

## $\beta_2$ -Adrenergic Receptor Lacking the Cyclic AMP-Dependent Protein Kinase Consensus Sites Fully Activates Extracellular Signal-Regulated Kinase 1/2 in Human Embryonic Kidney 293 Cells: Lack of Evidence for $G_s/G_i$ Switching.

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### ABSTRACT

Stimulation of the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) in human embryonic kidney (HEK) 293 cells causes a transient activation of Extracellular Signal-Regulated Kinase (ERK) 1/2. One of the mechanisms proposed for this activation is a PKA-mediated phosphorylation of the  $\beta_2$ AR that switches receptor coupling from  $G_s$  to  $G_i$  and triggers internalization of the receptor. To examine these phenomena, we characterized agonist activation of ERK1/2 in HEK293 cells by the endogenous  $\beta_2$ AR and in HEK293 cells stably overexpressing either the wild-type  $\beta_2$ AR or a substitution mutant  $\beta_2$ AR (PKA<sup>-</sup>) that lacks the cyclic AMP-dependent protein kinase (PKA) consensus phosphorylation sites (S261A, S262A and S345A, S346A). As the baseline, we established that epinephrine stimulation of the endogenous  $\beta_2$ AR in HEK293 cells (20–30 fmol/mg) caused a rapid and transient activation of ERK1/2 with an EC<sub>50</sub> of 5 to 6 nM. In contrast, the potency of epinephrine stimulation of ERK1/2 in cells stably overexpressing WT $\beta_2$ AR and PKA<sup>-</sup> (2–4 pmol of  $\beta_2$ AR/mg) was increased by over 100-fold relative to HEK293

cells, the EC<sub>50</sub> values being 20 to 60 pM. The nearly identical 100-fold shift in EC<sub>50</sub> for ERK1/2 activation in the PKA<sup>-</sup> and WT $\beta_2$ AR relative to that in the HEK293 showed that the PKA<sup>-</sup> are fully capable of activating ERK1/2. We also found maximal activation of ERK1/2 in the overexpressing cell lines at concentrations of epinephrine that cause no internalization (i.e., the EC<sub>50</sub> for internalization was 75 nM). Pertussis toxin pretreatment caused only a weak inhibition of epinephrine activation of ERK1/2 in the HEK293 (7–16%) and no inhibition in the PKA<sup>-</sup> cells. Finally we found that the Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (10  $\mu$ M) caused a >90% inhibition of epinephrine or forskolin activation of ERK1/2 in both cell lines. Our results indicate that the dominant mechanism of  $\beta_2$ AR activation of ERK1/2 does not require PKA phosphorylation of the  $\beta_2$ AR, receptor internalization or switching from activation of  $G_s$  to  $G_i$ , but clearly requires activation of a Src family member that may be downstream of PKA.

Early reports that purified  $\beta_2$ AR or a synthetic peptide corresponding to the third intracellular loop of the  $\beta_2$ AR could activate pure  $G_i$  in reconstituted preparations in vitro raised the possibility that the  $\beta_2$ AR could activate  $G_i$  in vivo (Cerione et al., 1985; Rubenstein et al., 1991), although with reduced efficiency relative to  $G_s$ . It was then demonstrated that PKA phosphorylation of a III<sub>1</sub> loop peptide of the  $\beta_2$ AR increased activation of  $G_i$  in reconstituted preparations and slightly reduced its activation of  $G_s$  (Okamoto et al., 1991), leading to the proposal that  $\beta_2$ AR activation could switch from  $G_s$  to  $G_i$  after PKA phosphorylation of the III<sub>1</sub> loop.

Recent support for this scheme was derived from studies showing that isoproterenol activation of ERK1/2 in HEK293 cells was blocked more than 85% by pretreatment with pertussis toxin, that isoproterenol activation of ERK1/2 was inhibited by H89, and that transient expression of a  $\beta_2$ AR lacking the PKA consensus sites (S261A, S262A, S345A, S346A) inhibited isoproterenol activation of ERK1/2 by 40% (Daaka et al., 1997). Other studies are not consistent with the switching hypothesis. First, it has been shown that forskolin activates ERK1/2 consistent with a PKA-mediated pathway of activation downstream of the receptor (Daaka et al., 1997; Schmitt and Stork, 2000). Second, a recent study found that isoproterenol activation of ERK1/2 in HEK293 cells was not inhibited by pertussis toxin (Schmitt and Stork,

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**ABBREVIATIONS:**  $\beta_2$ AR,  $\beta_2$ -adrenergic receptor; HEK, human embryonic kidney; ERK, extracellular signal-regulated kinase; PKA, cyclic AMP-dependent protein kinase; WT, wild-type; CGP-12177, 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one; PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; DMEM, Dulbecco's modified Eagle's medium; HA, hemagglutinin; AT, ascorbate/thiourea; HE, HEPES/EDTA; PAGE, polyacrylamide gel electrophoresis; ICI-118551, ( $\pm$ )-1-[2,3-(dihydro-5,7-methyl-1H-inden-4-yl)oxy]-3-[(methyl)amino]-2-butanol; EGF, epidermal growth factor.

2000). Third, we have shown that pertussis toxin pretreatment does not alter epinephrine-induced desensitization of HEK293 cells and S49 lymphoma cells, as would be expected if a  $G_s$  to  $G_i$  switch occurred (Clark et al., 1986; Seibold et al., 2000). Fourth, isoproterenol activation of ERK1/2 does not occur in the  $kin^-$  and  $cyc^-$  mutants of S49 mouse lymphoma cells (Wan and Huang, 1998).

Reports on the role of internalization of the  $\beta_2AR$  in ERK1/2 activation, studied through the use of transient expression of dominant negative dynamin (K44A) and mutant arrestins, have also been inconsistent. Several studies suggested that internalization was required for ERK1/2 activation by the  $\beta_2AR$  in HEK293 cells (Daaka et al., 1998; Luttrell et al., 1999b) and COS-7 cells (Pierce et al., 2000), whereas others found that internalization was not required for  $\beta_2AR$  activation of ERK1/2 in COS-1 cells (DeGraff et al., 1999).

Several lines of evidence suggest a role for Src in activation of ERK1/2 by the  $\beta_2AR$ : i) isoproterenol activation caused the formation of a multiprotein complex of receptor,  $\beta$ -arrestin, and Src, and dominant negative mutants of Src reduced ERK1/2 activation (Daaka et al., 1997; Luttrell et al., 1999b; Zou et al., 1999; Miller et al., 2000); ii) Src was shown to be activated by  $G_s\alpha$  and  $G_i\alpha$  in reconstituted *in vitro* preparations suggesting direct activation of Src by G proteins (Ma et al., 2000); and iii) it was suggested that Src binds to tyrosine 350 of the  $\beta_2AR$  after phosphorylation of this residue by an unidentified tyrosine kinase (Fan et al., 2001).

Obviously there seems to be considerable complexity in  $\beta_2AR$  activation of ERK1/2, and many issues are unresolved. In this article, we examine the role of receptor switching,  $G_i$ -internalization, and Src family kinases in  $\beta_2AR$  activation of ERK1/2 in HEK293 cells. We had previously generated a mutant  $\beta_2AR$  in which the two PKA consensus sites were eliminated by substitution of serines 261, 262, 345, and 346 with alanine, and this mutant receptor, termed PKA<sup>-</sup>, was stably overexpressed in the HEK293 cell line. We reported previously that the coupling efficiency of this mutant and the  $EC_{50}$  for activation of adenylyl cyclase were nearly identical to those of WT $\beta_2AR$  overexpressed at comparable levels (Seibold et al., 1998, 2000). Stable overexpression of the PKA<sup>-</sup> and the WT $\beta_2AR$  (~100-fold relative to endogenous receptor) caused a dramatic left-shift in the  $EC_{50}$  for epinephrine activation of adenylyl cyclase. This property of the  $\beta_2AR$  allows the analysis of mutant receptors overexpressed in HEK293 cells because the response of the endogenous receptor is overwhelmed if expression is high enough. Because the  $EC_{50}$  for epinephrine activation of adenylyl cyclase in cells expressing either the PKA<sup>-</sup> or WT $\beta_2AR$  is left-shifted relative to the endogenous receptor, it follows that activation of ERK1/2 should show a similar left-shift if the mechanism involves receptor activation of  $G_s$ . The prediction of the switching model in which the PKA-phosphorylated  $\beta_2AR$  is proposed to activate  $G_i$  is that there should be no left shift in ERK1/2 activation relative to the endogenous receptor; rather, there should be an inhibition of the activation by endogenous  $\beta_2AR$  (Daaka et al., 1997). The potency of agonist activation of ERK1/2 in HEK293 cells by transiently expressed wild-type or PKA<sup>-</sup> mutant  $\beta_2AR$ s was not examined in previous studies (Daaka et al., 1997; Schmitt and Stork, 2000).

In this article, we demonstrate that the stably overexpressed PKA<sup>-</sup> mutant of the  $\beta_2AR$  shows the predicted enormous left shift in the potency of activation of ERK1/2 by

epinephrine relative to that of the endogenous low level of expression in the HEK293 and that the shift is nearly identical to that found with overexpressed wild-type  $\beta_2AR$ . This demonstrated that the PKA consensus sites were not required for full activation of ERK1/2. Our data also show only a minor role for  $G_i$  in epinephrine activation of ERK1/2, no requirement for internalization (because the  $EC_{50}$  for epinephrine-induced internalization in the PKA<sup>-</sup> was about 1000-fold higher than that for activation of ERK1/2), and a requirement for Src family kinase activation.

## Materials and Methods

**Materials.** Antibodies to ERK1/2 and phospho-ERK1/2 were purchased from Cell Signaling Technology Inc. (Beverly, MA). [<sup>32</sup>P]NAD and [<sup>3</sup>H]CGP-12177 were obtained from PerkinElmer Life Sciences (Boston, MA). Pertussis toxin was from List Biological Laboratories Inc. (Campbell, CA). Enhanced chemiluminescence reagents and Hyperfilm were from Amersham Biosciences (Piscataway, NJ). Agonists and antagonists were purchased from Sigma-Aldrich. BA85 nitrocellulose was from Schleicher & Schuell (Keene, NH). Cell culture reagents were purchased from Mediatech (Herndon, VA). The Src family kinase inhibitor PP2 was from Alexis Corporation (Läufelfingen, Switzerland).

**Cell Culture.** HEK293 cells purchased from the American Type Culture Collection (Manassas, VA) were grown in 5% CO<sub>2</sub> at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. The stably transfected HEK293 cell lines used in this study expressing either WT or mutant receptors have been described previously (January et al., 1997; Seibold et al., 1998; Seibold et al., 2000), and were as follows: double epitope-tagged wild-type receptor (HA- $\beta_2AR$ -His<sub>6</sub>) and the double epitope-tagged PKA<sup>-</sup> (S261A, S262A, S345A, S346A), both with the hemagglutinin (HA) tag on the N terminus and His<sub>6</sub> tag on the C terminus; and HA-tagged wild-type (HA- $\beta_2AR$ ). The stably transfected cell lines were cultured in medium containing 400  $\mu$ g/ml G418. The levels of  $\beta_2AR$  in these transfect lines were 2 to 4 pmol/mg membrane protein, whereas the level of endogenous  $\beta_2AR$  was 30 to 40 fmol/mg.

**Cell Treatment and Preparation of Solubilized Extract.** Cells were grown to confluence in growth medium in 12-well plates coated with poly(L-lysine). The medium was removed 18 h before treatment, cells were incubated for 30 min with serum-free DMEM, and that medium was replaced with 2 ml of serum-free DMEM with or without 100 ng/ml of pertussis toxin. Cells were treated with hormones or carrier as indicated at 37°C with continuous rocking; pretreatments with  $\beta_2AR$  antagonists were for 2 min before agonist treatment, and PP2 was added 1 h before agonist treatment. Epinephrine was stored in 10 mM ascorbate/100 mM thiourea pH 7 (AT). Stock solutions were diluted 100-fold when additions were made to cells such that the final concentration of AT in all incubations (control and treated) was 0.1 mM ascorbate/1.0 mM thiourea. PP2 was dissolved and stored in DMSO and was diluted 100-fold for cell treatments. To terminate treatments, the medium was removed and 0.5 ml of solubilization buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.8% dodecyl maltoside, 1 mM EDTA, pH 7, 20 mM tetrasodium pyrophosphate, 10 mM NaF, 10  $\mu$ g/ml benzamidine, 10  $\mu$ g/ml trypsin inhibitor, 10  $\mu$ g/ml leupeptin, and 0.1  $\mu$ M okadaic acid) was added to each well. The plates were placed on ice, and the solubilized cells were pipetted into 1.5-ml microcentrifuge tubes. The tubes were rocked at 4°C for 30 min, and then centrifuged at 12,500 rpm for 15 min. The supernatants were removed and frozen at -80°.

**Measurement of ERK1/2 and Phospho-ERK1/2.** To measure activation of ERK1/2 by Western blotting, 20  $\mu$ l of the solubilized extracts were resolved by SDS-PAGE (10% gels) and blotted onto BA-85 nitrocellulose. The blots were blocked for 1 h in 5% nonfat dried milk in wash buffer (150 mM NaCl, 50 mM Tris pH 7.5, and 0.1% Tween 20). After two 10-min washes, the blots were incubated

overnight at 4°C on a rocker with a 1:1000 dilution of anti-phospho-ERK, washed twice for 10 min each, and incubated for 1 h at room temperature with a 1:5000 dilution of secondary antibody (goat anti-rabbit HRP-conjugate, Bio-Rad, Hercules, CA) After 2 washes, the blots were exposed to ECL reagents for 1 min, dried, and exposed to Hyperfilm (Amersham Biosciences) for 15 sec to 2 min. The blots were then stripped and reprobed identically with anti-ERK. Western blots were quantitated using Scion Image software (Scion Corp., Frederick, MD). All results with the anti-phospho-ERK were normalized to the corresponding anti-ERK.

**Membrane Preparation and Adenylyl Cyclase Assay.** Cells were plated into 100-mm dishes that had been precoated with poly(L-lysine). Treatments were administered at 37°C and were stopped by removal of media followed by six washes with 5 ml of ice-cold HE buffer (20 mM HEPES, pH 8.0, 1 mM EDTA, pH 7). The cells were scraped into HE containing 10 µg/ml leupeptin and 0.1 µM okadaic acid and homogenized with seven strokes in a type B Dounce homogenizer (Bellco Glass, Vineland, NJ). The homogenates were layered onto sucrose step gradients (23 and 43% prepared in HE buffer) and centrifuged at 25,000 rpm in a Beckman SW28.1 rotor for 35 min. The fraction at the 23/43% sucrose interface was removed, frozen in liquid nitrogen, and stored at -80°C. Adenylyl cyclase activity was measured as described previously (Seibold et al., 2000).

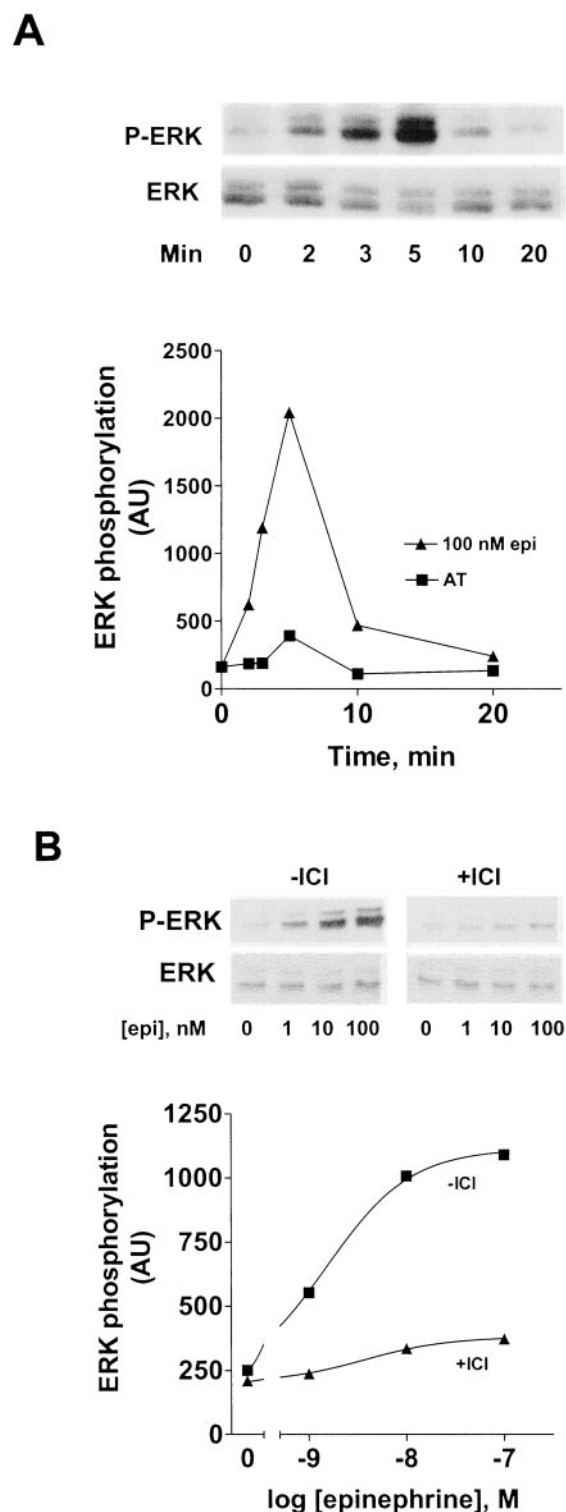
**ADP Ribosylation.** Membranes from control or pertussis toxin-treated cells were incubated for 30 min at 30°C with 10 µM NAD, 0.5 mM ATP, 0.2 mM GDP, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, pH 7, 20 mM Tris, pH 7.5, 5 mM dithiothreitol, 5 mM thymidine, 8 mM creatine phosphate, 8 U/ml creatine phosphokinase, and 5 µCi/tube [<sup>32</sup>P]NAD. The incubation mix was diluted in 5 ml of 20 mM Tris, pH 7.5, and centrifuged for 15 min at 30,000 rpm in a Beckman 50Ti rotor (Beckman Coulter, Fullerton, CA). The pellets were each dissolved in SDS sample buffer and resolved on 12% SDS-PAGE gels. The proteins were transferred to nitrocellulose and exposed to a Storm Phosphorimager screen (Molecular Dynamics, Sunnyvale, CA). The phosphorylated bands were quantitated using ImageQuant (Molecular Dynamics).

**Internalization.** The procedure for measuring internalization of the β<sub>2</sub>AR has been described in detail previously (Seibold et al., 2000). Cells in 12-well dishes were treated with epinephrine or AT for 5 min. Cells were then washed five times with ice-cold, serum-free DMEM, placed on ice, then incubated with 5 to 10 nM [<sup>3</sup>H]CGP-12177 with or without 1 µM alprenolol in serum-free DMEM. Dishes were then incubated for 1 h on ice, washed twice with ice-cold PBS to remove [<sup>3</sup>H]CGP-12177, and cells released by trypsin were transferred to scintillation vials for counting.

## Results

### Characterization of β<sub>2</sub>AR Agonist Activation of ERK1/2 in HEK293 Cells.

To examine the activation of ERK1/2 by the endogenous β<sub>2</sub>AR (≈20–30 fmol/mg membrane protein), HEK293 cells grown to confluence were placed in serum-free medium for 18 h before treatment with epinephrine. As shown in Fig. 1A, activation of ERK1/2 by 100 nM epinephrine showed a slight lag after which levels peaked at 5 min and then sharply declined, returning to near control levels by 20 min. To determine whether the activation of ERK1/2 by epinephrine was mediated by its binding to the β<sub>2</sub>AR, several approaches were taken. We found that 5 µM ICI-118551, a selective β<sub>2</sub>AR antagonist, added 2 min before a 5-min incubation with various concentrations of epinephrine, caused a nearly complete inhibition of ERK1/2 activation (Fig. 1B), consistent with the competitive action of ICI-118551. We also made the following observations (data not shown): i) the activation of ERK1/2 by 100 nM isoproterenol was equivalent to epineph-



**Fig. 1.** Characterization of epinephrine activation of ERK1/2 in HEK293 cells. A, time course of epinephrine activation of ERK1/2. HEK293 cells grown to confluence in 12-well plates were treated with 100 nM epinephrine or carrier (AT) for the indicated times. B, ICI-118551 inhibition of epinephrine-activated ERK1/2. HEK293 cells were pretreated for 2 min ± 5 µM ICI-118551, followed by 5-min treatments with the indicated concentrations of epinephrine or carrier (AT). After treatment, cells were solubilized and extracts were subjected to SDS-PAGE, transferred onto BA85 nitrocellulose membranes, and Western blotted with anti-phospho-ERK1/2 as described under *Materials and Methods*. Gels were then stripped and reprobed with anti-ERK1/2. Results with anti-phospho-ERK were normalized to the corresponding anti-ERK data and shown as arbitrary units (AU) on the y-axis.

rine and was also blocked by ICI-118551; ii) 20 nM salmeterol, a partial agonist and one of the most selective  $\beta_2$ AR agonists, showed  $\approx 80\%$  of the activation of ERK1/2 induced by epinephrine and was also blocked by ICI-118551; iii) 100 nM prazosin (an antagonist of both  $\alpha_1$ - and  $\alpha_2$ -adrenergic receptors) had no effect on epinephrine activation of ERK1/2. These data demonstrated that epinephrine activation of ERK1/2 was specific for the  $\beta_2$ AR.

To determine the  $EC_{50}$  for epinephrine activation of ERK1/2, HEK293 cells were incubated for 5 min with a range of epinephrine concentrations. Immunoblots from one representative experiment are shown in Fig. 2, as well as a data summary from five identical experiments. The  $EC_{50}$  for epinephrine activation of ERK1/2 was 5.7 nM. It should be noted that this  $EC_{50}$  is about 100- to 200-fold lower than that for epinephrine activation of adenylyl cyclase in membranes prepared from these cells (600–800 nM, data not shown).

**Epinephrine Stimulation of ERK1/2 in HEK293 Cells Overexpressing Either PKA<sup>-</sup>, HA- $\beta_2$ AR-His<sub>6</sub>, or HA- $\beta_2$ AR.** As discussed above, overexpression of  $\beta_2$ AR in a variety of cell types causes a progressive decrease in the  $EC_{50}$  (increased potency) for  $\beta_2$ AR activation of adenylyl cyclase (Whaley et al., 1994; Seibold et al., 1998; Clark et al., 1999). We have shown previously that the  $EC_{50}$  values for epinephrine activation of adenylyl cyclase in cells overexpressing either the WT $\beta_2$ AR or the PKA<sup>-</sup> were left-shifted 50- to 100-fold relative to the endogenous receptor in HEK293 cells. It follows that activation of ERK1/2 should show a similar left shift if the mechanism involved for both processes was

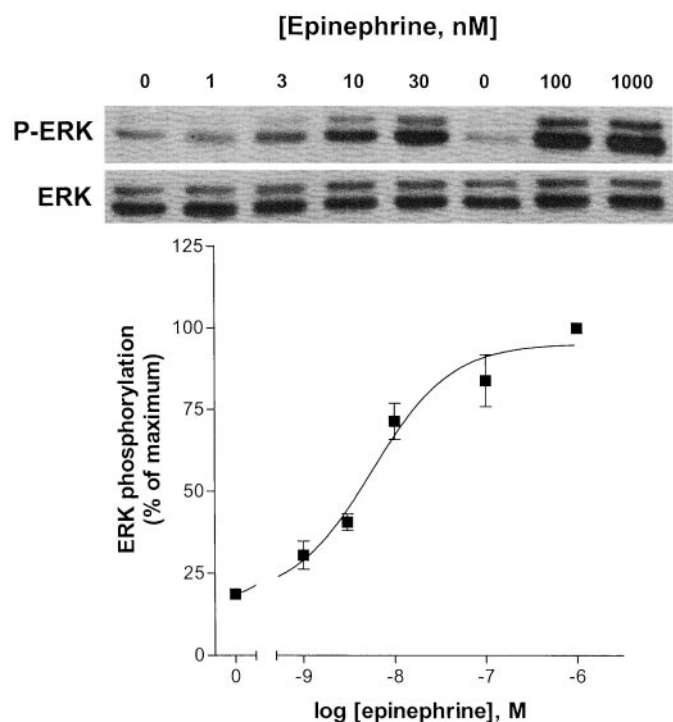
similar at the level of receptor coupling to G<sub>s</sub>/adenylyl cyclase.

To determine whether the  $EC_{50}$  for epinephrine activation of ERK1/2 in the PKA<sup>-</sup> overexpression line was left-shifted relative to the endogenous receptor and whether the shift in the potency of the PKA<sup>-</sup> was similar to that of two lines overexpressing the WT $\beta_2$ AR to similar levels, the experiments shown in Fig. 3 were performed. HEK293 cells stably overexpressing either the PKA<sup>-</sup>, HA- $\beta_2$ AR-His<sub>6</sub>, or HA- $\beta_2$ AR (2–4 pmol/mg) were incubated with a range of epinephrine concentrations for 5 min and the  $EC_{50}$  values for ERK1/2 activation determined. The  $EC_{50}$  for epinephrine activation of ERK1/2 in the PKA<sup>-</sup> cell line was  $\approx 36$  pM, more than 100-fold left-shifted relative to epinephrine activation of ERK1/2 by the endogenous receptor in HEK293 cells. Concentrations as low as 10 pM gave a significant increase in ERK1/2 phosphorylation. The  $EC_{50}$  values for epinephrine activation of ERK1/2 in the HA- $\beta_2$ AR-His<sub>6</sub> and HA- $\beta_2$ AR were in the range of 20 to 40 pM. These data demonstrated that the PKA<sup>-</sup>  $\beta_2$ AR was unimpaired in its activation of ERK1/2 because the potency of epinephrine activation was similar in the two lines overexpressing the wild-type receptors. Furthermore, as with the endogenous receptor, the left shift in the  $EC_{50}$  for ERK1/2 activation by the three overexpressing clones relative to their  $EC_{50}$  for adenylyl cyclase activation (January et al., 1998; Seibold et al., 2000) was more than 100-fold.

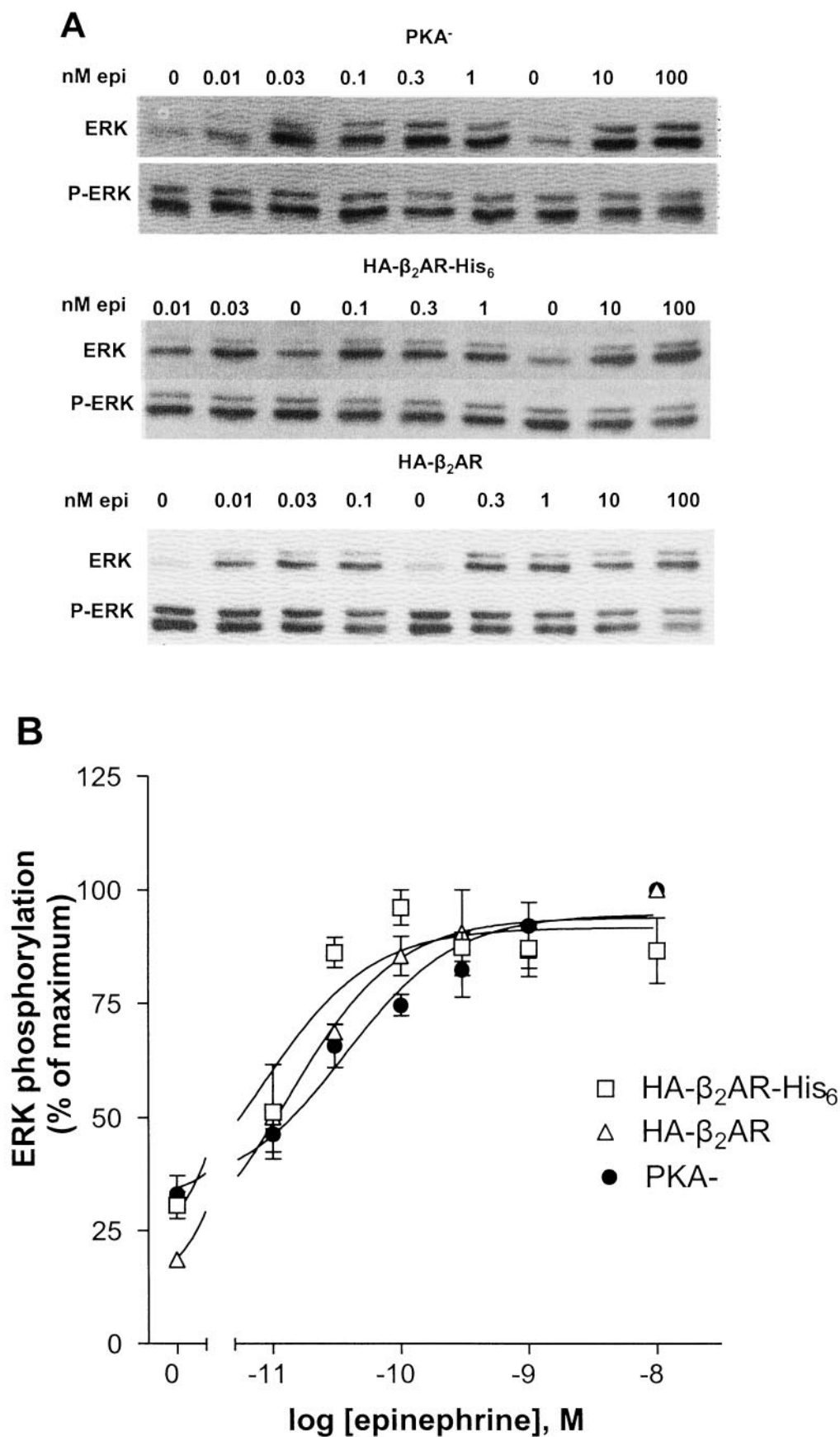
Because there was the possibility that the time course of ERK1/2 activation by epinephrine was altered at these very low concentrations, we followed the time course of ERK1/2 activation in the PKA<sup>-</sup> by 1.0 and 10.0 nM epinephrine as shown in Fig. 4. The data show that the time course of activation by 1.0 and 10.0 nM epinephrine in the PKA<sup>-</sup> cells was similar to that of the endogenous receptor in HEK293 cells. With lower concentrations of epinephrine, there is some indication that the lag is extended; however, the peak level of ERK1/2 remained at 5 min. A similar time course of ERK1/2 activation was found in the cells expressing the two wild-type receptors.

**Effect of Pertussis Toxin Pretreatment of HEK293 Cells on the  $\beta_2$ -Adrenergic Activation of ERK1/2.** To determine the role of G<sub>i</sub> in epinephrine activation of ERK1/2, we examined the effect of pertussis toxin pretreatment. HEK293 cells expressing only endogenous levels of receptor and the PKA<sup>-</sup> cell line were pretreated with or without 100 ng/ml of pertussis toxin for 18 h in serum-free medium. Cells were then incubated with various concentrations of epinephrine for 5 min. As shown in Fig. 5A (summary of seven independent experiments with a typical Western blot above), pertussis toxin pretreatment of HEK293 cells expressing the endogenous receptor caused only a 7 to 16% inhibition of ERK1/2 activation by the various concentrations of epinephrine. We also found only marginal pertussis toxin inhibition of either isoproterenol or salmeterol activation of ERK1/2 in the HEK293 cell line (data not shown). No significant pertussis toxin inhibition of epinephrine activation of ERK1/2 in the PKA<sup>-</sup>-expressing cells was observed (Fig. 5B).

Because pertussis toxin treatment caused only a weak inhibition of epinephrine activation of ERK1/2 by the endogenous receptor, and no inhibition of ERK1/2 activation by the PKA<sup>-</sup>, we performed several controls to determine the extent of toxin activity. First, cells were pretreated overnight with



**Fig. 2.** Dose response of epinephrine activation of ERK1/2 in HEK293 cells. HEK293 cells were treated for 5 min with the indicated concentrations of epinephrine. The cells were solubilized and ERK1/2 phosphorylation measured and quantitated as described under *Materials and Methods*. The data on the graph are the mean values  $\pm$  S.E.M. from five experiments, each performed in duplicate. The data from each experiment were converted to percentage of the maximum stimulation before determination of the means. The Western blot is from one representative experiment.

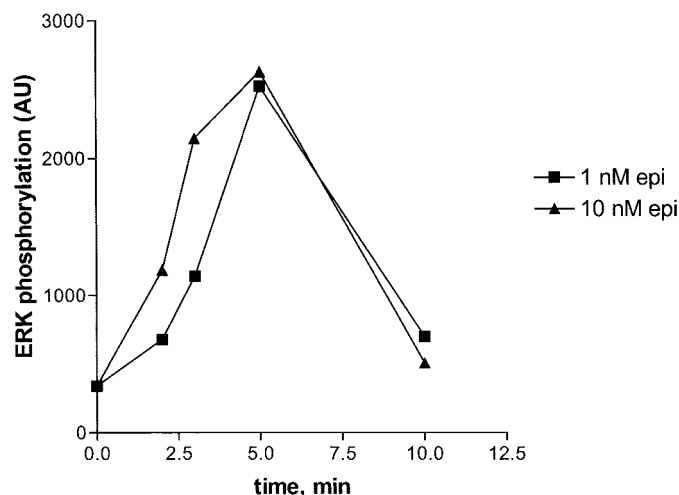


**Fig. 3.** Dose response of epinephrine activation of ERK1/2 in HEK293 cells stably expressing either the PKA<sup>-</sup>, HA-β<sub>2</sub>AR-His<sub>6</sub>, or HA-β<sub>2</sub>AR. Cells expressing the various receptors were treated for 5 min with the indicated concentrations of epinephrine. The cells were solubilized and ERK1/2 phosphorylation was measured and quantitated as described under *Materials and Methods*. A, Western blots are from one representative experiment for each cell line. B, the data are the mean ± S.E.M. of seven (PKA<sup>-</sup>) or four (HA-β<sub>2</sub>AR-His<sub>6</sub> and HA-β<sub>2</sub>AR) experiments, each performed in duplicate. The data from each experiment were converted to percentage of maximum epinephrine stimulation of ERK1/2 phosphorylation before calculating the means.

pertussis toxin, and membranes derived from control and pertussis toxin-treated HEK293 or PKA<sup>-</sup> cells were ADP-ribosylated in the presence of [<sup>32</sup>P]NAD. Overnight treatment with pertussis toxin resulted in a >90% inhibition of ADP-ribosylation of G<sub>i</sub>/G<sub>o</sub> in membrane preparations (Fig. 6A). As a second control, we examined pertussis toxin's effect on epinephrine stimulation of adenylyl cyclase. We found that pertussis toxin treatment of HEK293 cells caused an approximate doubling of epinephrine-stimulated adenylyl cyclase activity with no change in the EC<sub>50</sub> (Fig. 6B). This result is consistent with our prior study of this toxin's effects on epinephrine stimulation of adenylyl cyclase in the HEK293 cells overexpressing the wild-type and mutant  $\beta_2$ AR (Seibold et al., 2000) (i.e., a doubling of the V<sub>max</sub> and no change in the EC<sub>50</sub>).

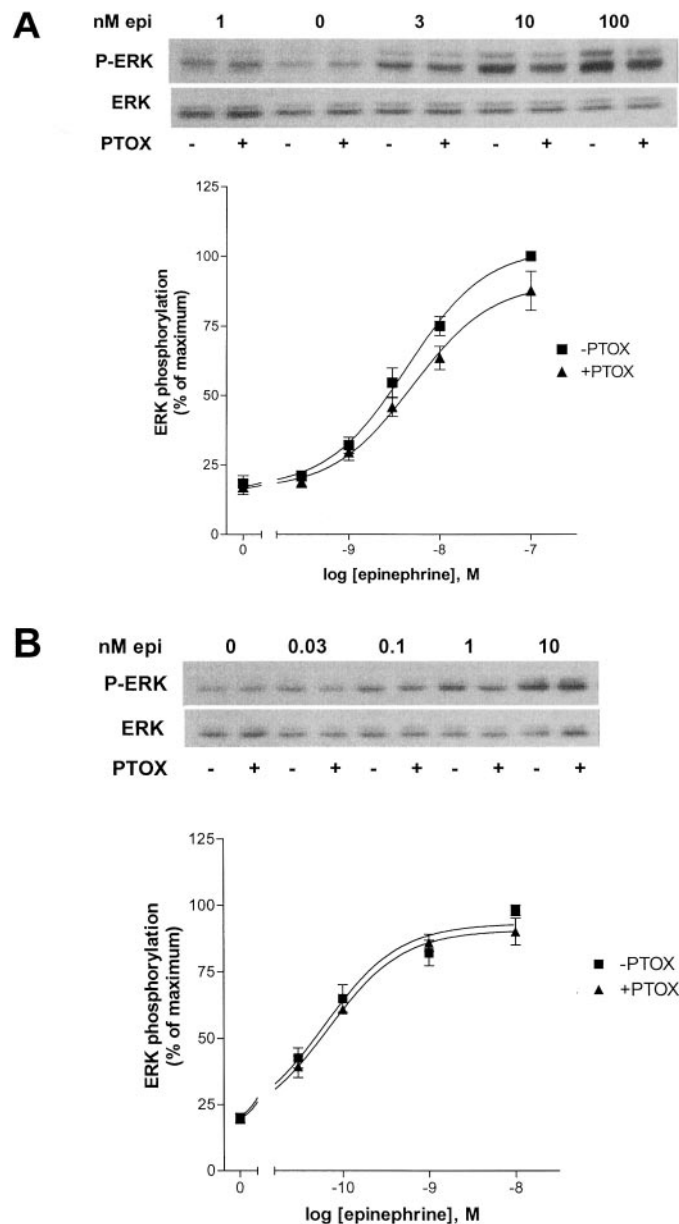
**Internalization of the  $\beta_2$ AR in PKA<sup>-</sup>.** As discussed, there have been some inconsistencies concerning the role of  $\beta_2$ AR internalization in activation of ERK1/2. It occurred to us that we could determine how closely these two processes were related by comparing their EC<sub>50</sub> values. To measure the EC<sub>50</sub> for epinephrine-induced internalization of the PKA<sup>-</sup>  $\beta_2$ AR, cells were treated for 5 min with or without various epinephrine concentrations as indicated in Fig. 7 (■). Internalization was measured using [<sup>3</sup>H]CGP-12177 as described under *Materials and Methods*. Epinephrine (10 nM) produced a barely detectable level of internalization, and the EC<sub>50</sub> for epinephrine-induced internalization was 75 nM. To compare the EC<sub>50</sub> for epinephrine-induced internalization with that for activation of adenylyl cyclase and ERK1/2, the PKA<sup>-</sup> data from Fig. 3 and the results from a typical adenylyl cyclase assay are also plotted in Fig. 7. It can be seen that the EC<sub>50</sub> for epinephrine activation of internalization is approximately 1000-fold higher than that for ERK1/2 and 10-fold higher relative to activation of adenylyl cyclase.

**Inhibition of ERK1/2 Activation by the Src Family Kinase Inhibitor PP2.** To assess the role of the Src family in ERK1/2 activation, PKA<sup>-</sup> cells were pretreated with the Src family inhibitor PP2 for 1 h before stimulation with



**Fig. 4.** Time course of epinephrine activation of ERK1/2 in PKA<sup>-</sup> cells. PKA<sup>-</sup> cells were treated with either 1.0 nM epinephrine (■) or 10.0 nM epinephrine (▲) for the times indicated on the graph. The value at zero time is the mean of controls determined at each of the time points. The cells were solubilized and ERK1/2 phosphorylation measured and quantitated as described under *Materials and Methods*. The data are from one representative experiment.

either epinephrine, forskolin, or EGF (Fig. 8). PP2 blocked 90 to 100% of epinephrine and forskolin activation of ERK1/2, but produced only a 33% inhibition of EGF stimulation. Similar results were obtained with the HEK293 cells expressing only endogenous  $\beta_2$ AR (data not shown). These data strongly suggest that Src family kinases play an essential role in  $\beta_2$ AR activation of ERK1/2. Furthermore, the inhibition of forskolin activation of ERK1/2 shows that it is unlikely that a direct receptor or G protein interaction with Src is required.



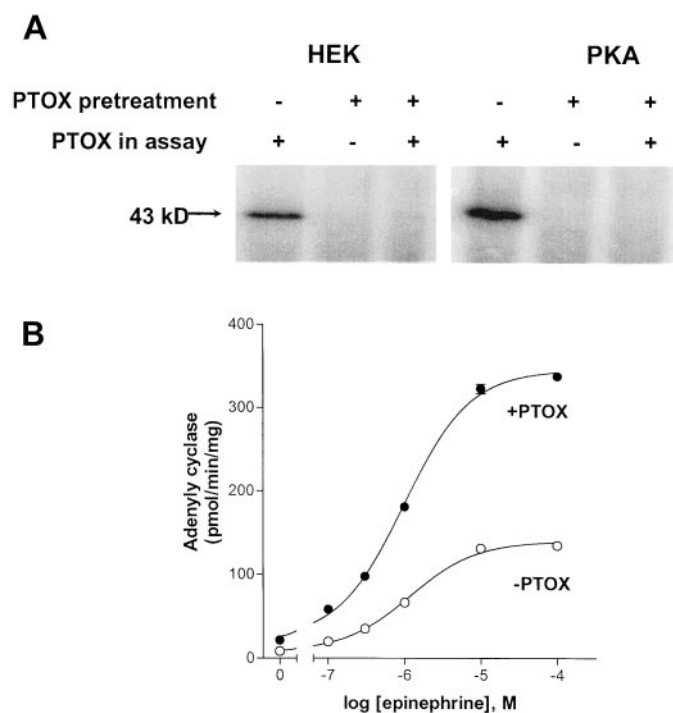
**Fig. 5.** Effect of pertussis toxin on epinephrine activation of ERK1/2 in HEK293 cells. HEK293 cells expressing either the endogenous  $\beta_2$ AR only (A) or the PKA<sup>-</sup>  $\beta_2$ AR (B) growing in 12-well plates were placed in serum-free medium and then treated with or without 100 ng/ml pertussis toxin (PTOX) for 18 h as described under *Materials and Methods*. Cells were incubated with increasing concentrations of epinephrine for 5 min as indicated. Phosphorylation of ERK1/2 was measured as described under *Materials and Methods*. The data are the means  $\pm$  S.E.M. of seven (HEK293) or three (PKA<sup>-</sup>) experiments. One representative Western blot is shown for each cell line.

## Discussion

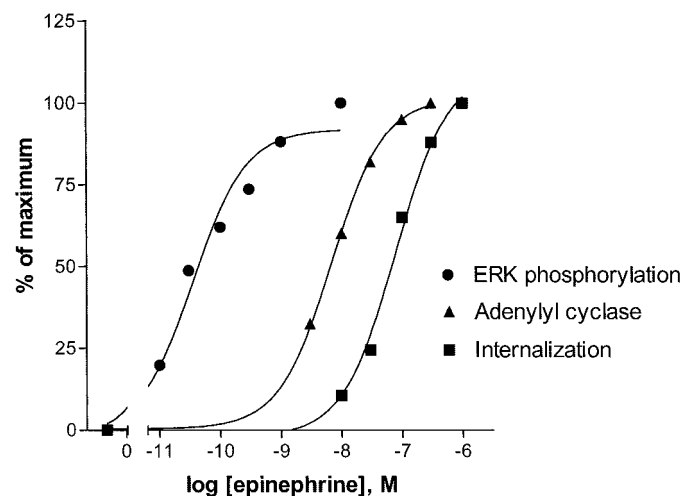
In this article, we focused on the proposal that PKA phosphorylation of the  $\beta_2$ AR switches receptor activation from  $G_s$  to  $G_i$  and that this switch of receptor activation of G proteins was required for ERK1/2 activation, as was internalization of the receptor. Two approaches were used to assess the capacity of the PKA<sup>-</sup> receptor to activate ERK1/2, both of which were based on our prior studies demonstrating that  $\beta_2$ AR overexpression predictably left-shifts the  $EC_{50}$  for agonist activation of adenylyl cyclase relative to that for the endogenous  $\beta_2$ AR. First, we found that the potencies ( $EC_{50}$  values) for epinephrine activation of ERK1/2 in the PKA<sup>-</sup> and the clones overexpressing WT $\beta_2$ AR were similar (20–60 pM) and about 100- to 200-fold lower than the  $EC_{50}$  for epinephrine activation of endogenous receptor in HEK293 cells (5–6 nM). Second, we determined whether the amplification of epinephrine activation of ERK1/2 relative to its activation of adenylyl cyclase for the overexpressed  $\beta_2$ ARs was similar to that for the endogenous receptor. For the endogenous receptor, the  $EC_{50}$  for epinephrine activation of adenylyl cyclase in cell-free membrane preparations ( $\approx 600$ –800 nM) was about 100-fold greater than the  $EC_{50}$  for ERK1/2 activation (5–6 nM), whereas the comparable ratio for the PKA<sup>-</sup> was about 200- to 300-fold (15 nM for adenylyl cyclase activation, and 30–60

pM for ERK1/2 activation). The shift in sensitivity of the PKA<sup>-</sup> for ERK1/2 activation relative to adenylyl cyclase was actually somewhat greater than that for the HEK293 cell line expressing only endogenous  $\beta_2$ AR.

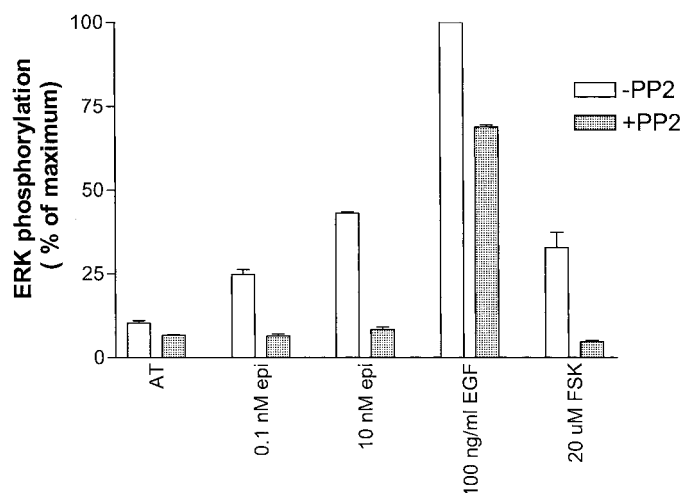
Our work also addressed the role of  $G_i$  in  $\beta_2$ AR activation of ERK1/2. Previous studies had found either a major role for  $G_i$  for agonist activation of ERK1/2 after stimulation of the endogenous  $\beta_2$ AR in HEK293 cells (Daaka et al., 1997) or none (Schmitt and Stork, 2000). In the latter study, activa-



**Fig. 6.** Evidence for the efficacy of pertussis toxin treatments. **A**, ADP-ribosylation of  $G_i/G_o$  in membranes from HEK293 and PKA<sup>-</sup> cells pretreated for 18 h  $\pm$  pertussis toxin. HEK293 or PKA<sup>-</sup> cells plated into 100-mm dishes were treated with or without pertussis toxin (100 ng/ml) and membranes were harvested and frozen as described under *Materials and Methods*. Thawed membranes were then incubated for 30 min in the presence or absence of pertussis toxin in the ADP-ribosylation mix containing [<sup>32</sup>P]NAD. Membranes were washed and aliquots resolved on 12% SDS-PAGE. **B**, epinephrine-stimulated adenylyl cyclase activity in membranes from HEK293 cells treated with pertussis toxin or not. HEK293 cells in 100-mm dishes were treated overnight with or without 100 ng/ml of pertussis toxin, membranes were prepared, and adenylyl cyclase stimulation by the indicated concentrations of epinephrine was measured in the membranes. The data are the means  $\pm$  S.E.M. of triplicate determinations from one representative experiment.



**Fig. 7.** Dose response of epinephrine-induced internalization in the PKA<sup>-</sup> and comparison with that for activation of ERK1/2 and adenylyl cyclase. Cells in 12-well dishes were treated with various concentrations of epinephrine for 5 min, and internalization of the  $\beta_2$ AR was measured by surface binding of [<sup>3</sup>H]CGP-12177 as described under *Materials and Methods*. The data shown are the average values of two identical experiments, each performed in triplicate at the various concentrations of epinephrine (variation in the values was less than 5% at each time point). Results are expressed as the percentage of the total surface binding that was internalized. The data for epinephrine activation of ERK1/2 for the PKA<sup>-</sup> expressing cells were taken from Fig. 3. The dose response for epinephrine stimulation of adenylyl cyclase in PKA<sup>-</sup> membrane preparations is from one representative assay.



**Fig. 8.** Inhibition of epinephrine-, EGF-, and forskolin-activated ERK1/2 by PP2. To assess a possible role for Src in ERK1/2 activation, PKA<sup>-</sup> cells were pretreated for 1 h at 37°C with 1% DMSO or 10  $\mu$ M PP2. The cells were then incubated for 5 min with 100 pM or 10 nM epinephrine, 100 ng/ml EGF, or 20  $\mu$ M forskolin. Phosphorylation of ERK1/2 was measured as described under *Materials and Methods*. The data from each experiment were normalized as percentage of maximum ERK1/2 stimulation. The data are the means  $\pm$  S.E.M. from four experiments.

tion of ERK1/2 in HEK293 cells by isoproterenol stimulation of the endogenous  $\beta_2$ AR involved  $G_s$ /PKA activation of Rap1 and B raf with no role for  $G_i$ . In agreement with this study, we found no pertussis toxin inhibition of epinephrine activation of ERK1/2 in HEK293 cells expressing the PKA<sup>-</sup> and only a slight inhibition of activation in HEK293 cells expressing only the endogenous  $\beta_2$ AR, suggesting that  $G_i$  played little if any role. In further support of our conclusion concerning the effects of pertussis toxin, we previously reported that conditions (low concentrations of epinephrine) that provoke a PKA-mediated desensitization of the  $\beta_2$ AR result in only a modest 2- to 3-fold increase in the EC<sub>50</sub> for epinephrine stimulation of adenylyl cyclase in L cells (Yuan et al., 1994). If the  $\beta_2$ AR switched to activation of  $G_i$ , a considerably larger loss of epinephrine-stimulated adenylyl cyclase activity would be expected. Also, we have not found that pertussis toxin treatment alters the extent of epinephrine-induced desensitization of HEK293 cells (Clark et al., 1996; Seibold et al., 2000) as would be predicted if PKA-mediated switching occurred.

There are a number of reasons that might account for the differences between our work and that of others (Daaka et al., 1997) that were also performed on clones of the HEK293 cells. Clonal cell lines are adapted for fast growth and it is possible that there are important differences in the expression of factors involved in the complex activation of ERK1/2 that shunt activation to alternate pathways in different clones, as has been suggested previously (Liebmann, 2001). That is, it is entirely possible that for unknown but potentially very interesting reasons, the clonal lines of HEK293 cells used by other groups (Daaka et al., 1998; Luttrell et al., 1999a; Maudsley et al., 2000) express such factors as reduced levels of  $G_i$ -specific RGS proteins and different levels of receptor binding proteins that alter localization to microdomains in the plasma membrane thus diminishing the coupling efficiency of  $\beta_2$ AR activation of  $G_i$  (Hall et al., 1998; Ostrom et al., 2001). For reasons such as these (and one could imagine many other scenarios) the role of  $G_i$  may vary significantly from cell line to cell line. Important experimental differences in the present work were that we used only stable transfection of the  $\beta_2$ ARs and determined EC<sub>50</sub> values for epinephrine activation of ERK1/2 with overexpression of the PKA<sup>-</sup> and wild-type  $\beta_2$ ARs. EC<sub>50</sub> values were not determined in the previous study of the PKA<sup>-</sup> mutant (Daaka et al., 1997). It is possible that the use of transient overexpression paradigms in previous studies actually resulted in much higher levels of receptor expression in a fraction of the cells and/or differential localization, and these factors could affect specificity and/or coupling efficiency of  $\beta_2$ AR activation of  $G_s$  versus  $G_i$ . Our studies with transiently expressed  $\beta_2$ ARs in HEK293 indicate that they couple poorly to  $G_s$ /adenylyl cyclase (i.e., they show almost no left shift in EC<sub>50</sub> for epinephrine activation of adenylyl cyclase with high expression). Finally, it is important not to overinterpret results after an 18-h pretreatment with pertussis toxin (Piiper et al., 2000), because cAMP levels are elevated for prolonged periods.

With regard to a role of internalization in ERK1/2 activation, previous studies based on the use of transient expression of dominant negative blockers of internalization have given somewhat ambiguous results. Taking a different approach that circumvents the need for expression of dominant-negative dynamin or arrestins, which may have nonspecific

effects, we found that activation of ERK1/2 in the PKA<sup>-</sup> overexpressing cells occurred at approximately 1000-fold lower concentrations (EC<sub>50</sub> = 30–60 pM) of epinephrine than those required for internalization (EC<sub>50</sub> = 75 nM), demonstrating a huge amplification of ERK1/2 activation relative to internalization. Our result contrasts with the prior study showing a correlation of the two processes (Daaka et al., 1998). Because we found full ERK1/2 activation with extremely low levels of  $\beta_2$ AR occupancy that do not cause internalization, there should be no GRK phosphorylation of the  $\beta_2$ AR and arrestin binding, because it is generally accepted that they are correlated with and required for internalization. The very small amplification of internalization (EC<sub>50</sub> = 75 nM) we observed relative to occupation of the  $\beta_2$ AR ( $K_d$  for epinephrine is ~500–600 nM) is entirely consistent with previous work that GRK-mediated phosphorylation and binding of  $\beta$ -arrestin occurs at much higher concentrations of epinephrine than those required for PKA activation.

A number of findings suggest that Src activation plays a role in  $\beta_2$ AR stimulation of ERK1/2 (Daaka et al., 1997; Ma et al., 2000; Maudsley et al., 2000; Fan et al., 2001), and it has been suggested that Src is activated by either formation of a  $\beta_2$ AR/ $\beta$ -arrestin/Src complex or by direct  $G_s$  or  $G_i$  activation of Src. Consistent with a role for Src, we found that the Src family kinase inhibitor PP2 (10  $\mu$ M) caused a >90% inhibition of epinephrine and forskolin activation of ERK1/2 in the HEK293 cells. However, that we see full activation of ERK1/2 by very low concentrations of both epinephrine (which are most unlikely to cause formation of a  $\beta_2$ AR/ $\beta$ -arrestin/Src complex) and forskolin (which bypasses the need for receptor or G proteins by direct binding to adenylyl cyclase), raises the question of the mechanism of Src activation. In this regard Schmitt and Stork (2002) recently reported that PKA activates Src by phosphorylation of serine 17 in NIH3T3 cells, as well as in HEK293 cells (P. Stork, personal communication). Whereas this phosphorylation site on Src has been known for some time (Collett et al., 1979), the physiological relevance has not been understood. These studies provide evidence that PKA phosphorylates and activates Src directly and, combined with our studies showing full activation of ERK1/2 by extremely low receptor occupancy in the PKA<sup>-</sup> cells, leads us to propose that Src is activated by the  $\beta_2$ AR by the classic  $G_s$ /adenylyl cyclase(AC)/PKA pathway and that the dominant pathway for  $\beta_2$ AR activation of ERK1/2 in HEK293 cells is as follows:  $\beta_2$ AR  $\rightarrow$   $G_s$   $\rightarrow$  AC  $\rightarrow$  PKA  $\rightarrow$  Src  $\rightarrow$  ERK. The similar amplification in the potency of ERK1/2 activation relative to adenylyl cyclase activation by the endogenous and overexpressed  $\beta_2$ ARs is further indication that  $G_s$  and PKA are probably major upstream players in ERK1/2 activation.

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