British Journal of Pharmacology (1997) 121, 317–323

Constitutive activity of human prostaglandin E receptor EP_3 isoforms

Jianguo Jin, Guang Fen Mao & [1]Barrie Ashby

Department of Pharmacology, Temple University School of Medicine, 3400 N. Broad Street, Philadelphia, PA 19140, U.S.A.

1 The human EP_3 prostaglandin receptor is a seven transmembrane, G protein-coupled receptor that couples to inhibition of adenylyl cyclase. The receptor occurs as at least six isoforms which result from alternative splicing. The isoforms are identical over the first 359 amino acids, comprising the seven transmembrane helices, but differ in the carboxyl terminal tail which ranges in length from 6 to 65 amino acids beyond the common region.

2 We have stably expressed in CHO-K1 cells four of the isoforms (EP_{3I}-EP_{3IV}) and a form of the EP_3 receptor (T-359) truncated at the carboxyl-terminal region defined by the alternative splicing site at amino acid number 359.

3 Isoforms EP_{3I} and EP_{3II} showed concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase in CHO-K1 cells by the EP_3 receptor agonist, sulprostone. The IC_{50} calculated for sulprostone inhibition was 0.2 nM for EP_{3I} and 0.15 nM for EP_{3II}. The maximum extent of inhibition was 80% for both isoforms.

4 Isoforms EP_{3III} and EP_{3IV} showed marked constitutive activity, inhibiting forskolin-stimulated adenylyl cyclase in the absence of agonist. EP_{3IV} also displayed some agonist-dependent inhibition whereas EP_{3III} was fully constitutively active.

5 The truncated receptor T-359 was fully constitutively active, inhibiting forskolin-stimulated adenylyl cyclase by about 70% in the absence of agonist, and showed no agonist-dependent inhibition, in agreement with a similar truncation of the mouse EP_3 receptor.

6 To confirm that differences in cyclic AMP level between isoforms represent constitutive activity, we treated cells with pertussis toxin for 6 h to abolish G_i function. Pertussis toxin reversed sulprostone-mediated inhibition of cyclic AMP formation in EP_{3I} and EP_{3II} and abolished constitutive activity of EP_{3III}, EP_{3IV} and T-359 so that the level of forskolin-stimulated cyclic AMP produced was the same in all cells and similar to that obtained in mock-transfected cells. In mock-transfected cells, sulprostone had no effect on forskolin-stimulated cyclic AMP formation.

7 For these experiments we chose clones that showed similar expression levels of each isoform, as determined by binding of [^{3}H]-prostaglandin E_2 (PGE_2) (EP_{3I}, 0.71; EP_{3II}, 1.47; EP_{3IV}, 1.59 pmol mg^{-1} protein). Mock-transfected cells showed no detectable binding of [^{3}H]-PGE_2. In addition, we performed a detailed study of the effects of expression level on constitutive activity. Over a six fold range of expression there was no change in the properties of each isoform with regard to whether it was constitutively active or not.

8 The degree of constitutive activity correlated with the inverse of the length of the C-terminal tail of the isoforms. However, no correlation was found between isoforms from human and mouse: whereas EP_{3II} shows no constitutive activity, its mouse homologue, $EP_{3\gamma}$, shows almost complete constitutive activity, even though the C-terminal domains of the receptors following the splice site differ in only 7 of 29 amino acids.

Keywords: Prostaglandin; prostaglandin receptor; adenylyl cyclase; Cyclic AMP; constitutive activity; Isoforms; splice variants; carboxyl terminus; G protein; cDNA

Introduction

Prostaglandin E_2 (PGE_2) is involved in many physiological and pathophysiological events and exerts its actions through binding to at least four distinct EP receptors that are members of the G protein-linked receptor superfamily. The EP_1 receptor couples to phospholipase C, the EP_2 and EP_4 receptors couple to stimulation of adenylyl cyclase, and the EP_3 receptor couples to inhibition of adenylyl cyclase. All of the receptors have been cloned from human cells. In addition, six isoforms of the human EP_3 receptor have been identified that are identical over the first 359 amino acids but differ in the carboxyl-terminal region which varies in length from 6–65 amino acids beyond the splice site (An *et al*., 1994; Regan *et al*., 1994; Schmid *et al*., 1995; Kotani *et al*., 1995). The isoforms also differ in their functional properties, including coupling to G proteins other than G_i, indicating that the C-terminal region is an important determinant in G protein coupling.

In addition to the isoforms identified in human cells, three isoforms ($EP_{3\alpha}$, $EP_{3\beta}$, $EP_{3\gamma}$) have been cloned from mouse (Negishi *et al*., 1993; Irie *et al*., 1993). However, all three inhibit adenylyl cyclase, the α-form desensitizes towards PGE_2 whereas the β-form does not desensitize (Negishi *et al*., 1993), and the γ-form can also couple to G_s (Irie *et al*., 1993). The α-form is partially constitutively active when compared with the β form (Hasegawa *et al*., 1996), whereas the γ form is almost fully constitutively active at G_i, but shows agonist-dependent activity at G_s (Negishi *et al*., 1996). Truncation of the mouse EP_3 receptor at the splice variant site leads to a fully constitutively active form of the receptor, which inhibits forskolin-stimulated adenosine 3′:5′-cyclic monophosphate (cyclic AMP) formation in the absence of prostaglandins (Hasegawa *et al*., 1996).

[1] Author for correspondence.

We have cloned the human EP_3 receptor (Kunapuli *et al*., 1994) and stably expressed four of the isoforms in Chinese hamster ovary (CHO)-K1 cells. In this paper we have compared the properties of the four isoforms of EP_3 with a mutated receptor truncated at the splice variant site common to all of the isoforms. We have used the nomenclature of An *et al*. (1994) to designate the human isoforms EP_{3I}-EP_{3IV}. We show that, in agreement with studies on the mouse receptor (Hasegawa *et al*., 1996), truncation of the receptor results in fully constitutive (agonist-independent) inhibition of adenylyl cyclase. We expressed the isoforms in CHO-K1 cells at levels similar to the mouse receptors (about 1 pmol mg^{-1} protein). EP_{3I} and EP_{3II} both inhibit adenylyl cyclase in an agonist-dependent manner, with little or no constitutive activity, whereas EP_{3III} and EP_{3IV} show marked constitutive activity. The degree of constitutive activity correlates with the inverse length of the C-terminal domain. However, no correlation was found between isoforms from human and mouse: whereas EP_{3II} showed no constitutive activity, its mouse homologue, $EP_{3\gamma}$, showed almost complete constitutive activity, even though the C-terminal domains of the receptors following the splice site differ in only 7 of 29 amino acids.

Methods

Preparation and expression of EP_3 receptor isoforms and a truncated form of the EP_3 receptor

We have previously cloned the EP_3 receptor isoform I (EP_{3I}) from human erythroleukemia (HEL) cells (Kunapuli *et al*., 1994). The EP_{3I} receptor was ligated into the vector pRc/RSV (Invitrogen, San Diego, CA). Other isoforms were constructed by removing the C-terminal tail of EP_{3I} making use of a unique BamHI site in the seventh transmembrane domain region and an XbaI site in the 3′ non-coding region and substituting a polymerase chain reaction (PCR) fragment for each of the isoforms $EP_{3II}-EP_{3IV}$. C-terminal regions of isoforms were obtained by reverse transcriptase PCR from HEL cell total RNA with a common sense primer and antisense primers in the 3′ non-coding region specific for each isoform. PCR fragments corresponding to the C-terminus of EP_{3III} and EP_{IV} were obtained with the same primers and were separated by electrophoresis on a 1% agarose gel. The sense primer for all isoforms was 5′-GAC ACACACGGAGAAGCAGAAAG-3′ (Bases 948–970 of the EP_3 receptor). The antisense primer for EP3II was 5′-GGATTCTAGAGGTA CCAAAA GCTGTGCAACTGC-3′ and the antisense primer for EP3III and IV was 5′-GGAT TCTAGA CTTCCAGTGATGTGATCCTGGCA-G-3′ where the underlined region defines the XbaI site. The PCR fragments were cut with BamHI and XbaI, purified and ligated into the EP3I-pRC/RSV in which the C-terminal region was removed by BamHI-XbaI digestion.

The truncated receptor was prepared by PCR of the full-length EP_3 receptor by use of a sense primer in the 5′ non-coding region and an antisense primer inserting stop codons after the common C-terminal sequence at the splice variant site. The sense primer was 5′-TCTTGGATCCTTGGGTT-TACCTGC-3′ where the underlined region defines a BamHI site. The antisense primer was designed to insert a stop codon after amino acid 359 which marks the end of the common sequence of all of the isoforms. Three stop codons were inserted in each of the three coding frames as shown. The antisense primer was 5′-GGATCTAGACTATTTATTCAC-TGGCAAAACTTTCGAAG-3′ where underlined regions define an XbaI site and three stop codons. The PCR reactions (final volume 100 μl) contained 10 μl of the reverse transcription reaction from HEL cell total RNA, 10 μl of 10X PCR buffer, 10 μl of a mixture of 100 mM of deoxynucleotide triphosphates, 10 μl dimethyl sulphoxide, 5 μl sense primer, 5 μl antisense primer, 1 μl pfu polymerase (Stratagene) and 49 μl sterile water. The following programme was used for PCR: 30 cycles of 94°C for 5 min, 56°C for 5 min, 72°C for 2 min followed by one cycle of 72°C for 3 min. The PCR fragments were cut with BamHI and XbaI, purified and ligated into the EP_{3I}-pRC/RSV in which the C-terminal region was removed by BamHI-XbaI digestion.

The DNA sequences of the splice variant and truncated constructs were confirmed by sequencing according to the method of Sanger *et al*. (1977). Constructs in pRc/RSV were transfected into CHO-K1 cells by use of Lipofectin reagent from GIBCO BRL (Gaithersburg, MD). Cells expressing the vector were selected with Geneticin (500 μg ml^{-1}) and single cell clones obtained by serial dilution. Mock transfected cells were transfected with pRc/RSV vector alone.

Radioligand binding studies

Receptor expression levels were determined with [^{3}H]-PGE_2 by filtration on a Brandel Cell Harvester (Brandel, Gaithersburg, MD). For each of the isoforms, and for T-359 and mock-transfected cells, membranes were prepared from two 100 mm tissue culture dishes of CHO-K1 cells. The cells were removed from the dishes and lysed by sonication in 50 mM Tris-HC1 (pH 7.5), 1 mM EGTA, 10 μM leupeptin and 10 μg ml^{-1} soybean trypsin inhibitor. The lysate was centrifuged at $36{,}000 \times g$ for 20 min at 4°C. The pellet was washed once and resuspended in 50 mM Tris-HC1 (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA and 10 μM leupeptin. Protein

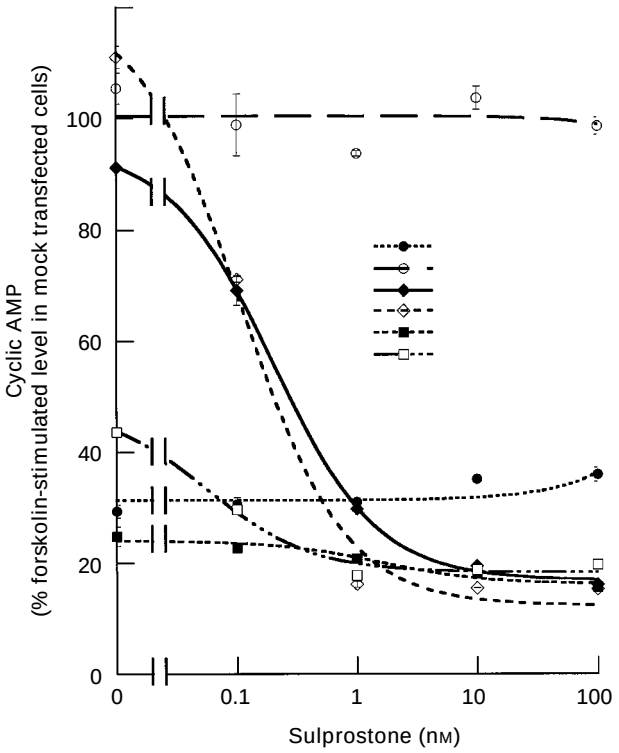

Figure 1 Inhibition of adenylyl cyclase by EP_{3I}, EP_{3II}, EP_{3III}, EP_{3IV} and T-359. CHO-K1 cells expressing EP_{3I} (◆), EP_{3II} (◇), EP_{3III} (■), EP_{3IV} (□) isoforms and the truncated receptor T-359 (●) were incubated with 20 μM forskolin for 10 min in the presence of the indicated concentrations of sulprostone and 1 mM 3-isobutyl methylxanthine (IBMX). Mock-transfected cells (○) were treated in a similar manner. Cyclic AMP was measured as described under Methods. Values are means ± s.e.mean of duplicates and the experiment was repeated three times with similar results. The curves represent best fits to a hyperbola determined by Kaleidagraph (Adelbeck Software) with the following equation: Cyclic AMP as % forskolin-stimulated level = 100-I_{max} [sulprostone]/(IC_{50} + [sulprostone]) where I_{max} is the maximum % inhibition and IC_{50} is the concentration of sulprostone that gives 50% inhibition.

concentrations were determined with the Coomassie Plus protein assay reagent (Pierce, Rockford, IL). Membrane suspensions (0.2–0.8 mg protein ml^{-1}) were incubated with [^{3}H]-PGE_2 for 60 min at room temperature, rapidly filtered through Whatman GF/C filters and washed twice with 5 ml of ice cold buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA before liquid scintillation counting. Experiments were performed by displacement of

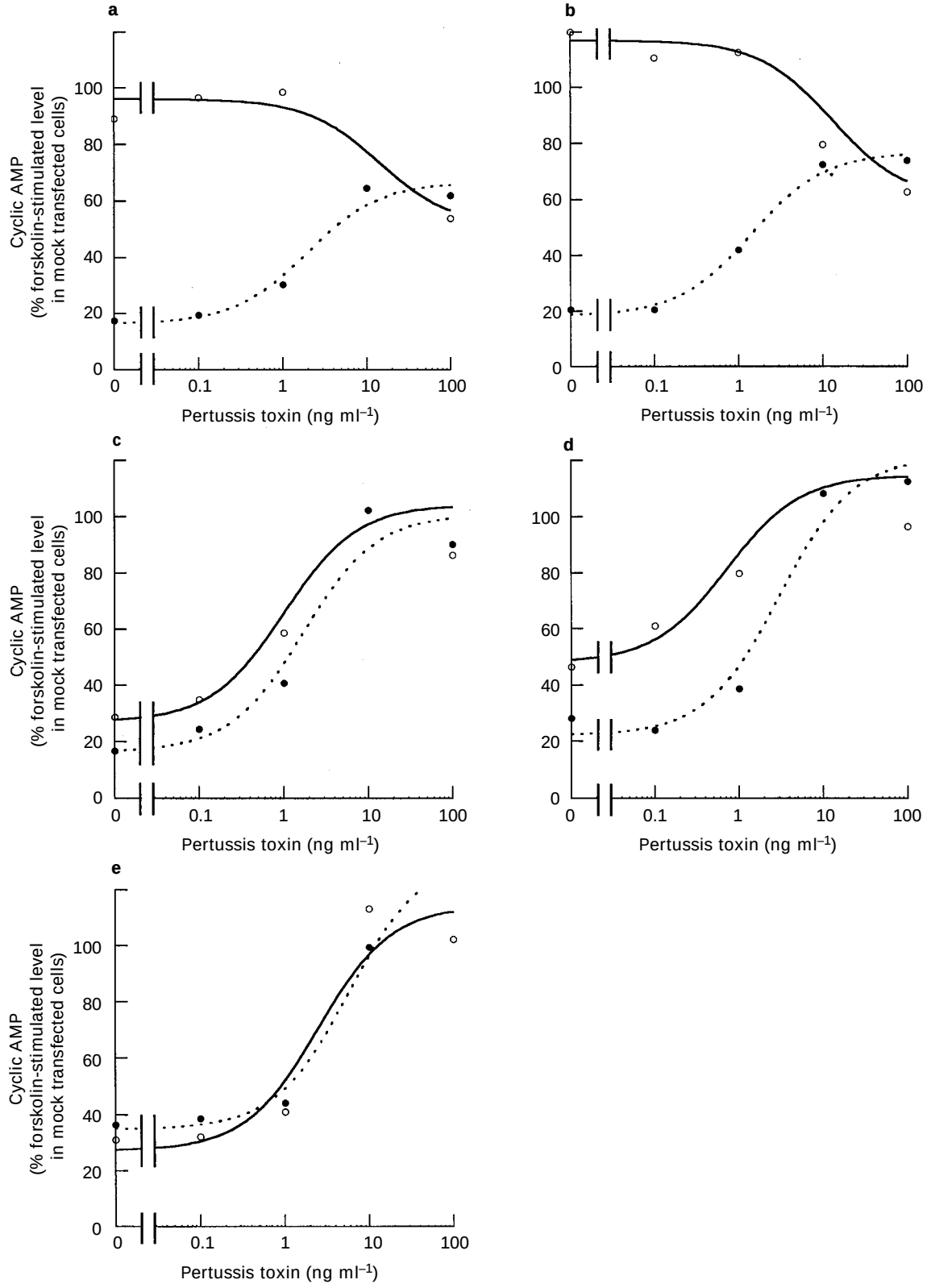

Figure 2 Effect of pertussis toxin concentration on adenylyl cyclase activity of (a) EP_{3I}, (b) EP_{3II}, (c) EP_{3III}, (d) EP_{3IV} and (e) T-349. CHO-K1 cells expressing EP_{3I}, EP_{3II}, EP_{3III}, EP_{3IV} isoforms and the truncated receptor T-359 were incubated with the indicated concentrations of pertussis toxin for 6 h before the assay of forskolin-stimulated cyclic AMP formation in the absence (○) and presence (●) of 100 nM sulprostone. Mock-transfected cells were treated in a similar manner. Cyclic AMP was measured as described under Methods. Values are means of duplicates and the experiment was repeated three times with similar results.

2.5 nM [^{3}H]-PGE_2 with increasing concentrations of non-radioactive PGE_2 (0–1000 nM). Data were analysed with the programme LIGAND (Munson & Rodbard, 1980). The dissociation constant for PGE_2 determined by this method was calculated to be in the range 5–7 nM for all of the isoforms and T-359.

Cyclic AMP measurements

To measure cyclic AMP formation, transfected and mock-transfected cells grown in six-well 35 mm culture dishes were incubated for 16 h with 2 μCi of [^{3}H]-adenine (25 Ci mmol^{-1}) to label uniformly the adenine nucleotide pool.

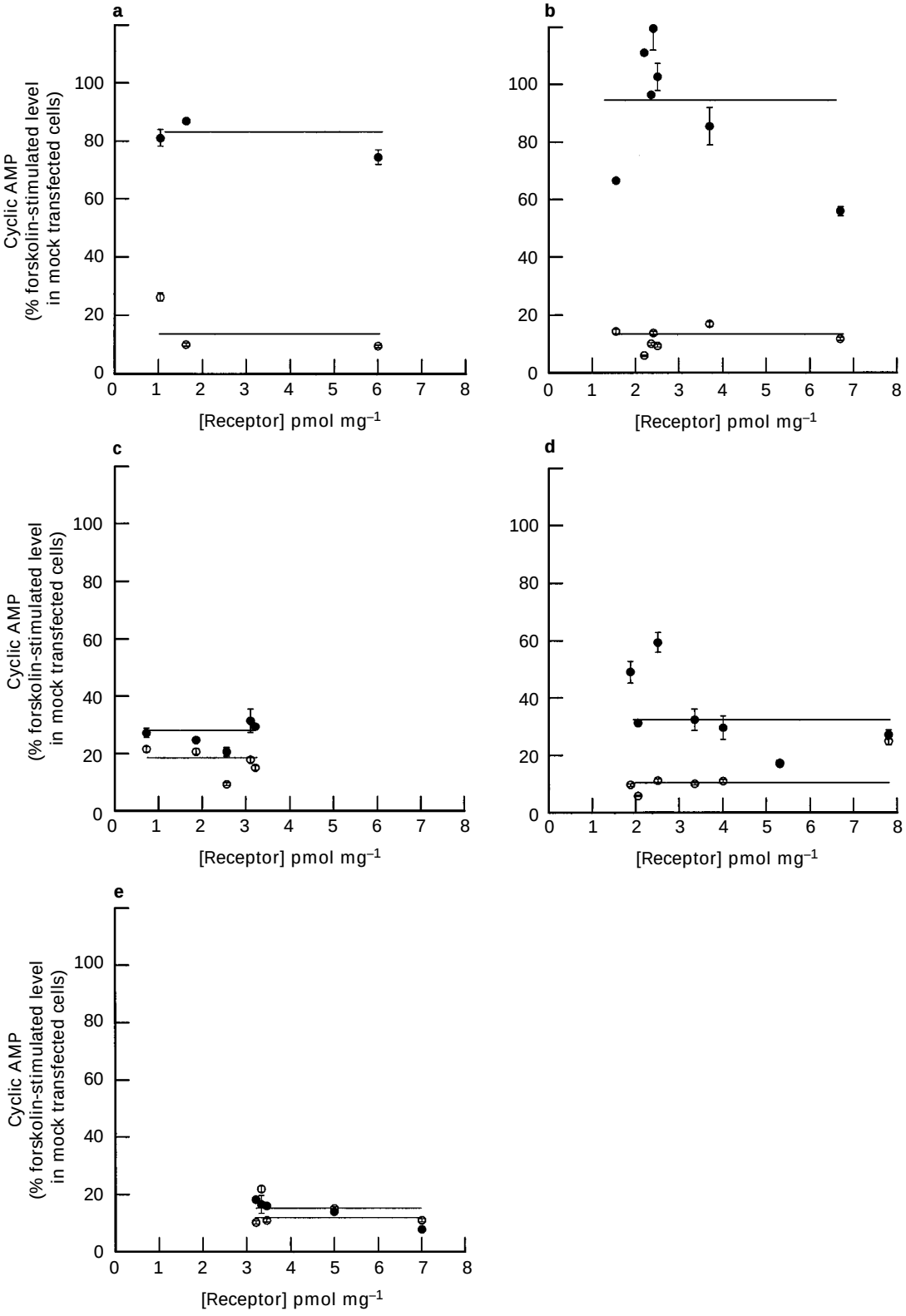

Figure 3 Effect of receptor expression level on constitutive activity of (a) EP_{3I}, (b) EP_{3II}, (c) EP_{3III}, (d) EP_{3IV} and (e) T-359. Several clones of CHO-K1 cells each expressing different levels of EP_{3I}, EP_{3II}, EP_{3III}, EP_{3IV} isoforms and the truncated receptor T-359 were challenged with forskolin in the absence (○) and presence (●) of 100 nM sulprostone. Mock-transfected cells were treated in a similar manner. Cyclic AMP was measured as described under Methods. Values are means ± s.e.mean of triplicates. The horizontal lines represent the mean value of each data set.

Table 1 Carboxyl-terminal sequences of isoforms of the human and mouse EP_3 receptors

Isoform	*C-terminal sequence*
Human EP_3I	...FCQIRYHTNNYASSSTSLPCQCSSTLMWSDHLER*
Human EP_3II	...FCQVANAVSSCSNDGQKGQPISLSNEIIQTEA*
Human EP_3III	...FCQEEFWGN*
Human EP_3IV	...FCQMRKRRLREQEEFWGN*
Human T-359	...FCQ*
	↑ Splice site
Mouse $EP_3\alpha$	...FCQIRDHTNYASSSTSLPCPGSSALMWSDQLER*
Mouse $EP_3\beta$	...FCQMMNNLKWTFIAVPVSLGLRISSPREG*
Mouse $EP_3\gamma$	...FCQVANAVSSCSSDGQKGQAISLSNEVVQPGP*
Mouse T-335	...FCQ*
	↑ Splice site

Amino acid sequences of the isoforms of the EP_3 receptor from human and mouse are compared. *Indicates the end of the sequence.

Where indicated cells were incubated for 6 h with pertussis holotoxin. Medium was removed and replaced by medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) for 10 min. The medium was replaced with medium containing 1 mM IBMX and various concentrations of the EP_3 receptor agonist, sulprostone, for 15 min at 37°C. Cyclic AMP levels were elevated by simultaneous addition of 20 μM forskolin to observe inhibition by sulprostone. At the end of the incubation, medium was replaced by a stopping solution containing 2,000 c.p.m. of [^{14}C]-cyclic AMP as recovery standard. Cyclic AMP was determined by the method of Salomon (1979) and expressed relative to the amount of cyclic AMP generated by mock-transfected cells challenged with 20 μM forskolin for 15 min.

Materials

PGE_2 was obtained from Biomol (Plymouth Meeting, PA) and sulprostone from Cayman Chemical (Ann Arbor, MI). Restriction enzymes, kits for RNA extraction, first strand synthesis and pfu DNA polymerase were from Stratagene (La Jolla, CA). PCR primers were synthesized by Life Technologies (Grand Island, NY). [2, 8-^{3}H]-adenine and [^{14}C]-cyclic AMP were obtained from DuPont NEN (Boston, MA). [^{3}H]-PGE_2 (182 Ci mmol^{-1}) was from Amersham (Arlington Heights, IL). Pertussis toxin, forskolin and other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO). CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD).

Results

Effect of sulprostone on forskolin-stimulated cyclic AMP formation

We examined the effect of the EP_3 specific agonist, sulprostone, on inhibition of forskolin-stimulated adenylyl cyclase in transfected cells. Concentration-response curves are presented in Figure 1 for cells expressing each of the isoforms and the truncated receptor T-359. For these experiments we chose clones that showed similar expression levels of each isoform, as determined by binding of [^{3}H]-PGE_2 (EP_{3I}, 0.71 pmol mg^{-1} protein; EP_{3II}, 1.22 pmol mg^{-1} protein; EP_{3III}, 1.47 pmol mg^{-1} protein; EP_{3IV}, 1.59 pmol mg^{-1} protein). Mock-transfected cells showed no detectable binding of [^{3}H]-PGE_2. The dissociation constant for [^{3}H]-PGE_2 binding was calculated to be between 5 and 7 nM for all of the isoforms and T-359.

In the absence of sulprostone, EP_{3I} and EP_{3II} showed similar forskolin-stimulated cyclic AMP levels to mock-transfected cells, indicating that the receptors are not constitutively active. Increasing concentrations of sulprostone led to a decrease in forskolin-stimulated cyclic AMP level in both EP_{3I} and EP_{3II} with no change in the mock-transfected cells. The IC_{50} calculated for sulprostone inhibition was 0.2 nM in the case of EP_{3I} and 0.15 nM in the case of EP_{3II} (Figure 1). By contrast, EP_{3III} and EP_{3IV} showed lower forskolin-stimulated cyclic AMP levels in the absence of sulprostone than mock transfected cells, indicating agonist-independent inhibition. EP_{3III} and EP_{3IV} also showed agonist-dependent inhibition. The truncated form of the receptor, T-359, showed constitutive activity, characterized by a forskolin-stimulated cyclic AMP level that was the same in the absence or presence of sulprostone.

Effect of pertussis toxin on forskolin-stimulated cyclic AMP formation

To confirm that differences in cyclic AMP level between isoforms represent constitutive activity, we examined the effect of sulprostone on cells treated with pertussis toxin for 6 h to abolish G_i function. In mock-transfected cells, pertussis toxin over the range 0.1 – 100 ng ml^{-1} led to a 20% reduction in the amount of cyclic AMP (not shown). The inhibitory effect of pertussis toxin is contrary to the expected effect of pertussis toxin which generally leads to an increase in cyclic AMP which is ascribed to the fact that G_i is tonically active, displaying a low level of inhibition even in the absence of agonists that can be abolished by pertussis toxin. However, similar inhibition of forskolin-stimulated cyclic AMP formation by pertussis toxin in CHO cells has been observed by Hasegawa *et al.* (1996).

With isoforms EP_{3I-IV} and T-359, forskolin-stimulated cyclic AMP levels were measured in the absence or presence of 100 nM sulprostone (Figure 2). Pertussis toxin reversed sulprostone-mediated inhibition of cyclic AMP formation in EP_{3I} and EP_{3II} and abolished constitutive activity of EP_{3III}, EP_{3IV} and T-359.

In the case of cells expressing EP_{3I} or EP_{3II} receptors, pertussis toxin reduced cyclic AMP formation in the absence of sulprostone in the same manner as in mock transfected cells. Pertussis toxin increased the amount of cyclic AMP in the presence of sulprostone by abolishing agonist-dependent inhibition of cyclic AMP formation.

By contrast, in cells expressing EP_{3III}, the pattern of cyclic AMP formation indicated that EP_{3III} is almost completely constitutively active; and in cells expressing EP_{3IV}, the result was between that obtained with EP_{3I} and EP_{3III}, confirming that EP_{3IV} is partially constitutively active.

In cells expressing the truncated receptor EP_3 T-359, pertussis toxin increased the amount of cyclic AMP generated in a concentration-dependent manner. The final level of cyclic AMP was similar to that attained in mock-transfected cells. Sulprostone had no effect on cyclic AMP formation. The result can be explained by reversal of G_i-mediated EP_3 T-359 constitutive activity which is completely independent of agonist binding.

Effect of receptor expression level on forskolin-stimulated cyclic AMP formation

Because of the possibility that constitutive activity may result from over- or under-expression of receptors, we examined the effect of receptor expression level on constitutive activity of the receptor isoforms. We chose clones expressing different levels of each isoform and the truncated receptor. Figure 3 shows forskolin-stimulated cyclic AMP formation in the absence and presence of 100 nM sulprostone as a function of receptor expression level. It can be seen that both the level of forskolin-stimulated cyclic AMP formation and the level of sulprostone inhibition were independent of receptor expression level.

Discussion

Following a study by Hasegawa *et al.* (1996) that truncation of the mouse EP_3 receptor at the splice variant site resulted in agonist-independent constitutive activity (inhibition of adenylyl cyclase in the absence of prostaglandins), we have prepared a truncated form of the human EP_3 receptor (T-359) and have shown that it too is constitutively active. We have also examined the activity of four isoforms of EP_3 that vary in length of the C-terminal tail from 6 to 31 amino acids (Table 1). Our studies show that isoforms III and IV of human EP_3 receptor are markedly constitutively active whereas isoforms I and II show little or no constitutive activity. The degree of constitutive activity appears to correlate with the inverse of the length of the C-terminal tail of the isoforms. Constitutive activity was abolished by pertussis toxin.

Other investigators have studied the properties of the isoforms of the human prostaglandin EP_3 receptor (An *et al.*, 1994; Regan *et al.*, 1994; Schmid *et al.*, 1995; Kotani *et al.*, 1995) but none has described constitutive activity. An *et al.* (1994) showed that EP_{3II} displays greater agonist-induced inhibition of forskolin-stimulated adenylyl cyclase than either EP_{III} or EP_{3IV}, which agrees with our findings. Regan *et al.* (1994) measured agonist-mediated inhibition of cyclic AMP formation indirectly by use of a reporter gene linked to a cyclic AMP response element and normalized their data to % forskolin-stimulated reporter activity, which would mask constitutive activity. Schmid *et al.* (1995) also normalize their data in such a way as to mask constitutive activity. Finally, Kotani *et al.* (1995) did not obtain data on the inhibition of forskolin-stimulated cyclic AMP formation by the isoforms except in the case of EP_{IV}, which, when stably expressed in CHO cells, gave only 38% inhibition of forskolin-stimulated cyclic AMP formation, in agreement with our findings. There is no evidence of constitutive activity because the effects of pertussis toxin were not investigated.

In the work presented here we normalized all of the data to the forskolin-stimulated cyclic AMP level in mock-transfected cells, which gives a measure of the amount of adenylyl cyclase present in CHO-K1 cells. The method is validated by the fact all of the isoforms and T-359 gave the same level of forskolin-stimulated cyclic AMP formation after treatment with pertussis toxin, which eliminates EP_3 receptor effects on adenylyl cyclase. To examine whether constitutive activity was an artifact, perhaps resulting from high or low levels of receptor expression, we studied in detail the correlation between constitutive activity and receptor expression level. There was no change in constitutive activity over a six fold range of receptor expression for isoforms I–IV or for T-359.

Our results can be compared with those obtained with the mouse EP_3 isoforms. The α isoform of the mouse EP_3 receptor is constitutively active compared with the β isoform, displaying about 50% inhibition of forskolin-stimulated cyclic AMP formation in the absence of prostaglandins (Hasegawa *et al.*, 1996), whereas the γ form is fully constitutively active at G_i, but shows agonist-dependent activity at G_s (Negishi *et al.*, 1996). Comparison of the deduced amino acid sequences of the C-terminal domains of the human and mouse EP_3 receptors (Table 1) reveals that human EP_{3I} is homologous to mouse $EP_{3\alpha}$ and that human EP_{3II} is homologous to mouse $EP_{3\gamma}$. Expression levels of the human isoforms were comparable with those of the mouse isoforms (about 1 pmol mg^{-1} protein). However, there is no correlation between the constitutive behaviour of the homologous isoforms. Human EP_{3II} shows no constitutive activity whereas its mouse homologue, $EP_{3\gamma}$, is almost completely constitutively active. Human EP_{3I}, shows little constitutive activity whereas its mouse homologue, $EP_{3\alpha}$, shows marked constitutive activity.

The observation that receptors display constitutive activity has led to a model that postulates that receptors exist in an equilibrium between inactive and active conformational states and that agonists bind preferentially to and stabilize the active state (Lefkowitz *et al.*, 1993). However, both the free and agonist occupied states can couple to and activate G proteins so that the degree of constitutive activity is determined by how far the equilibrium is shifted towards the active state in the absence of agonist.

Changes in the equilibrium between inactive and active states may be engendered by subtle changes in receptor structure. For example, the native D_{1B} dopamine receptor shows high agonist-independent activation of adenylyl cyclase whereas the D_{1A} dopamine receptor shows no constitutive activity. Recently Charpentier *et al.* (1996) have shown that swapping two variant amino acids (phenylalanine to isoleucine and arginine to lysine) in the third intracellular loop of the two receptors converted the D_{1A} receptor to a constitutively active form and the D_{1B} receptor to a non-constitutively active form.

By contrast with the dopamine receptors, the isoforms of the EP_3 prostaglandin receptor have identical third intracellular loops and differ in their C-terminal regions. We hypothesize that the core of the EP_3 receptor, comprising the seven transmembrane domains, couples constitutively to G_i (i.e. the receptor is stabilized in an active conformation) and that the C-tail blocks this interaction. Agonist binding causes a conformational change that moves the C-tail away from the G protein interaction region and allows receptor-G protein coupling. The fact that the human EP_{3II} receptor is not constitutively active whereas its mouse homologue, $EP_{3\gamma}$, is constitutively active may be related to differences in sequence in their C-terminal sequences which are compared in Table 1. The most significant differences are in the last three amino acids in which the sequence threonine-glutamic acid-alanine of the human receptor becomes proline-glycine-proline in the mouse. We hypothesize that these changes disrupt the C-terminal domain in the mouse receptor so that it does not interact efficiently with the G protein coupling domain. In future work, we intend to swap the three C-terminal amino acids of the human and mouse EP_{3II} receptors and determine whether we can switch constitutive and non-constitutive activities from one to the other.

Constitutive activity has been observed in G protein-coupled receptor systems of all types. In the rhodopsin (Rim & Oprian, 1995) and thyroid-stimulating hormone receptor (Zhang *et al.*, 1995) systems, naturally-occurring activating mutations lead to pathological manifestations such as retinitis pigmentosa and hyperthyroidism, respectively. There is clear evidence for constitutive activity of native opioid receptors linked to inhibition of adenyl cyclase in NG108-15 cell membranes (Costa *et al.*, 1992). It is not known whether constitutively active native receptors have any physiological significance. However, their presence in cells would clearly alter responses to endogenous agonists. In this regard, we have previously postulated that the EP_3 receptor is co-localized with prostaglandin receptors coupled to stimulation of adenylyl cyclase in cells such as platelets (Ashby, 1990) and vascular smooth muscle cells (Ashby, 1992). We postulate that co-localization of opposing receptors for the same agonist would buffer cellular response to transient increases in autacoid

concentration. According to this model, differential expression of EP_3 isoforms with different levels of constitutive activity in different tissues would lead to varying degrees of homeostatic regulation and effectively alter the activity of prostaglandin receptors coupled to stimulation of adenylyl cyclase.

This work was supported by United States Public Health Service, National Institutes of Health grant RO1 HL 48114.

References

AN, S., YANG, J., SO, S.W., ZENG, L. & GOETZL, E.J. (1994). Isoforms of the EP3 subtype of human prostaglandin E2 receptor transduce both intracellular calcium and cAMP signals. *Biochemistry*, **33,** 14496 – 14502.

ASHBY, B. (1990). Novel mechanism of heterologous desensitization of adenylate cyclase: Prostaglandins bind with different affinities to both stimulatory and inhibitory receptors on platelets. *Mol. Pharmacol.*, **38,** 46 – 53.

ASHBY, B. (1992). Comparison of iloprost, cicaprost and prostacyclin effects on cyclic AMP metabolism in intact platelets. *Prostaglandins*, **43,** 255 – 261.

CHARPENTIER, S., JARVIE, K.R., SEVERYNSE, D.M., CARON, M.G. & TIBERI, M. (1996). Silencing of the constitutive activity of the dopamine D1B receptor: reciprocal mutations between D1 receptor subtypes delineate residues underlying activation properties. *J. Biol. Chem.*, **271,** 28071 – 28078.

COSTA, T., OGINA, Y., MUNSON, P.J., ONARAN, H.O. & RODBARD, D. (1992). Drug efficacy at guanine nucleotide-binding regulatory protein-linked receptors: thermodynamic interpretation of negative antagonism and of receptor activity in the absence of ligand. *Mol. Pharmacol.*, **41,** 549 – 560.

HASEGAWA, H., NEGISHI, M. & ICHIKAWA, A. (1996). Two isoforms of the prostaglandin E receptor EP3 subtype different in agonist-independent constitutive activity. *J. Biol Chem.*, **271,** 1857 – 1860.

IRIE, A., SUGIMOTO, Y., NAMBA, T., HARAZONO, A., HONDA, A., WATABE, A., NEGISHI, M., NARUMIYA, S. & ICHIKAWA, A. (1993). Third isoform of the prostaglandin E receptor EP3 subtype with different C-terminal tail coupling to both stimulation and inhibition of adenylate cyclase. *Eur. J. Biochem.*, **217,** 313 – 318.

KOTANI, M., TANAKA, I., OGAWA, Y., USUI, T., MORI, K., ICHIKAWA, A., NARUMIYA, S., YOSHIMI, T. & NAKAO, K. (1995). Molecular cloning and expression of multiple isoforms of human prostaglandin E receptor EP3 subtype generated by alternative messenger RNA splicing: multiple second messenger systems and tissue-specific distributions. *Mol. Pharmacol.*, **48,** 869 – 879.

KUNAPULI, S.P., MAO, G.F., BASTEPE, M., LIU-CHEN, L., LI, S., CHEUNG, P.P., DERIEL, J.K. & ASHBY, B. (1994). Cloning and expression of a prostaglandin E receptor EP3 subtype from human erythroleukemia cells. *Biochem. J.*, **298,** 263 – 267.

LEFKOWITZ, R.J., COTECCHIA, S., SAMAMA, P. & COSTA, T. (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.*, **14,** 303 – 307.

MUNSON, P.J. & RODBARD, D. (1980). LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.*, **107,** 220 – 239.

NEGISHI, M., HASEGAWA, H. & ICHIKAWA, A. (1996). Prostaglandin E receptor EP3gamma isoform, with mostly full constitutive Gi activity and agonist-dependent Gs activity. *FEBS Lett.*, **386,** 165 – 168.

NEGISHI, M., SUGIMOTO, Y., IRIE, A., NARUMIYA, S. & ICHIKAWA, A. (1993). Two isoforms of prostaglandin E receptor EP3 subtype. Different COOH-terminal domains determine sensitivity to agonist-induced desensitization. *J. Biol. Chem.*, **268,** 9517 – 9521.

REGAN, J.W., BAILEY, T.J., DONELLO, J.E., PIERCE, K.L., PEPPERL, D.J., ZHANG, D., KEDZIE, K.M., FARBAIRN, C.E., BOGARDUS, A.M., WOODWARD, D.F. & GIL, D.W. (1994). Molecular cloning and expression of human EP3 receptors: evidence of three variants with differing carboxyl termini. *Br. J. Pharmacol.*, **112,** 377 – 385.

RIM, J. & OPRIAN, D.D. (1995). Constitutive activation of opsin: interaction of mutants with rhodopsin kinase and arrestin. *Biochemistry*, **34,** 11938 – 11945.

SOLOMON, Y. (1979). Adenylate cyclase assay. *Adv. Cyc. Nucl. Res.*, **10,** 35 – 55.

SANGER, F., NICKLEN, S. & COULSON, A.R. (1977). DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.*, **74,** 5463 – 5468.

SCHMID, A., THIERAUCH, K.-H., SCHLEUNING, W.-D. & DINTER, H. (1995). Splice variants of the human EP3 receptor for prostaglandin E2. *Eur. J. Biochem.*, **228,** 23 – 29.

ZHANG, M.-L., SUGAWA, H., KOSUGI, S. & MORI, T. (1995). Constitutive activation of the thyrotropin receptor by deletion of a portion of the extracellular domain. *Biochem. Biophys. Res. Commun.*, **211,** 205 – 210.

(Received October 22, 1996
Revised January 13, 1997
Accepted February 5, 1997)