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Pharmacological characterization and identification of EP_3 prostanoid receptor binding sites in hamster uterus homogenates

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Abstract

The pharmacological properties of [^{3}H]-prostaglandin E_2 ([^{3}H]-PGE_2) binding to washed homogenates of hamster uterus were determined. Scatchard analysis of competition data yielded dissociation constants (K_ds) of 30.9 ± 5.6 nM (n = 3) and apparent receptor density (B_{max}) of 25.25 ± 1.89 pmol g^{-1} wet weight tissue (74 ± 8% specific binding). Competition studies yielded the following affinity parameters (K_i) for various prostanoids: GR63799X = 13 ± 4 nM; PGE_2 = 17 ± 3 nM; sulprostone = 64 ± 5 nM; enprostil = 67 ± 3 nM; misoprostol = 124 ± 15 nM; cloprostenol = 187 ± 33 nM; carba-prostacyclin = 260 ± 167 nM; iloprost = 555 ± 162 nM; $PGF_{2\alpha}$ = 767 ± 73 nM; PGD_2 > 3560 nM; fluprostenol = $11\,790 \pm 2776$ nM; RS93520 = $21\,558 \pm 14\,228$ nM. These data closely matched the pharmacological profile of previously described EP_3 receptors such as in bovine corpus luteum (BCLM) and the cloned mammalian EP_3 receptors. The high correlation between the current hamster uterus pharmacology data vs the EP_3 receptor binding in BCLM ($r = 0.94$; $P < 0.0001$), vs cloned human EP_3 receptor ($r = 0.94$, $P < 0.0001$), vs the cloned mouse EP_3 receptor binding ($r = 0.78$; $P < 0.002$), vs cloned rat EP_3 receptor ($r = 0.9$, $P < 0.0004$), and vs EP_3 receptor-mediated functional responses ($r = 0.72$, $P < 0.02$) substantiated the conclusion that the hamster uterus contains EP_3 receptor binding sites.

Introduction

Prostanoids such as the prostaglandins (PGs), including PGD_2, PGE_2 and $PGF_{2\alpha}$, are arachidonic acid derivatives that are found endogenously in the body and have a multitude of physiological, pharmacological and pathological functions in various mammalian cells and tissues (Coleman et al 1994; Narumiya 1994). For instance, PGD_2 induces sleep and hypothermia, and inhibits platelet aggregation; PGE_2 has hyperthermic, anti-diuretic and algesic effects; $PGF_{2\alpha}$ inhibits oxytocin release and induces labour (Coleman et al 1994; Narumiya 1994). Furthermore, these prostanoids have either relaxant or contractile (sometime both) effects on smooth muscles, and ester or amide derivatives of certain prostanoids, such as travoprost (Sharif et al 1999; Hellberg et al 2001, 2002), latanoprost (Stjernschantz et al 1995) and bimatoprost (Woodward et al 2003), lower intraocular pressure and are used therapeutically to treat ocular hypertension and glaucoma.

The receptors mediating the effects of the prostanoids have been classified as DP, EP, FP, IP and TP (with subtypes for EP known as EP_1, EP_2, EP_3 and EP_4) corresponding in general terms with the names of the natural prostanoids (Coleman et al 1994; Narumiya 1994). All endogenous and recombinant prostanoid receptors are heptahelical membrane-spanning proteins coupled to their effector mechanisms via G-proteins, with DP, EP_2, EP_4 and IP receptors preferentially coupling to adenylyl cyclase via G_s, and EP_1, EP_3, FP and TP receptors preferentially coupling to phospholipase C via G_q or $G_{q/11}$ (Coleman et al 1994; Narumiya 1994; Abramovitz et al 2000). The different splice variants of the EP_3 receptor couple to a variety of signal transduction mechanisms (Narumiya 1994; Ushibuki et al 1995).

Prostanoids potently contract and/or relax various smooth muscles of different tissues either to elicit direct relaxation or contraction of the whole tissue, such as in

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the uterus (e.g. Senior et al 1993; Fernandes & Crankshaw 1995), or to lead to vasodilation or vasoconstriction as in the vascular system (e.g. Giles et al 1989). Low concentrations of PGE_2 are potent stimulants of uterine contractility in-vitro (Senior et al 1993) and in-vivo (Martin & Bygdeman 1975) with relaxant effects being observed at high concentrations (Senior et al 1993). In the pregnant human myometrium, the excitatory phase is mediated by EP_3 receptors while the inhibitory phase is mediated by EP_2 receptors (Senior et al 1993). Hamster uterus strips in-vitro respond to PGE_2 (Yeardley et al 1992) and like uterine membranes of various species (Crankshaw et al 1979; Hodam et al 1989) hamster uterus binds [^{3}H]-PGE_1 (Wakeling & Wyngarden 1974). However, the binding studies of Wakeling & Wyngarden (1974) only used some natural prostanoids and a few analogues of PGE_1 to characterize the binding sites using [^{3}H]-PGE_1 as the radioligand. Similarly, early functional receptor studies of Yeardley et al (1992) on hamster uterus were inconclusive about the nature of the EP-class receptors present in this tissue. In an effort to resolve the latter issues and extend these early preliminary studies, we therefore sought to use [^{3}H]-PGE_2 binding techniques, employing a broad range of potent and selective synthetic prostanoids and some natural prostanoids, to determine the pharmacological characteristics of the EP-receptor binding sites that mediate the inhibition of hamster uterine contractions (Yeardley et al 1992). The aims of the present study were to demonstrate the presence and define the pharmacology of [^{3}H]-PGE_2 binding sites on adult hamster uterus homogenates using a large variety of prostanoids having some degree of selectivity for the various prostanoid receptor subtypes known today.

Materials and Methods

Methods

Washed total particulate hamster uterus homogenates (20 mg mL^{-1}) were prepared by standard homogenization and centrifugation procedures as previously described for bovine corpus luteum (BCLM) (Sharif et al 1998). Aliquots (400 μL) of the washed hamster uterus homogenate were incubated with [^{3}H]-PGE_2 (0.9–2 nM) in Krebs buffer (pH 7.4) for 1 h at 23 °C in a total volume of 500 μL. Non-specific binding was defined with 1 μM unlabeled PGE_2. When affinities of various prostanoids were determined they were tested for their ability to compete for specific [^{3}H]-PGE_2 binding over the 10 pM–100 μM concentration range. The ligand binding assays were terminated by rapid vacuum filtration using Whatman GF/B glass fibre filters previously soaked in 0.3% polyethyleneimine.

Data analysis and statistical methods

Competition data were analysed using a nonlinear, iterative curve-fitting computer program (Bowen & Jerman 1995; Sharif et al 1999; Sharif & Davis 2002) and the binding inhibition data (IC_{50} values) converted to equilibrium inhibition constants (K_i) according to Cheng and Prusoff (1973). Another suite of programs (EBDA) (McPherson 1983) incorporating the LIGAND program (Munson & Rodbard 1980) was also used to perform further analyses of the data in terms of Scatchard analysis of competition data as previously described (Davis & Sharif 2000; Sharif & Davis 2002).

Data were represented as mean ± s.e.m. When pharmacological data from the current studies were compared with literature data to construct correlation plots, all the data were converted to –log of the binding inhibition constants (K_i) and represented as pK_i values, or in the case of functional responses the data were presented as pEC_{50} values. The correlation plots of the data sets were constructed using the Origin Scientific Graphics software package (Microcal Software, Northampton, MA). The statistical analysis of the data sets used for the correlation plots was performed by the aforementioned software package and as previously documented for other receptor systems (Davis & Sharif 2000; Sharif & Davis 2002). A significance level of $P < 0.05$ denoted significance in all cases.

Materials

Frozen uteri obtained from adult female hamsters were obtained from Pel-freez (Rogers, AR), EDTA (disodium salt), Tris base, BSA, digitonin, formic acid, ammonium formate, LiCl and polyethyleneimine from Sigma Chemical Co. (St Louis, MO) and EGTA from Fluka BioChemika (Buchs, Germany). Origin Scientific Graphics software package was purchased from Microcal Software (Northampton, MA). [^{3}H]-PGE_2 (171 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). All prostanoids were purchased from Cayman Chemical Co. (Ann Arbor, MI) or synthesized in the Medicinal Chemistry department at Alcon Research Ltd or by contract using published methods, except the following, which were generous gifts from the companies cited: ZK118182 from Schering Ag (Berlin and Bergkamen, Germany); BW245C, AH23848, AH22921 and GR63799X from Glaxo-SmithKline (Stevenage, UK); RS93520 from Hoffman La-Roche (Basel, Switzerland) and SC-19220 from G.D Searle (Skokie, IL). The chemical structures of some of the key prostanoids used in these studies were: ZK118182 ((5*Z*,13*E*)-(9*R*,11*R*,15*S*)0-9-chloro-15-cyclohexyl-11,15-dihydroxy-3-oxo-16,17,18,19,20-pentanor-5,13-prostadienoic acid)); ZK110841 ((5*Z*,13*E*)-9*R*,11*R*,15*S*)-9-chlor-15-cyclohexyl-11,15-dihydroxy-16,17,18,19,20-pentanor-5,13-prostadienoic acid)); *RS*-93520((C3′*S*,1*R*,2*R*,3*S*,6*R*)-2-C3′-cyclohexyl-3′hydroxyprop-1-ynyl)-3-hydroxybicyclo-[4.2.0]oct-7-ylidene)butyrate); SQ27986 ([1*S*-[1*B*,2*B*(5*Z*),3*A*(1*E*,3*S*), 4*B*]]-7-[3-(3-cyclohexyl-3-hydroxy-1-propenyl)-7-oxabi-cyclo -[2.2.1]hept-2-yl]-5-heptenoic acid)); BW245C (5-(6-carboxyhexyl)-1-(3-cyclohexyl-3-hydroxypropylhydantoin); cloprostenol (16-*m*-chlorophenoxy tetranor prostaglandin $F_{2\alpha}$); fluprostenol (16-*m*-trifluorophenoxy tetranor $PGF_{2\alpha}$). Chemical structures and the names of the

other most commonly used prostanoids can be found in Coleman et al (1994).

Results

Specific [^{3}H]-PGE_2 binding to washed hamster uterus homogenates constituted 74 ± 8% (n = 9) of the total binding when 2 nM of radioligand was utilized to label the high-affinity binding sites. Scatchard analysis of data from unlabelled PGE_2 competing for [^{3}H]-PGE_2 binding, thus dilution of specific activity method, yielded a mean dissociation constant (K_d) of 30.9 ± 5.6 nM (n = 3) and apparent receptor density (B_{max}) of 25.25 ± 1.89 pmol g^{-1} wet weight tissue (Figure 1). Competition studies with various classes of prostanoids yielded the following affinity (K_i) parameters (n 3 experiments): GR63799X = 13 ± 4 nM; PGE_2 = 17 ± 3 nM; sulprostone = 64 ± 5 nM; enprostil = 67 ± 3 nM; misoprostol = 124 ± 15 nM; cloprostenol = 187 ± 33 nM; carba-prostacyclin = 260 ± 167 nM; iloprost = 555 ± 162 nM; $PGF_{2\alpha}$ = 767 ± 73 nM; PGD_2 > 3560 nM; fluprostenol = 11 790 ± 2776 nM; RS93520 = 21 558 ± 14 228 nM, BW245C > 19 000 nM; latanoprost > 62 000 nM; SQ29548 > 87 000 nM, etc. (Figure 2; Table 1).

Various degrees of statistical significance between the pharmacological data from hamster uterus and other preparations were obtained: correlation coefficients for hamster uterus data vs the EP_3 receptor binding in bovine corpus luteum (BCLM), r = 0.94, $P < 0.0001$ (Figure 3A); for hamster uterus data vs the cloned human EP_3 receptor, r = 0.94, $P < 0.00001$ (Figure 3B); for hamster uterus data vs the cloned rat EP_3 receptor, r = 0.9, $P < 0.0004$ (Figure 3C); and for hamster uterus data vs the functional activity at cloned human and rat EP_3 receptors, r = 0.72, $P < 0.02$ (Figure 3D). Other levels of significance of data correlations were: for hamster uterus data vs the cloned mouse EP_1 receptor, r = 0.5, $P < 0.05$; for hamster uterus data vs the cloned mouse EP_2 receptor, r = 0.3, $P < 0.28$; for hamster uterus data vs the cloned mouse EP_3 receptor, r = 0.78, $P < 0.002$; for hamster uterus data vs the cloned mouse EP_4 receptor, r = 0.4, $P < 0.2$ (data not shown). Hamster uterus binding data correlated with the cloned rat EP receptors (Boie et al 1997) as follows: vs cloned rat EP_1 (r = 0.69, $P < 0.01$), vs cloned rat EP_2 (r = 0.12), vs cloned rat EP_3 (r = 0.9, $P < 0.0004$; Figure 3C), vs cloned rat EP_4 (r = 0.5, $P < 0.09$) (data not shown). Similarly, when compared with

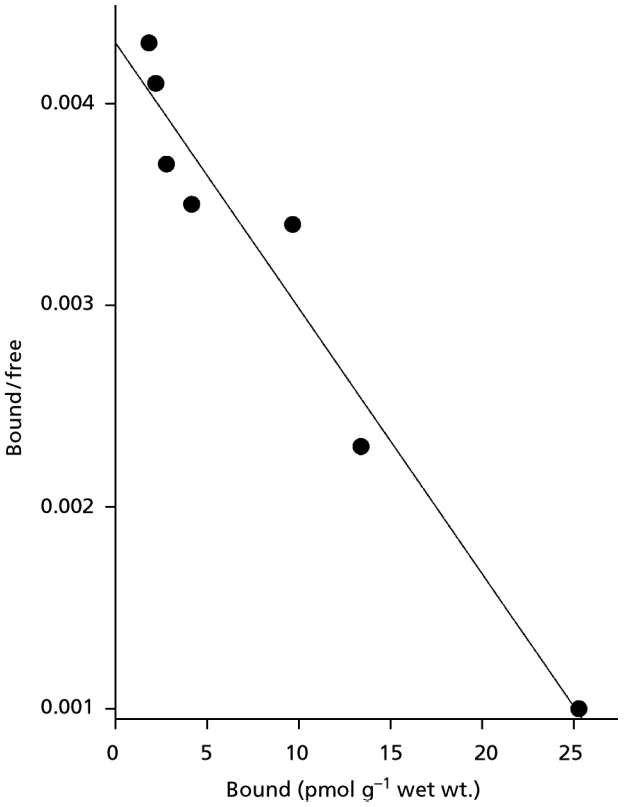

Figure 1 Scatchard plot of specific [^{3}H]-PGE_2 binding to hamster uterus homogenates. Data are from a representative experiment of three. The binding parameters obtained from such studies were: K_d = 30.9 ± 5.6 nM, B_{max} = 25.25 ± 1.89 pmol g^{-1} wet weight (n = 3).

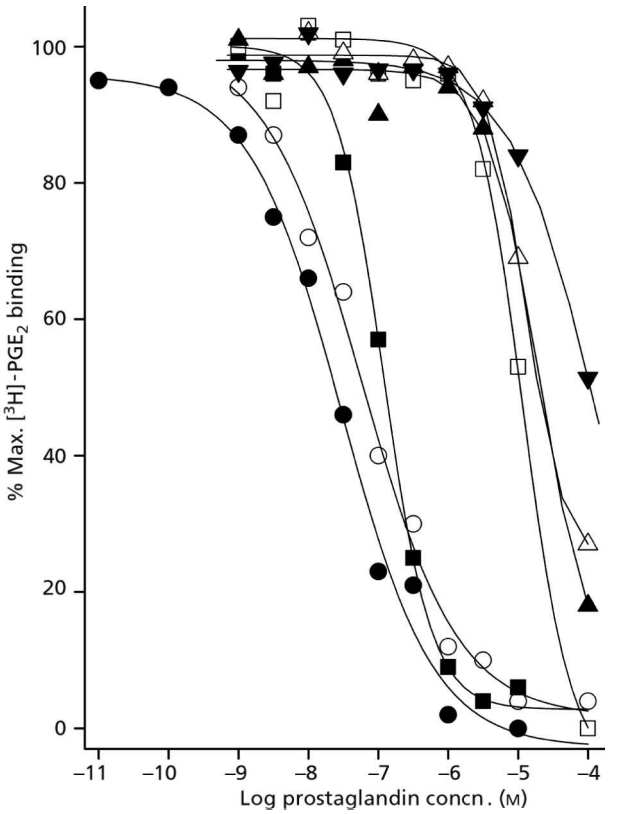

Figure 2 Competition curves for various prostanoids displacing specific [^{3}H]-PGE_2 binding to hamster uterus homogenates. Data are from a representative experiment. Mean ± s.e.m. of the data from several experiments are shown in Table 1. ●, GR 63799X; ○, enprostil; ■, misoprostil; □, AH 23848; ▲, ZK 118182; △, butaprost acid; ▼, AH 22921.

Table 1 Affinities of selected prostanoids competing for specific [^{3}H]-PGE_2 binding sites on hamster uterus and BCLM homogenates.

Compound	[^{3}H]-PGE_2 binding inhibition in hamster uterus (K_i, nM)	[^{3}H]-PGE_2 binding inhibition in BCLM (K_i, nM)[1]	Functional potency in EP_3 receptor functional assays (EC_{50}, nM)
GR63799X	13 ± 4	11 ± 2 [1.9 nM]	
PGE_2	17 ± 3	3 ± 0.2 [0.9 nM]	0.41[2]
17-Phenyl-trinor PGE_2	37 ± 5	7 ± 1 [3.7 nM]	
Sulprostone	64 ± 5	11 ± 2 [0.6 nM]	1.4 ± 0.5[3]; 0.42[2]
Enprostil	67 ± 3	25 ± 17	3.7 ± 1.1[3]
Misoprostol	124 ± 15	54 ± 3 [67 nM]	
Cloprostenol	187 ± 33	91 ± 12	221 ± 60[3]
Carba-prostacyclin	260 ± 167		
Iloprost	555 ± 162	265 ± 12 [22 nM]	0.63[2]
$PGF_{2\alpha}$	767 ± 73	24 ± 11 [75 nM]	4.2[2]
PGD_2	3566 ± 356	1114 ± 118 [280 nM]	13[2]
Bimatoprost acid (17-Phenyl-trinor $PGF_{2\alpha}$)	5302 ± 822	388 ± 126	
AH23848	9286 ± 1885	7943 ± 2189	
(±)-Fluprostenol	11 790 ± 2776	3501 ± 461	> 10 μM[3]
Latanoprost acid (PHXA85)	15 866 ± 5244	7944 ± 990	> 10 μM[3]
BW245C	19 974 ± 9658		
ZK118182	20 569 ± 3405	8738 ± 2567	
RS-93520	21 558 ± 14 228		
Butaprost acid	27 707 ± 8735	> 75 000	
Latanoprost (PHXA41)	62 019 ± 31 660	> 100 000	
AH22921	83 026 ± 19 505	39 810 ± 13 019	
SQ29548	87 018 ± 36 019		
SC-19920	125 854	39 916	

Data are mean ± s.e.m. from up to five experiments. Affinity values shown in square brackets are adapted from Kiriyama et al (1997) for [^{3}H]-PGE_2 binding to the cloned mouse EP_3 receptor for reference. [1]Adapted from Sharif et al (1998) for comparison. Functional data are adapted from: [2]Boie et al (1997) and [3]Sharif et al (1998). BCLM = bovine corpus luteum.

the ligand binding data obtained for cloned human EP_{1-4} receptors (Abramovitz et al 2000), the following data correlations became apparent: hamster uterus vs cloned human EP_1 ($r = 0.7$, $P < 0.0001$), vs cloned human EP_2 ($r = 0.15$), vs cloned human EP_3 ($r = 0.94$, $P < 0.0001$; Figure 3B), vs cloned human EP_4 ($r = 0.59$, $P < 0.02$) (data not shown).

Discussion

In the current studies, specific [^{3}H]-PGE_2 binding to hamster uterus homogenates reflected interaction with an apparent single population of nanomolar affinity and apparent high receptor density sites. The hamster uterus [^{3}H]-PGE_2 binding parameters compared well with those previously described for baboon, rabbit, tree shrew and human uterine membranes (Crankshaw et al 1979; Hodam et al 1989). Using tissue slices and [^{3}H]-PGE_1 as the radioligand, Wakeling & Wyngarden (1974) also found high-affinity EP binding sites in hamster uterus preparations. These [^{3}H]-PGE_1 binding sites represented EP-class PG receptor binding sites since unlabelled PGE_2, PGE_1 and its various acid and ester analogues more potently displaced [^{3}H]-PGE_1 binding than $PGF_{2\alpha}$. However, whether these [^{3}H]-PGE_1 binding sites (Wakeling & Wyngarden 1974) represented EP_1, EP_2, EP_3 or EP_4 classes remained undefined due to the unavailability of more PG receptor-selective agents and at that time the latter sub-types were unknown. However, in the current studies we believe we have pharmacologically identified the [^{3}H]-PGE_2 binding sites in the hamster uterus as the EP_3 receptor sites (Figure 2; Table 1) as was also recently demonstrated in bovine corpus luteum (Sharif et al 1998). In support of this conclusion, we found that prostanoids known to have high affinities for the EP_3 receptor (such as GR63799X, sulprostone and enprostil) were potent competitors of [^{3}H]-PGE_2 binding to hamster uterus homogenates, while compounds selective for DP (ZK118182, BW245C, RS93520), EP_1 (iloprost), EP_2 (e.g. butaprost, misoprostol), EP_4 (e.g. AH23848, AH22921), FP (fluprostenol, latanoprost acid), IP (iloprost, carba-prostacyclin) and TP (SQ-29548) receptors (Coleman et al 1994) exhibited low micromolar affinities (Figure 2; Table 1). The EP_3 receptor-like nature of the hamster uterus [^{3}H]-PGE_2 binding sites from the current studies was further substantiated by the similarity of the rank order of potency and magnitude of affinities of numerous prostanoids tested against [^{3}H]-PGE_2 binding sites in the bovine corpus luteum expressing EP_3 receptor

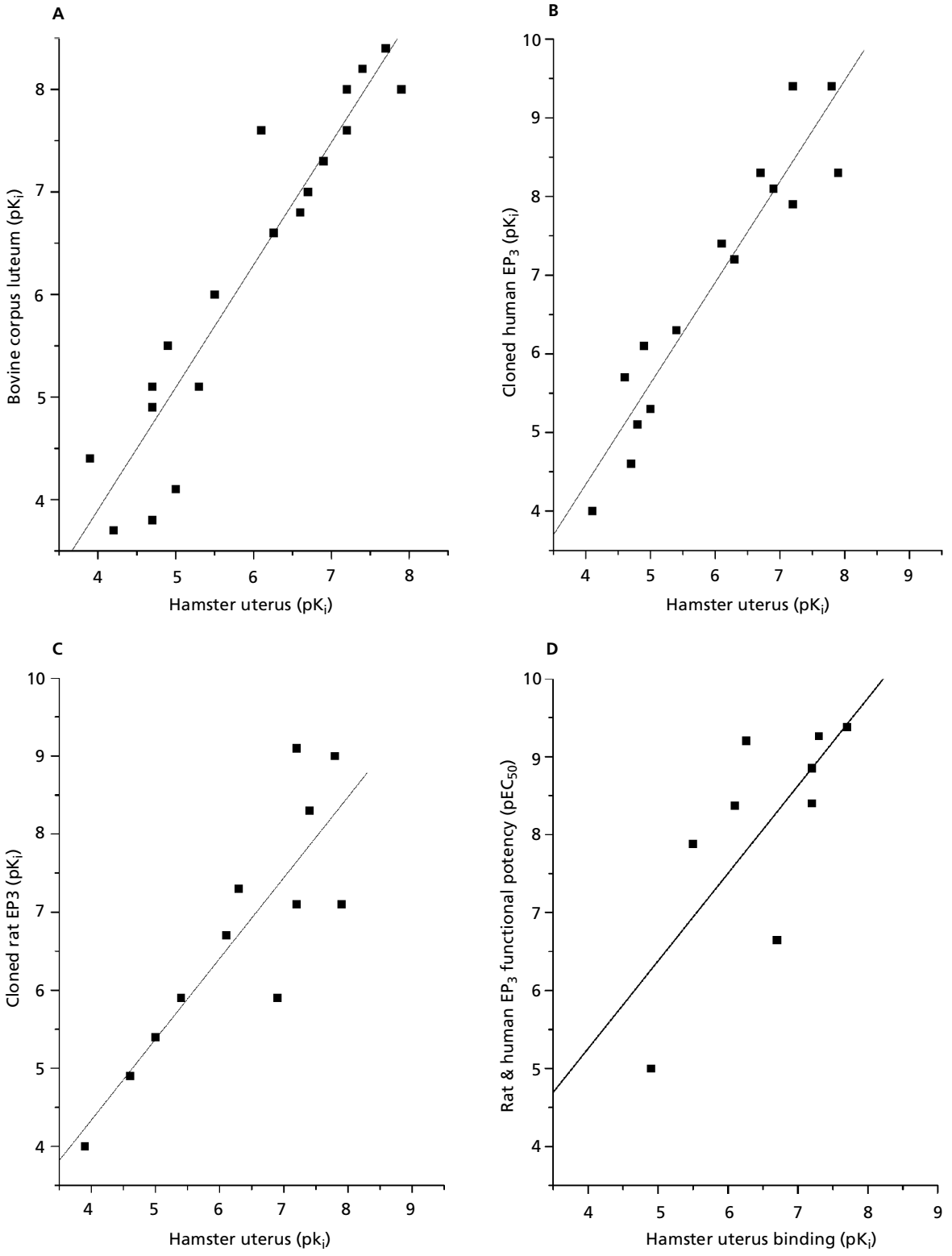

Figure 3 Correlation plots of [^{3}H]-PGE_2 binding data from hamster uterus vs (A) bovine corpus luteum ($r = 0.94$, $P < 0.0001$, $n = 19$), vs (B) cloned human EP_3 receptor ($r = 0.94$, $P < 0.0001$, $n = 15$), vs (C) cloned rat EP_3 receptor ($r = 0.9$, $P < 0.0004$, $n = 10$), and vs (D) functional data for rat and human EP_3 receptors ($r = 0.72$, $P < 0.02$, $n = 9$). The source of the comparative data for the cloned EP_3 receptors was as follows: for cloned rat EP_3 receptors (Boie et al 1997) and for cloned human EP_3 receptor (Abramovitz et al 2000). The bovine corpus luteum data were from Sharif et al (1998).

protein and mRNA (Tsai et al 1996; Sharif et al 1998; Table 1), and the pharmacological profile of the cloned mouse (Kiriyama et al 1997), rat (Boie et al 1997) and human (Abramovitz et al 2000) EP_3 receptors. Since the mouse, rat and hamster are closely related, it was of particular significance that the hamster uterus binding data best correlated with the cloned mouse and rat EP_3 receptor pharmacology and not with the cloned mouse or rat EP_1, EP_2 and EP_4 receptors (see Results). The close correlation between the PG affinities in the hamster uterus binding studies and the functional potencies (and their rank orders) of numerous prostanoids determined in cells expressing recombinant EP_3 receptors (Boie et al 1997; Sharif et al 1998; Table 1) also lends strong credence to the identification of EP_3 receptor sites in the hamster uterus. In addition, it was noteworthy that the classic FP-receptor agonist cloprostenol, which has been also shown previously to exhibit EP_3 agonist activity in a number of assays (Coleman et al 1994; Sharif et al 1998), also exhibited a reasonably high affinity for [^{3}H]-PGE_2 binding to hamster uterus homogenates.

cDNA clones encoding four different EP_3 receptor splice variants have been reported (Negishi et al 1993; Kotani et al 1995) and their mRNAs detected in numerous tissues, including mouse uterus (Ushikubi et al 1995) and the human uterus (Kotani et al 1995). Our homogenate-based ligand binding studies do not provide direct information about which EP_3 receptor isoform(s) are present in the hamster uterus, and to our knowledge hamster uterus EP_3 receptor mRNAs have not yet been isolated. Interestingly, even though EP_3 receptors contract the hamster uterus while EP_4 receptors relax this tissue (Yeardley et al 1992; Coleman et al 1994), the pharmacological properties of the binding sites detected by [^{3}H]-PGE_2 binding resemble more closely the EP_3 receptor rather than the EP_4 receptor. This observation is probably related to a higher density of EP_3 receptors in this tissue than EP_4 receptors. However, further work is needed to elaborate on these findings.

Conclusion

It is concluded that hamster uterus contains a high density of specific, high affinity [^{3}H]-PGE_2 binding sites that have the pharmacological characteristics of EP_3 receptors. Since EP_3 receptor mRNA is also abundantly expressed in the uterus, the present study lends further support to the importance of EP_3 receptor-mediated signalling in uterine function.

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