0026-895X/93/040802-08$03.00/0

MOLECULAR PHARMACOLOGY, 44:802-809

Selective Coupling of α_2-Adrenergic Receptor Subtypes to Cyclic AMP-Dependent Reporter Gene Expression in Transiently Transfected JEG-3 Cells

DAVID J. PEPPERL and JOHN W. REGAN

Department of Pharmacology and Toxicology (D.J.P., J.W.R.), Department of Physiology (J.W.R.), and the Program in Neuroscience (J.W.R.), College of Pharmacy, University of Arizona, Tucson, Arizona 85721

Received January 8, 1993; Accepted July 21, 1993

SUMMARY

A cAMP-dependent reporter gene has been used in transiently transfected human choriocarcinoma (JEG-3) cells to examine the second messenger coupling of the human α_2-adrenergic receptor subtypes. The reporter gene consists of a cAMP response element linked to the gene for chloramphenicol acetyltransferase (CAT). Plasmids encoding the α_2-C10 (α_{2A}), α_2-C2 (α_{2B}), or α_2-C4 (α_{2C}) receptor subtypes were co-transfected with a plasmid containing the reporter gene, and the ability of α_2 receptor agonists to influence forskolin-stimulated CAT expression was examined. For α_2-C10, agonists had a biphasic effect on forskolin-stimulated CAT expression. Thus, low (nanomolar) concentrations of agonist inhibited CAT expression by ~60%, whereas high (micromolar) concentrations reversed this inhibition and could even potentiate CAT expression by as much as 140%. A significantly different pattern of coupling was observed for the other α_2 receptor subtypes. For α_2-C4, agonists only inhibited forskolin-stimulated CAT expression, whereas for α_2-C2 only potentiation of expression was seen. Each of these responses was specifically blocked by α_2- but not α_1- or β-adrenergic receptor antagonists. For α_2-C4, the inhibition of forskolin-stimulated CAT expression was prevented by pretreatment of the cells with pertussis toxin. This was also true for the inhibition obtained with α_2-C10. The potentiation of CAT expression, however, was not prevented by pertussis toxin pretreatment in cells transfected with either α_2-C2 or α_2-C10. In this transient expression system, each α_2-adrenergic receptor subtype had access to the same complement of G proteins, adenylyl cyclase, and other second messengers. It would appear, therefore, that the potential for the activation of unique intracellular responses exists even among closely related receptor subtypes.

The α_2ARs, along with the α_1ARs and βARs, mediate the physiological effects of the catecholamines epinephrine and norepinephrine (1). The α_2ARs are found throughout the body, particularly in platelets, where they mediate aggregation, and the brain, where α_2AR activation inhibits further norepinephrine release. At present, three subtypes of α_2ARs have been identified by molecular cloning (2). The α_2-C10, α_2-C2, and α_2-C4 subtypes, so named for their chromosomal locations, correspond to the pharmacologically defined α_{2A}, α_{2B}, and α_{2C} subtypes, respectively (3).

Typically, α_2ARs have been thought to couple to intracellular second messenger pathways by activating an inhibitory G protein and thereby inhibiting adenylyl cyclase. However, the α_2ARs may couple to other intracellular pathways involving Na^+/H^+ exchange, phospholipase A_2, and phosphatidylinositol hydrolysis (4). With regard to more specific studies with the cloned α_2AR subtypes, it was shown in stably transfected CHO cells that activation of α_2-C10 could decrease intracellular cAMP at low concentrations of agonist and increase it at high concentrations of agonist (5). Similar studies showed that α_2-C4 and α_2-C10 could both inhibit adenylyl cyclase and weakly activate inositol phosphate turnover when stably transfected into Chinese hamster lung fibroblasts (CCL39) (6). Less is known about the coupling of α_2-C2, although recently it was shown that all three α_2AR subtypes could both inhibit and stimulate adenylyl cyclase in membranes prepared from stably transfected CHO cells (7). To directly compare the function of these three α_2AR subtypes in an intact cell system, we have coexpressed each subtype with a cAMP-responsive reporter gene in human choriocarcinoma cells (JEG-3). Using this system, we have demonstrated marked functional differences between the α_2AR subtypes with respect to their ability to activate second messenger pathways. In doing this we also demonstrated

This work was supported by a grant from the National Institutes of Health (R29 EY09355-02) and by additional support from the Pharmaceutical Manufacturers Association, Allergan Inc., and Eli Lilly & Co.

ABBREVIATIONS: AR, adrenergic receptor; CHO, Chinese hamster ovary; CRE, cAMP response element; CAT, chloramphenicol acetyltransferase; FBS, fetal bovine serum; PTX, pertussis toxin; PAC, *p*-aminoclonidine; DMEM, Dulbecco's modified Eagle's medium.

the possibility of α_2AR subtypes differentially controlling the activity of genes that are regulated by CREs.

Materials and Methods

Reagents. Epinephrine bitartrate, norepinephrine, oxymetazoline, propranolol, yohimbine, PTX, and butyryl-CoA were from Sigma Chemical Co. (St. Louis, MO). Prazosin and UK-14304 were from Pfizer, Inc. (New York, NY). Forskolin was from Hoechst-Roussel Pharmaceutics (Somerville, NJ), medetomidine from Farmos Group Ltd. (Oulu, Finland), and PAC from Research Biochemicals, Inc. (Wayland, MA). Cell culture reagents were from GIBCO (Grand Island, NY), except for FBS, which was from Hyclone Laboratories (Logan, UT). [^{3}H]Chloramphenicol (30–36 Ci/mmol) was from NEN-DuPont (Boston, MA), and mixed xylenes were from Aldrich Chemical Co. (Milwaukee, WI).

Expression plasmids. The reporter plasmid used in these studies, TES*Bgl*IICRE(+)ΔNHSE, was generously provided by Dr. Pamela Mellon (Salk Institute, La Jolla, CA). This plasmid contains an 18-base CRE from the promotor of the α subunit gene for the human glycoprotein hormones, which is linked to the herpes simplex virus thymidine kinase promoter, which in turn is linked to DNA encoding bacterial CAT (8). In JEG-3 cells transfected with this plasmid, increases in intracellular cAMP stimulate the expression of CAT. The possibility of using this as a reporter for receptors that are coupled to adenylyl cyclase has been shown in a study with dopamine receptors (9). Plasmids encoding the α_2-C2, α_2-C4, and α_2-C10 AR subtypes were prepared from pBC12BI as described previously (10).

Cell culture. Human choriocarcinoma cells (JEG-3) were obtained from the American Type Culture Collection (HTB 36) and cultured in 5% CO_2 at 37° in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in 10-cm dishes 1–2 days before transfection. JEG-3 cells were transfected with 10 μg of reporter plasmid and 10 μg of α_2AR plasmid using the calcium phosphate precipitation technique (11). Briefly, cells were incubated at 37° in DMEM/5% FBS, pH 7.1, for 2 hr, at which time the DNA-calcium phosphate mixtures were added and incubated for 5 hr. The plates were incubated at 37° in DMEM/10% dimethylsulfoxide for 3 min and then maintained in DMEM/5% FBS for 36–40 hr. Cells were rinsed twice with DMEM and drugs were added in 5 ml of DMEM. The cells were incubated for 4 hr at 37° and then harvested.

Cell harvest and CAT assay. Immediately after the drug incubations, the cells were rinsed with cold PBS, pH 7.4, and scraped into 1 ml of 40 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl. Cells were centrifuged and lysed by three cycles of freeze-thaw in 200 μl of 250 mM Tris, pH 7.5. CAT assays (12) were performed using 50 μl of cytosol, 200 nCi of [^{3}H]chloramphenicol, and 300 μM butyryl-CoA. Assays were for 1 hr at 37° and were stopped by the addition of 200 μl of mixed xylenes. Butyrylated chloramphenicol was extracted into the xylenes, which were back-extracted twice with 200 μl of 10 mM Tris, pH 8.0, 1 mM EDTA. Radiolabeled product was measured by liquid scintillation counting, using a Packard Tri-Carb 460C counter at 50–52% efficiency.

Results

Preliminary studies showed that, using the calcium phosphate precipitation technique, each of the α_2AR subtypes could be expressed transiently in JEG-3 cells at levels ranging from 0.2 to 2 pmol/mg of protein. In six separate experiments, the following levels of expression were obtained for the three α_2AR subtypes (mean ± standard error): α_2-C2, 0.75 ± 0.23; α_2-C4, 0.31 ± 0.05; α_2-C10, 1.16 ± 0.33 pmol/mg of protein. These studies also indicated that untransfected JEG-3 cells and cells transfected with control plasmids did not express α_2ARs. In cells transfected with α_2-C10 and the reporter plasmid, a forskolin dose-response curve was obtained. Fig. 1 shows that

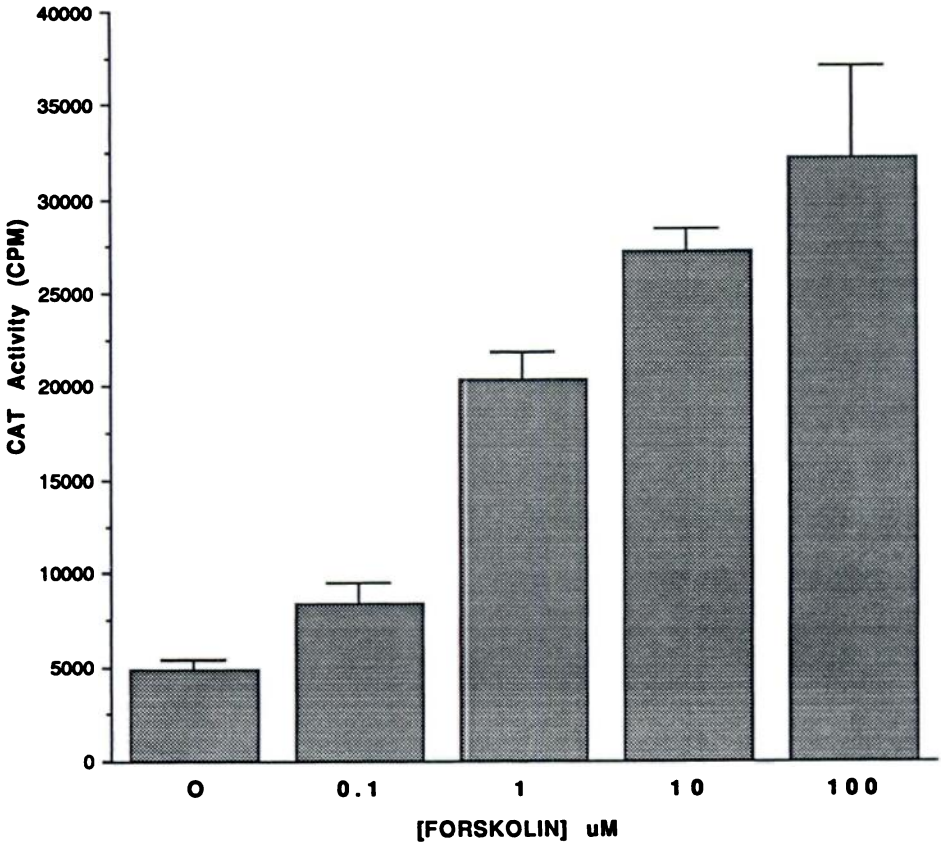


Fig. 1. Stimulation of CAT activity by forskolin in JEG-3 cells transfected with α_2-C10 and the CRE-CAT reporter gene. Cells were transfected with 10 μg of reporter plasmid and 10 μg of pBCα_2-C10, as described in Materials and Methods. Cells were incubated with forskolin for 4 hr. [^{3}H]CAT assays were done for 1 hr at 37°. Each *point* is the mean ± standard error from three plates of transfected cells. The experiment is representative of two that were done.

forskolin caused a significant dose-dependent stimulation of CAT activity, with a maximum 6-fold stimulation occurring at 100 μM forskolin. Other experiments showed that a concentration of 1 μM forskolin, which stimulated CAT activity ~4-fold above basal levels, was optimal for studying the inhibition of CAT activity by coincubation with α_2AR agonists. At higher concentrations of forskolin (100 μM) the α_2AR-mediated inhibition of CAT activity was difficult to observe, and thus 1 μM forskolin was used for all subsequent experiments.

Fig. 2 shows dose-response curves for the effects of α_2AR agonists on forskolin-stimulated CAT activity in JEG-3 cells co-transfected with plasmids encoding α_2-C10 and the reporter gene. The agonists epinephrine, PAC, medetomidine, and UK-14304 each produced a biphasic response, with low concentrations of agonist inhibiting CAT activity and high concentrations either returning activity to the 100% level (PAC, medetomidine, and UK-14304) or increasing activity ~1.4-fold to 240% (epinephrine). Norepinephrine also produced a biphasic response in α_2-C10-transfected cells, with 80% potentiation of CAT activity at the highest dose (data not shown). EC_{50} values for inhibition of forskolin-stimulated CAT activity (Table 1) ranged from 0.06 nM (PAC) to 6.3 nM (UK-14304). The maximal inhibition obtained for each agonist was approximately 60%. This inhibition could be blocked by coincubation with the α_2AR-selective antagonist yohimbine but not by the α_1AR antagonist prazosin or the βAR-selective antagonist propranolol (Fig. 3). Similarly, the potentiation of forskolin-stimulated CAT expression was also selectively blocked by α_2AR but not by α_1AR or βAR antagonists.

A different pattern for the agonist dose-response curve was found in cells transfected with α_2-C4. Thus, in forskolin-stimulated JEG-3 cells that had been co-transfected with α_2-C4 and

TABLE 1

Agonist potencies for forskolin-stimulated CAT expression in JEG-3 cells transfected with the human α_2AR subtypes

Potencies for either the potentiation (α_2-C2) or the inhibition (α_2-C4 and α_2-C10) of forskolin-stimulated CAT expression were determined from the data shown in Figs. 2, 4, and 6 for epinephrine, PAC, medetomidine, and UK-14304. Data for norepinephrine and oxymetazoline were obtained from similar dose-response experiments (data not shown).

Compound	EC_{50}		
	α_2-C2	α_2-C4	α_2-C10
		nM	
Epinephrine	40	20	0.2
Norepinephrine	200	3.2	3.2
PAC	16	3.2	0.06
Medetomidine	0.6	0.2	0.2
UK-14304	500	2.0	6.3
Oxymetazoline	6.3	10	ND*

* ND, not determined.

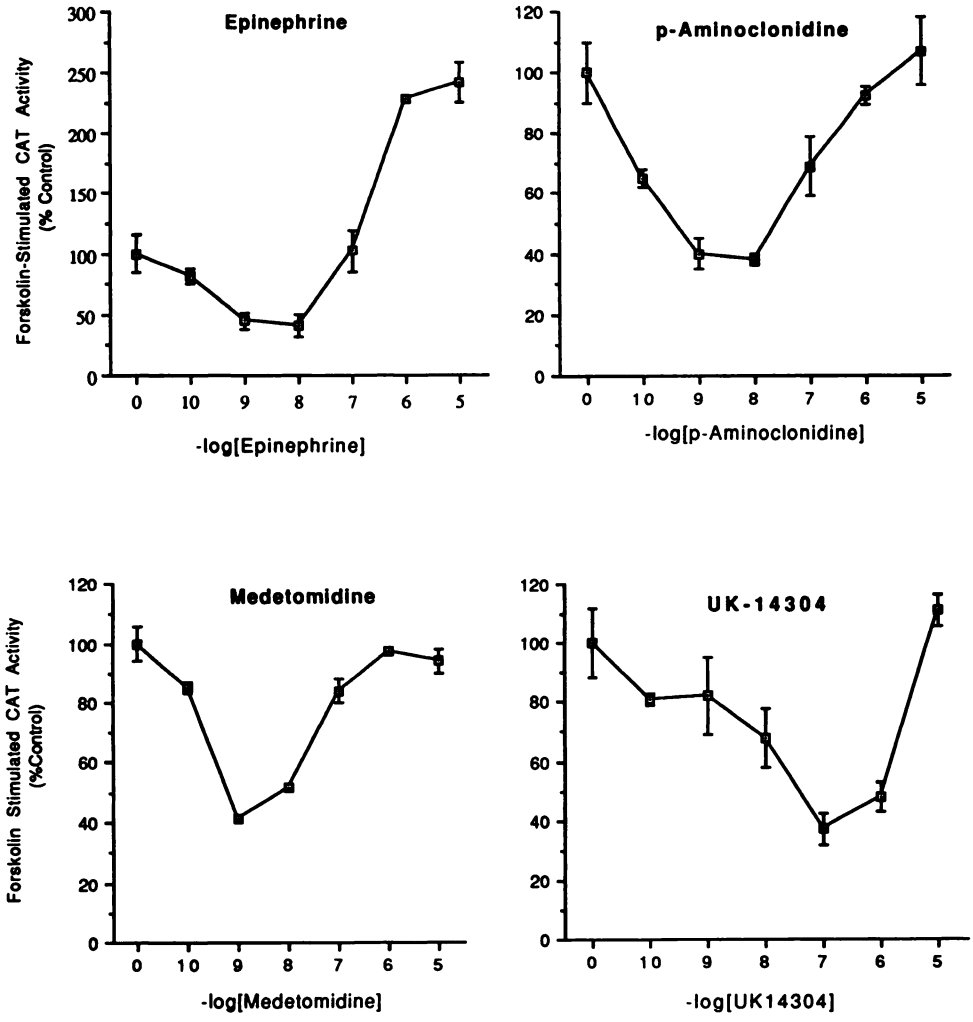


Fig. 2. Effect of α_2AR agonists on forskolin-stimulated CAT activity in JEG-3 cells transfected with reporter plasmid and α_2-C10. Cells were co-transfected with 10 μg of each plasmid, as described in Materials and Methods. Cells were incubated with 1 μM forskolin and the indicated concentrations of agonists for 4 hr. [^{3}H]CAT assays were done for 1 hr at 37°. Each *point* is the mean ± standard error from three plates of transfected cells. The experiment is representative of four that were done with epinephrine and three with medetomidine.

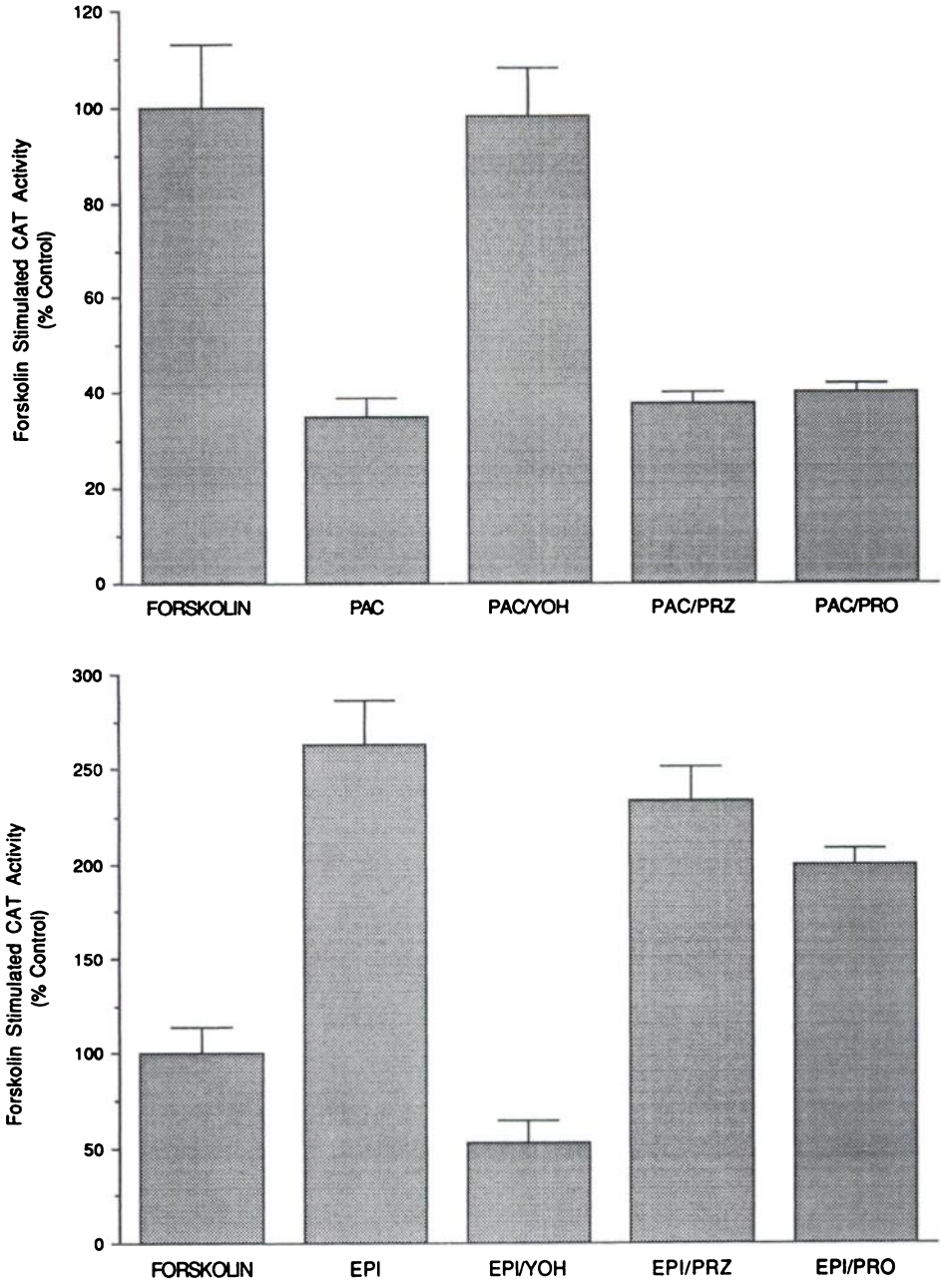


Fig. 3. Effect of the AR antagonists yohimbine (*YOH*), prazosin (*PRZ*), and propranolol (*PRO*) on the inhibition of CAT activity by α_2AR agonists (*upper*) and on the potentiation of CAT activity by α_2AR agonists (*lower*) in JEG-3 cells co-transfected with the reporter plasmid and α_2-C10. Cells were incubated with 1 μM forskolin and either 100 nM PAC (*upper*) or 10 μM epinephrine (*EPI*) (*lower*). Antagonists were used at a final concentration of either 1 μM (*upper*) or 10 μM (*lower*). Each *point* is the mean ± standard error from three plates of transfected cells.

the reporter plasmid there was predominantly inhibition of reporter gene expression (Fig. 4) and, compared with α_2-C10, the inhibition was nearly complete (80–98%). The order of potency for this inhibitory response was medetomidine > UK-14304 > norepinephrine = PAC > oxymetazoline > epinephrine (Table 1). This inhibition was completely blocked by coincubation with yohimbine but not with prazosin or propranolol (Fig. 5).

For α_2-C2 a purely stimulatory pattern of α_2AR agonist effects on forskolin-stimulated CAT activity was found. Fig. 6 shows that all the agonists produced dose-dependent increases in CAT activity that were similar to the maximal stimulation observed for cells transfected with α_2-C10 (~1.4-fold). Medetomidine was again the most potent agonist at this subtype, followed by oxymetazoline, PAC, and epinephrine. Norepinephrine and UK-14304 were much less potent, with EC_{50} values of 200 nM and 500 nM, respectively (Table 1). PAC (100 nM) elicited a 70–80% potentiation of forskolin-stimulated CAT activity (Fig. 7), which could be specifically blocked by yohimbine but not by either prazosin or propranolol.

Finally, PTX was used to examine the possible role of G_i with respect to the effects of α_2AR agonists on the inhibition and potentiation of forskolin-stimulated CAT activity observed in the previous experiments. Fig. 8 shows the effects of PTX on epinephrine dose-response curves in JEG-3 cells co-transfected with either α_2-C2, α_2-C4, or α_2-C10 and the reporter gene and then stimulated with forskolin. For α_2-C4 and α_2-C10, pretreatment of the cells with PTX abolished the inhibitory effects of epinephrine on CAT activity. For α_2-C10, epinephrine stimulated CAT activity in the PTX-treated cells another 2–3-fold, whereas for α_2-C4 there was a modest 0.5-fold stimulation. PTX pretreatment of the α_2-C2-transfected cells had no effect

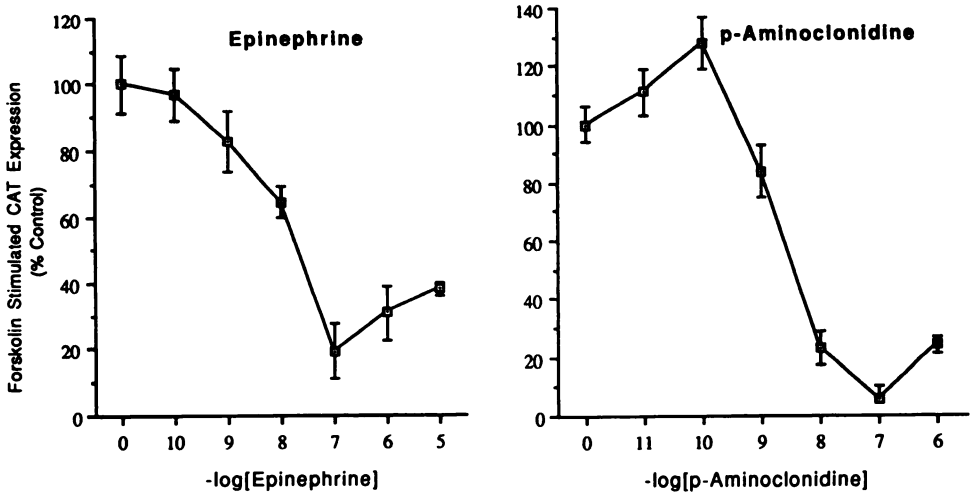
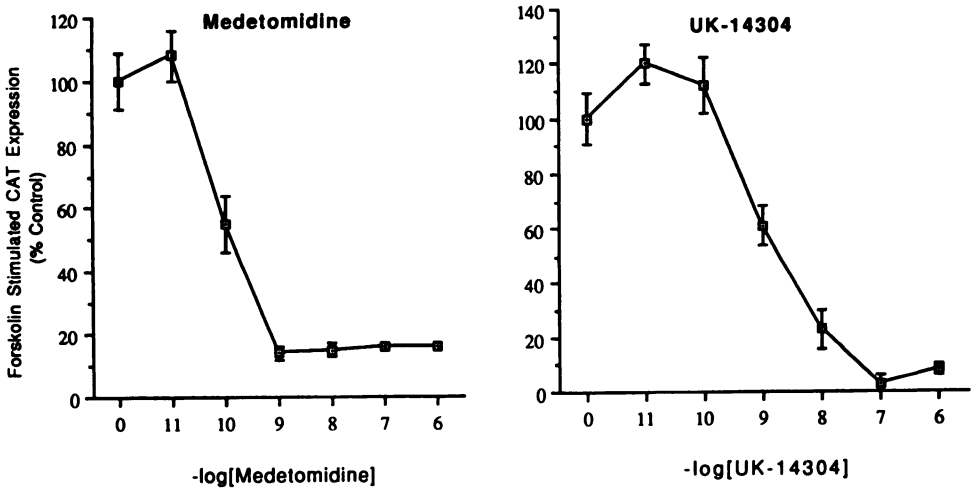


Fig. 4. Effect of α_2AR agonists on forskolin-stimulated CAT activity in JEG-3 cells co-transfected with the reporter plasmid and α_2-C4. Transfections and CAT assays were performed as described in Materials and Methods and in the legend to Fig. 2. Each *point* is the mean ± standard error from three plates of transfected cells. The experiment is representative of three that were done with each of the agonists.



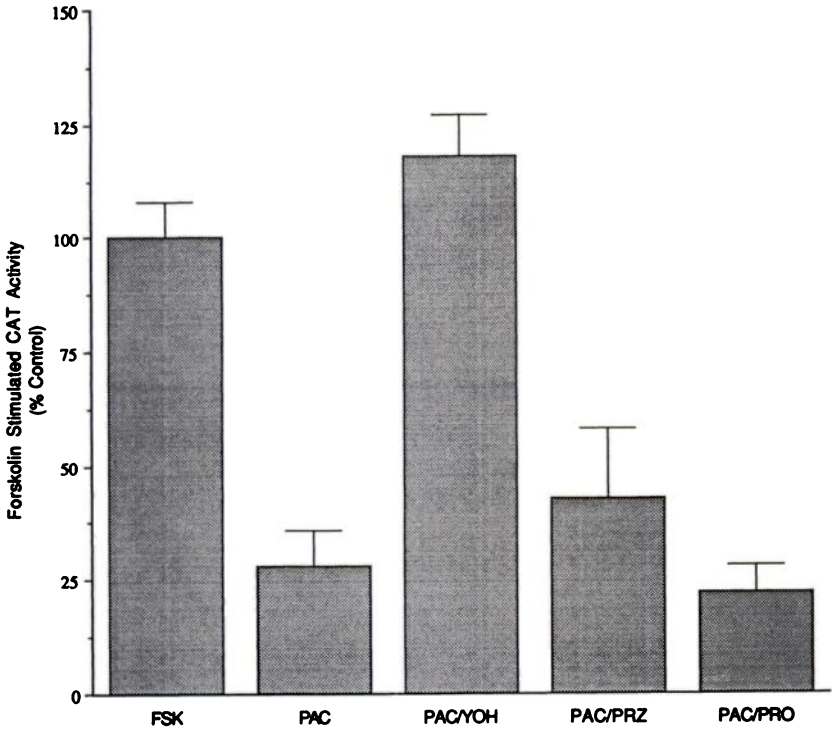


Fig. 5. Effect of the AR antagonists yohimbine (*YOH*), prazosin (*PRZ*), and propranolol (*PRO*) on the inhibition of CAT activity by α_2AR agonists in JEG-3 cells co-transfected with the reporter plasmid and α_2-C4. Cells were incubated with 1 μM forskolin (*FSK*) and 100 nM PAC. Antagonists were present at a final concentration of 1 μM. Each *point* is the mean ± standard error from three plates of transfected cells.

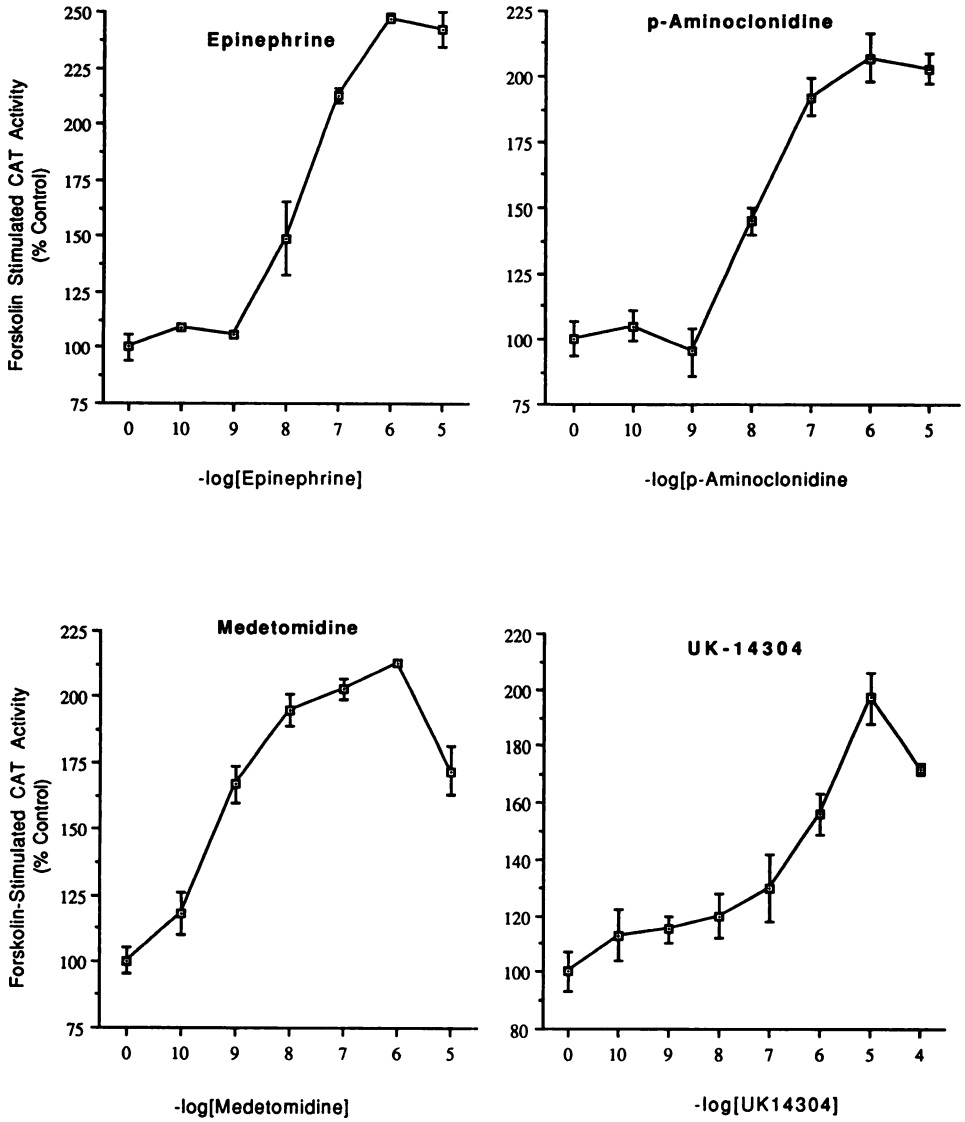


Fig. 6. Effect of α_2AR agonists on forskolin-stimulated CAT activity in JEG-3 cells co-transfected with the reporter plasmid and α_2-C2. Transfections and CAT assays were performed as described in Materials and Methods and in the legend to Fig. 2. Each *point* is the mean ± standard error from three plates of transfected cells. The experiment is representative of three that were done with each of the agonists.

on the epinephrine potentiation of forskolin-stimulated CAT activity. The effects on the cells of the PTX treatment itself were minimal. Forskolin produced a 4 ± 1-fold increase in CAT activity in JEG-3 cells transfected with α_2-C10 (12 experiments), whereas forskolin produced a 3 ± 1-fold increase in CAT activity in JEG-3 cells pretreated with PTX and transfected with α_2-C10 (five experiments).

Discussion

The present experiments show that, although the subtypes are closely related in structure (10), in binding properties (3), and in coupling to G proteins (5–7, 13, 14), the activation of individual α_2AR subtypes can, nevertheless, result in the production of unique intracellular responses. The results also suggest that, for a given receptor subtype, different agonists have different abilities to influence either the inhibition or the potentiation of CAT activity. Thus, with α_2-C10 epinephrine and PAC produce similar degrees of inhibition but differ in their ability to potentiate forskolin-stimulated CAT activity. This has been confirmed with other agonists; norepinephrine potentiates CAT activity 1.5-fold, whereas UK-14304 and medetomidine at high doses only return CAT activity to the control level. This difference in the ability of agonists to maximally potentiate CAT activity with α_2-C10 suggests a possible difference in the way in which catecholamines (epinephrine and norepinephrine) and imidazolines (PAC, UK-14304, and medetomidine) interact with the receptor. Thus, these two classes of α_2AR agonists may share overlapping but nonidentical binding sites, the consequence of which might be that the binding of catecholamines to the receptor produces a slightly different conformational change that allows more effective coupling of the receptor to the cAMP-potentiating pathway.

Although the reporter plasmid used for these studies is ultimately driven by cAMP, we do not know how closely our CAT measurements reflect actual cAMP concentrations in these cells. One reason for this is that direct measurements of cAMP in this transient expression system are of little value. Typically, only a small fraction of the cells are actually transfected (<10%). Thus, when cells are stimulated by forskolin, the concentration of cAMP increases in all the cells but is decreased only in those cells expressing α_2ARs. The present system works because the same population of cells takes up both the receptor plasmid and the reporter plasmid.

It is interesting that our results for α_2-C10 closely resemble the results of other studies in which cAMP measurements were

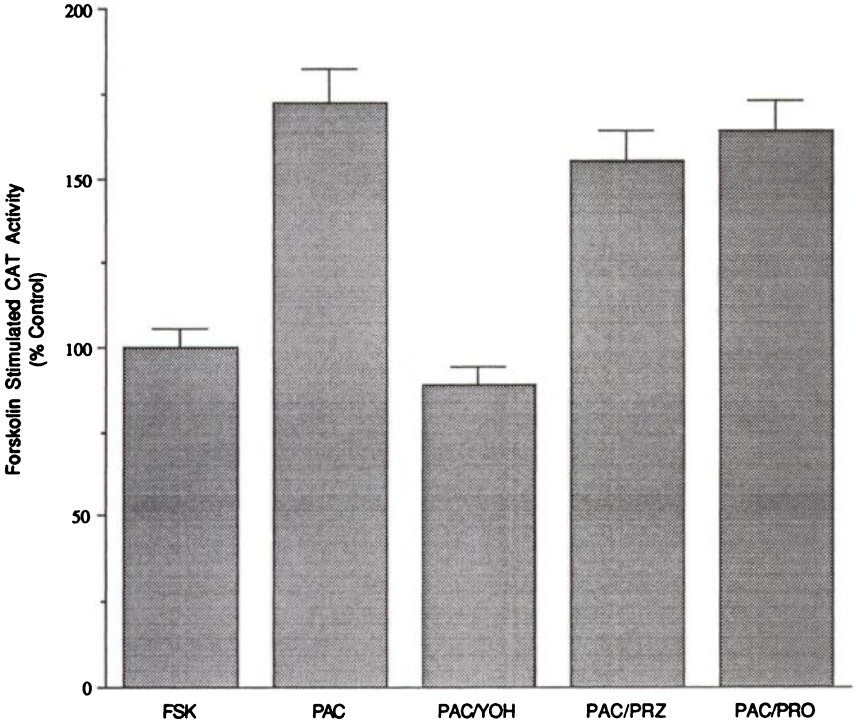


Fig. 7. Effect of the AR antagonists yohimbine (*YOH*), prazosin (*PRZ*), and propranolol (*PRO*) on the potentiation of CAT activity by α_2AR agonists in JEG-3 cells co-transfected with the reporter plasmid and α_2-C2. Cells were incubated with 1 μM forskolin (*FSK*) and 100 nM PAC. Antagonists were present at a final concentration of 1 μM. Each *point* is the mean ± standard error from three plates of transfected cells.

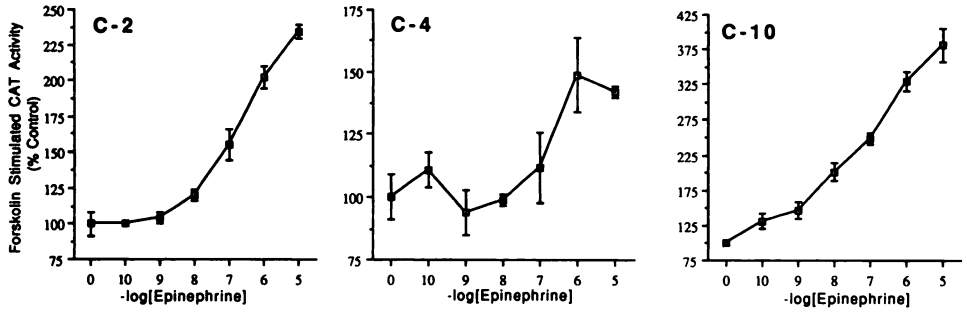


Fig. 8. Effect of epinephrine on forskolin-stimulated CAT activity in JEG-3 cells co-transfected with reporter plasmid and either α_2-C2 (*left*), α_2-C4 (*center*), or α_2-C10 (*right*) and pretreated with PTX. The transfected cells were incubated with 200 ng/ml PTX for 18 hr before drug incubation. Each *point* is the mean ± standard error from three plates of transfected cells.

made with stably transfected cell lines (5, 13). Thus, epinephrine produced a biphasic dose-response curve for cAMP in CHO cells that had been stably transfected with human α_2-C10 and stimulated with forskolin, i.e., low concentrations of epinephrine inhibited cAMP formation and high concentrations stimulated formation. Both the extent of inhibition and that of potentiation were similar to the results obtained here with forskolin-stimulated CAT activity. In addition, agonist potencies in our system are comparable to those from other studies. For example, the EC_{50} of epinephrine for decreasing cAMP at α_2-C10 in stably transfected CHO cells was 3 nM, whereas the EC_{50} for increasing cAMP is in the micromolar range (5). For epinephrine the EC_{50} for the inhibition of forskolin-stimulated CAT activity in JEG-3 cells was 0.2 nM, and the EC_{50} for the potentiation of CAT activity is also in the micromolar range.

Our results are also comparable to measurements made of adenylyl cyclase activity in CHO cells stably transfected with α_2-C10 but not to results obtained for CHO cells transfected with either α_2-C4 or α_2-C2 (7). In particular, the latter study reported that α_2-C2 was just as effective as either α_2-C10 or α_2-C4 in inhibiting adenylyl cyclase but was the least effective in terms of stimulating adenylyl cyclase activity. If the results of our experiments reflect adenylyl cyclase activity, then α_2-C2, at least in JEG-3 cells, would appear to couple predominantly to the stimulation of cyclase. With respect to agonist potencies the present results also compare favorably with measurements of adenylyl cyclase activity in stably transfected CHO cells (7). Thus, for UK-14304 the EC_{50} for inhibiting adenylyl cyclase with α_2-C10 was 90 nM, whereas the EC_{50} for the inhibition of forskolin-stimulated CAT activity was 6 nM. For both the stimulation of adenylyl cyclase activity in CHO cells and the potentiation of CAT activity in JEG-3 cells, the EC_{50} of UK-14304 with α_2-C10 was in the micromolar range. It is interesting that in contrast to α_2-C10, where the potentiation of CAT activity by agonists is in the micromolar range, for α_2-C2 the EC_{50} values are in the nanomolar range. This could indicate that, at least for α_2-C2, the ability to stimulate cAMP formation is physiologically significant.

We cannot completely exclude the possibility, with respect to α_2-C4, that the poor to absent potentiation of CAT activity is not a result of its level of expression. For example, it has been clearly shown in the case of α_2-C10 that the potentiation of cAMP formation, and of adenylyl cyclase activity, is a function of its level of expression (5, 7). In stably transfected

CHO cells, 'low' levels of expression of α_2-C10 (0.5–1.0 pmol/mg of protein) yielded primarily inhibition and high levels of expression (>1.0 pmol/mg) yielded a biphasic dose-response curve, with low concentrations of epinephrine inhibiting cAMP formation and high concentrations stimulating formation. In 10 separate experiments with α_2-C4, however, we have never seen a marked potentiation, even though the levels of receptor expression varied by 5-fold (0.1–0.5 pmol/mg). Furthermore, inhibitory activity as a result of receptor activation has not been observed in any of our experiments with α_2-C2, and receptor expression levels have not been reported as a factor affecting the inhibitory activity of α_2AR activation on cAMP formation or adenylyl cyclase activity (5, 7). To examine this further, two experiments were performed in which the level of α_2-C2 receptor expression was altered by varying the amount of plasmid DNA used in the transfection. Although the receptor expression could be altered over a 3-fold range (0.07–0.22 pmol/mg of protein) and was as much as 23 times lower than the highest level of expression that we observed for α_2-C2 (1.6 pmol/mg), the pattern of the epinephrine dose-response curve was still purely stimulatory (data not shown).

The transient functional expression system that we have described, with its use of a CRE-reporter gene, is clearly measuring a functional response that is initiated by the activation of α_2ARs. Although the use of the reporter gene itself is artifical, it is important to realize that changes in cAMP can ultimately affect the expression of genes that are regulated by CREs. Our experiments clearly reflect this potential regulation of gene expression. With respect to the activation of CAT itself, much happens in the 4-hr incubation; cAMP increases, protein kinase A is activated, cAMP response element binding protein is phosphorylated, the CAT gene is transcribed, etc. With the possible exception of the stimulation of adenylyl cyclase activity discussed earlier (7), marked differences in the coupling of α_2AR subtypes to second messengers have not been described. It is possible that functional differences between the α_2AR subtypes might not be appreciated by biochemical determinations that are either too close in the pathway (e.g., GTPase or adenylyl cyclase) or too close in time with respect to receptor activation. A possible advantage of the present system is that it is more downstream and it may reflect a summation of more than one signaling pathway. It is significant that, in otherwise identical systems, the activation of closely related subtypes can produce unique intracellular responses. Should the receptors be present in different cells, with a different complement of G proteins, adenylyl cyclase isozymes, etc., then the potential for unique signaling by the same endogenous agonist would be even greater. For pharmacologists the potential to influence this signal transduction is significant, because we are not limited in our choice of agonist.

References

1. Ruffolo, R. R., Jr., A. J. Nichols, and J. P. Hieble. Functions mediated by *alpha*-2 adrenergic receptors, in *The Alpha-2 Adrenergic Receptors* (L. E. Limbird, ed.). Humana Press, Clifton, NJ, 187–280 (1988).
2. Regan, J. W., and S. Cotecchia. The α-adrenergic receptors: new subtypes, pharmacology, and coupling mechanisms, in *Molecular Biology of G-Protein-Coupled Receptors* (M. R. Brann, ed.). Birkhauser Inc., Boston, MA, 76–112 (1992).
3. Bylund, D. B., H. J. Blakall, L. J. Iversen, M. G. Caron, R. J. Lefkowitz, and J. W. Lomasney. Pharmacological characteristics of α_2-adrenergic receptors: comparison of pharmacologically defined subtypes with subtypes identified by molecular cloning. *Mol. Pharmacol.* **42**:1–5 (1992).
4. Limbird, L. E. Receptors linked to inhibition of adenylate cyclase: additional signalling mechanisms. *FASEB J.* **2**:2686–2695 (1988).
5. Fraser, C. M., S. Arakawa, R. W. McCombie, and C. J. Venter. Cloning, sequencing analysis and permanent expression of a human α_2-adrenergic receptor in Chinese hamster ovary cells. *J. Biol. Chem.* **264**:11754–11761 (1989).
6. Cotecchia, S., B. K. Kobilka, K. W. Daniel, R. D. Nolan, E. Y. Lapetina, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Multiple second messenger pathways of α-adrenergic receptor subtypes expressed in eukaryotic cells. *J. Biol. Chem.* **265**:63–69 (1990).
7. Eason, M. G., H. Hurose, B. D. Holt, J. R. Raymond, and S. B. Liggett. Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects. *J. Biol. Chem.* **267**:15795–15801 (1992).
8. Delegeane, A. M., L. H. Ferland, and P. L. Mellon. Tissue-specific enhancer of the human glycoprotein hormone α-subunit gene: dependence on cyclic AMP-inducible elements. *Mol. Cell. Biol.* **7**:3994–4002 (1987).
9. Montmayeur, J. P., and E. Borrelli. Transcription mediated by a cAMP responsive promoter element is reduced upon activation of dopamine D_2 receptors. *Proc. Natl. Acad. Sci. USA* **88**:3135–3139 (1991).
10. Lomasney, J. W., W. Lorenz, L. F. Allen, K. King, J. W. Regan, T. L. Yang-Feng, M. G. Caron, and R. J. Lefkowitz. Expansion of the α_2-adrenergic receptor family: cloning and characterization of a human α_2-adrenergic receptor subtype, the gene for which is located on chromosome 2. *Proc. Natl. Acad. Sci. USA* **87**:5094–5098 (1990).
11. Grahm, F. L., and A. J. van der Eb. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456–467 (1973).
12. Seed, B., and J. Y. Sheen. A simple phase extraction assay for chloramphenicol acetyl transferase activity. *Gene* **67**:271–277 (1988).
13. Jones, S. B., S. P. Halenda, and D. B. Bylund. α_2-Adrenergic receptor stimulation of phospholipase A_2 and of adenylate cyclase in transfected Chinese hamster ovary cells is mediated by different mechanisms. *Mol. Pharmacol.* **39**:239–245 (1991).
14. Kurose, H., J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Functional interactions of recombinant α_2-adrenergic receptor subtypes and G-proteins in reconstituted phospholipid vesicles. *Biochemistry* **30**:3335–3341 (1991).

Send reprint requests to: John W. Regan, Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721.