor subtypes were transfected into PC12 cells, with the β and α_1, but not α_2Rs eliciting ERK phosphorylation (20).

Combined with the PC12 cell data, our findings suggest the existence of potent neuron-specific inhibition of ERK phosphorylation via G_{i/o}-coupled receptor activation in the adult mouse, which is likely of physiological importance. The ERK signaling cascade participates in neuronal damage induced by ischemia (49) and in the generation of seizure activity in cultured hippocampal neurons (50). G_{i/o}-coupled receptors typically reduce neurotransmitter release and hyperpolarize postsynaptic cells, actions that would be expected to reduce these pathologies. Thus, if these receptors coupled to the ERK cascade in neurons, it would be predicted that the activation of these receptors might exacerbate rather than ameliorate ERK-dependent forms of ischemic damage and seizure.

Although significant enhancement of ERK phosphorylation was not detected with the activation of the α_2AR in adult mouse CA1, we did observe clear activation of ERK by this receptor in CA1 from 2–3-week-old mice. In addition, the basal level of phospho-ERK in CA1 is developmentally regulated, with the adult animal expressing over 4-fold more phospho-ERK2 than the young animal. Interestingly, cAMP/PKA plays a role in basal phospho-ERK levels, at least in the adult mouse CA1, as PKA inhibition slightly increased ERK phosphorylation. However, basal cAMP concentrations do not differ between the two age groups, suggesting developmental changes in signaling pathways regulating ERK activity.

Although the α_2AR displayed differences in ERK coupling depending on the age of the animal, the nonhomologous GABA_B receptor activated ERK in both young and adult mouse CA1. Interestingly, this receptor has recently been shown to physiologically interact with ATF4 and CREB2 in central nervous system neurons (22, 23) and to promote CREB2-mediated transcription through an ERK-dependent mechanism (23). Thus, GABA_B receptor activation of ERK may regulate CREB2-mediated transcriptional processes in area CA1. CREB2 has been demonstrated to play a critical role in regulating the transition from short to long term forms of synaptic facilitation and memory in invertebrate preparations (51, 52), suggesting the possibility that GABA_B receptor activation may locally influence these processes in vertebrates. The specificity of the G_{i/o}-linked receptor coupling to ERK that we demonstrate here is not surprising given that the GABA_B receptor shares no homology with the α_2AR and 5HT_{1A} receptor (47). Furthermore, the degree of ERK activation elicited by GABA_B receptors is relatively small in comparison to the G_{s} and G_{i/o}-adrenergic receptor responses. In future studies, it will be important to determine whether the difference in ERK activation levels is due to a subpopulation of these receptors on distinct cell populations or subcellular locations and/or reflects mechanistic differences in the signaling of G_{i/o}-coupled receptors versus the G_{s} and G_{i/o}-coupled receptors.
Acknowledgments—We thank Jason Moore for advice on statistical analysis and Roger Colbran for critically reading the manuscript.

REFERENCES
