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Affinity of cholecystokinin receptor antagonists for the gastrin-binding protein

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Abstract

A 78 kDa gastrin-binding protein is a likely target for the anti-proliferative effects of the cholecystokinin (CCK) receptor antagonists D,L-4-benzamido-*N*,*N*-dipropylglutaramic acid (proglumide) and *N*-4-chlorobenzoyl-L-tryptophan (benzotript) on colorectal carcinoma cell lines [Baldwin, G.S., 1994. Antiproliferative gastrin/cholecystokinin receptor antagonists target the 78-kDa gastrin-binding protein. Proc. Natl. Acad. Sci. USA 91, 7593–7597.]. Definition of the physiological role of the gastrin-binding protein has been hampered by the very low affinity of benzotript for the gastrin-binding protein. Benzotript analogues were therefore tested for their ability to inhibit the binding of iodinated gastrin to the gastrin-binding protein. The affinity of the most potent analogue (the D-isomer of benzotript, CR 665) was similar to the value reported previously for the L-isomer. In order to isolate more potent binding inhibitors, several selective CCK receptor antagonists were also tested as inhibitors of the binding of gastrin to the gastrin-binding protein. The affinity of the most potent binding inhibitor PD 149164 (benzenebutanoic acid, 4-fluoro-!b/-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo-[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-, [*R*-(*R**,*S**)]-) was approximately 10-fold higher than the L-isomer of benzotript. PD 149164 may serve as the lead compound for the future development of more potent and selective gastrin-binding protein inhibitors.

Keywords: Gastrin; CCK receptor antagonist; Gastrin-binding protein

1. Introduction

Several receptors for peptides of the gastrin/cholecystokinin (CCK) family have been described (Silvente-Poirot et al., 1993; Shulkes and Baldwin, 1997; Wank, 1998). The CCK_1 receptor, which is predominantly found on pancreatic acini, binds sulphated forms of CCK preferentially. The gastric mucosa and brain are the major sites of expression of the CCK_2 receptor, which has equal affinity for both gastrin and CCK, and does not require peptide sulphation for binding. Both the CCK_1 and CCK_2 receptors belong to the family of seven transmembrane receptors, and both activate similar signalling pathways.

A third gastrin receptor (the 78 kDa gastrin-binding protein) was originally identified in detergent extracts of porcine gastric membranes by its ability to bind ^{125}I-labelled gastrin (Baldwin et al., 1986). The gastrin-binding protein is structurally unrelated to the CCK_1 and CCK_2 receptors, but is similar in amino acid sequence to the α-subunit of a mitochondrial trifunctional protein (Baldwin, 1993; Mantamadiotis et al., 1993). Like the α-subunit, which catalyses the second and third steps in the oxidation of long chain fatty acids (Uchida et al, 1992), the gastrin-binding protein sequence has both an enoyl-CoA hydratase and a 3-hydroxyacyl-CoA dehydrogenase domain. The exact role of the gastrin-binding protein has not been determined, but a population is present on the surface of gastrointestinal carcinoma cell lines, and appears to be the target for the anti-proliferative effects of CCK receptor antagonists (Baldwin, 1994). The gastrin-binding protein may also have a role in fatty acid oxidation, as both crotonyl-CoA, a substrate for enoyl CoA hydratase, and acetoacetyl-CoA, the product of 3-hydroxyacyl-CoA dehydrogenase, inhibit the binding of gastrin to the gastrin-binding protein (Baldwin, 1994).

Many antagonists have been described for CCK receptors (Silvente-Poirot et al., 1993; Shulkes and Baldwin,

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1997; Wank, 1998). The first antagonists, D,L-4-benzamido-*N*,*N*-dipropylglutaramic acid (proglumide) and *N*-4-chlorobenzoyl-L-tryptophan (benzotript), which were developed as inhibitors of gastric acid secretion, suffered from the twin disadvantages of being weak and non-selective (Hahne et al., 1981). A major breakthrough came with the isolation of asperlicin from the fungus Aspergillus alliaceus by a group at Merck, Sharp and Dohme Research Laboratories (Chang et al., 1985). Asperlicin was 70-fold more potent than benzotript for the CCK_1 receptor, and was not recognized by the CCK_2 receptor (Chang et al., 1985). Extensive structure/activity studies on compounds containing the 1,4-benzodiazepine ring system of asperlicin yielded the potent antagonists L364,718 (Chang and Lotti, 1986) and L365,260 (Lotti and Chang, 1989), which are specific for the CCK_1 and CCK_2 receptors, respectively. Several unrelated compounds have since been developed which bind either CCK_1 or CCK_2 receptors with high selectivity and affinities in the pM-nM range (Silvente-Poirot et al., 1993; Shulkes and Baldwin, 1997; Wank, 1998). The discovery of such potent and selective antagonists has clarified the physiological roles of CCK receptors considerably.

In a similar manner specific antagonists for the gastrin-binding protein might help to define its physiological role. Although the non-selective CCK receptor antagonists proglumide and benzotript both target the gastrin-binding protein, the concentrations required for 50% inhibition (IC_{50}) of cross-linking of gastrin to the gastrin-binding protein are high (5.1 mM and 200 μM, respectively) (Baldwin, 1994). In order to investigate the requirements for binding of benzotript to the gastrin-binding protein, several benzotript analogues were tested as inhibitors of the binding of iodinated gastrin to the gastrin-binding protein. In addition, as a first step in defining antagonists selective for the gastrin-binding protein, the interaction between a panel of selective CCK receptor agonists and antagonists and the gastrin-binding protein was assessed. Because of the similarity between the gastrin-binding protein and the α-subunit of the mitochondrial trifunctional protein, the antagonists were also tested for their effects on fatty acid oxidation in normal human fibroblasts.

2. Materials and methods

2.1. Materials

Agonists and antagonists, the names and structures of which are given in Table 1 and Fig. 1, respectively were

Table 1
Names of CCK receptor agonists and antagonists mentioned in this study

Code	Name	Supplier
Benzotript	*N*-4-chlorobenzoyl-L-tryptophan	
A-57696	Boc-Leu-Met-Asp-Phe-NH_2	Abbott
A-71378	Desamino-Tyr (SO_3H)-Nle-Gly-Trp-Nle-(*N*-methyl)Asp-Phe-NH_2	Abbott
PD 132461	Carbamic acid, 2-[1-(hydroxymethyl)-2-phenylethyl-amino]-1-(1*H*-indol-3-ylmethyl)-1-methyl-2-oxoet-tricyclo-[3,3,1,1,^{3,7}]dec-2yl ester, [*R*-(*R**,*S**)]-	Parke-Davis
PD 134308	Butanoic acid, 4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo-[3,3,1,1,^{3,7}]dec-2-yloxy)carbonyl] amino]propyl]amino]-1-phenylethyl]amino]-4-oxo-, [*R*-(*R**,*R**)]-, *N*-methyl-D-glucamine salt	Parke-Davis
PD 142898	!b/-Alanine, *N*-[!a/-methyl-*N*-[[(2-methylcyclohexyl) oxy]carbonyl]-D-tryptophyl]-L-3-(phenylmethyl)-, (1*S*-trans)-, compd. 1-deoxy-1-(methylamino)-D-glucitol (1:1)	Parke-Davis
PD 149164	Benzenebutanoic acid, 4-fluoro-!b/-[[3-(1*H*-indol -3-yl)-2-methyl-1-oxo-2-[[(tricyclo-[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-, [*R*-(*R**,*S**)]-	Parke-Davis
PD 149650	Benzenebutanoic acid, 4-fluoro-!b/-[[3-(1*H*-indol -3-yl)-2-methyl-1-oxo-2-[[(tricyclo-[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-, ethyl ester, [*R*-(*R**,*S**)]-	Parke-Davis
CR 1409	D,L-4-(3,4-dichlorobenzoylamino)-5-(di-*N*-pentylamino)-5-oxo-pentanoic acid	Rotta
SR 27897B	1-{[2-(4-(2-chlorophenyl) thiazol-2-yl)-aminocarbonyl]-indolyl}-acetic acid	Sanofi
LY 288513	(4S,5R)-*trans*-*N*-(4-bromophenyl)-3-oxo-4,5-diphenyl-1-pyrazolidinecarboxamide	Eli Lilly
RP 73870A	{{[*N*-(methoxy-3-phenyl)-*N*-(*N*-methyl-*N*-phenyl-carbamoylmethyl)-carbamoylmethyl]-3-ureido}-phenyl}-2-ethylsulphonate, K^+ salt	Rhone-Poulenc Rorer

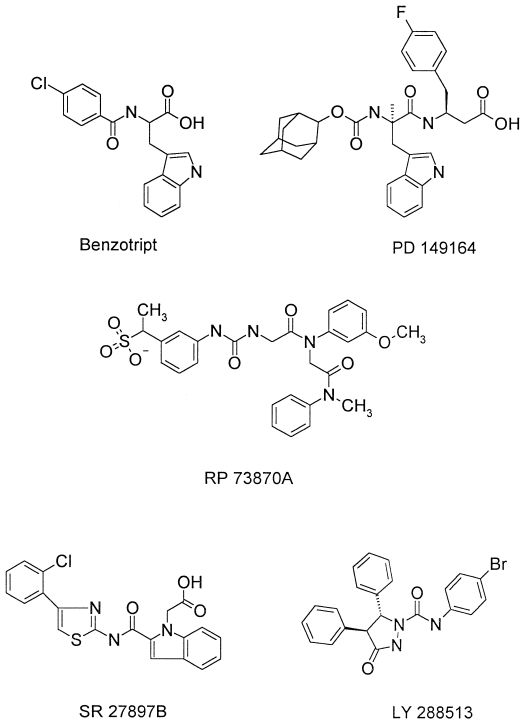

Fig. 1. Structures of some CCK receptor antagonists. The chemical names of the CCK receptor antagonists benzotript, PD 149164 (Parke-Davis), RP 78370A (Rhone-Poulenc Rorer), LY 288513 (Eli Lilly), and SR 27897B (Sanofi Recherche) are given in Table 1.

generous gifts from the following companies: A 57696 and A 71378 were from Abbott Laboratories (Abbott Park, IL); LY 288513 was supplied by Eli Lilly and Co. (Indianapolis, IN); PD 132461, PD 134308 (CI 988), PD 142898, PD 149164 and PD 149650 were from Parke Davis (Cambridge, England); RP 73870A was from Rhone-Poulenc Rorer (Vitry sur Seine, France); CR 449, CR 505, CR 512, CR 517, CR 546, CR 617, CR 665, CR 1409, CR 1436, CR 1955 and CR 2850 were from Rotta Research Laboratorium (Milan, Italy); SR 27897B was from Sanofi Recherche (Toulouse, France).

2.2. Purification of the gastrin-binding protein

The porcine 78 kDa gastrin-binding protein was purified from detergent extracts of gastric mucosal membranes by chromatography on concanavalin-A-Sepharose and diethylaminoethyl-Sepharose as described previously (Baldwin, 1994; Baldwin et al., 1986).

2.3. Measurement of antagonist affinities

Affinities of CCK receptor agonists and antagonists for the gastrin-binding protein were measured in a covalent cross-linking assay as described previously (Baldwin, 1994; Baldwin et al., 1986). Briefly, [^{125}I]–[Nle^{15}]gastrin-(2–17) (approx. 30,000 cpm, 10 fmol) in 50 mM Na^+ HEPES, pH 7.6 was incubated with 0.2 mM disuccinimidyl suberate on ice for 15 min. A 25μl aliquot of this gastrin-disuccinimidyl suberate mix was then added to the gastrin-binding protein in the presence of increasing concentrations of each receptor antagonist, and the incubation continued for a further 20 min at 0°C. The reactions were stopped by the addition of an equal volume of loading buffer containing 50 mM dithiothreitol, and the samples were boiled for 5 min before being separated on a 10% sodium dodecyl sulphate-polyacrylamide gel. The protein bands were visualised by exposing the gels to phosphorimager plates (Fuji-Bas) or X-ray film.

2.4. Curve fitting

Estimates of IC_{50} values, and of the levels of [^{125}I]-[Nle^{15}]gastrin-(2–17) bound in the absence of competitor, were obtained with the program SIGMASTAT (Jandel Scientific, San Rafael, CA) by nonlinear regression to the equation $y = a/(1 + x/b)$, where y is the amount of iodinated gastrin bound expressed as a percentage of the value a observed in the absence of binding inhibitor, x is the concentration of binding inhibitor, and b is the IC_{50} value.

2.5. Fatty acid oxidation assays

Long chain fatty acid oxidation was measured as described previously (Yang et al., 1998). In brief, normal human skin fibroblasts (10^4 per well) were seeded in 96 well plates in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, with and without each receptor antagonist (50 μM). After 20 h the cells were washed with phosphate-buffered saline before [9,10 (n)-^{3}H] palmitic acid was added, and the plates were incubated for a further 3 h. The supernatant was passed through an AG1-X8 anion exchange column to remove unreacted palmitic acid, and the tritiated water released was quantified by scintillation counting of the percolate. The cells were lysed in 2 M NaOH and the protein concentrations in the lysate were determined by the Bradford assay (Reed and Northcote, 1981).

3. Results

3.1. Affinity of benzotript derivatives for the gastrin-binding protein

The most potent inhibitor of the binding of gastrin to the gastrin-binding protein reported to date is the non-selective CCK receptor antagonist benzotript (*N*-4-chlorobenzoyl-L-tryptophan) (Fig. 1) (Baldwin, 1994). In order to determine what effect alteration of the substituents on the benzoyl and tryptophan ring systems had on binding, several benzotript derivatives were tested for their ability to inhibit the cross-linking of [^{125}I]gastrin to the gastrin-bi-

Table 2
Comparison of affinities of benzotript analogues for the gastrin-binding protein
[^{125}I]–[Nle^{15}]gastrin-(2–17) was cross-linked to the porcine gastrin-binding protein with disuccinimidylsuberate in the absence (control) or presence of the indicated analogues of benzotript (*N*-4-chlorobenzoyl-L-tryptophan) at a concentration of 200 μM as previously described (Baldwin, 1994; Baldwin et al., 1986). The analogues differed in the substituents on the tryptophan amino group, and in the stereochemistry of tryptophan, as indicated. Reaction products were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the radioactivity associated with the gastrin-binding protein in the presence of a given analogue was detected and quantified with a phosphorimager, and expressed as a percentage of the radioactivity associated with the gastrin-binding protein in the absence of the analogue. The IC_{50} value for CR 665 (mean ± standard error, $n = 3$) was obtained as described in the Fig. 2 legend.

Compound	Substituent	Isomer	% Control at 200 μM	IC_{50} value (μM)
Benzotript	4-chlorobenzoyl	L	50	200
CR 449	4-[(chlorophenyl)methoxy]carbonyl	L	82.5 ± 4.5	
CR 505	4-trifluoromethyl	L	77.0 ± 1.0	
CR 512	4-cyanobenzoyl	L	88.5 ± 0.5	
CR 517	(diphenylmethoxy)carbonyl	L	84.5 ± 3.5	
CR 546	3-chlorobenzoyl	L	77.0 ± 2.0	
CR 617	2-chlorobenzoyl	L	129.5 ± 1.5	
CR 665	4-chlorobenzoyl	D	78.5 ± 2.5	450 ± 90
CR 1436	3,4-dichlorobenzoyl	L	102.0 ± 2.0	
CR 1955	3,5-dimethylbenzoyl	D,L	118.0 ± 5.0	
CR 2850	3,5-dichlorobenzoyl	D,L	82.5 ± 6.5	

nding protein (Table 2). None of the 10 compounds tested inhibited crosslinking by more than 30% at a concentration of 200 μM (Table 2). Compound CR 665 (*N*-4-chlorobenzoyl-D-tryptophan, the D-isomer of benzotript) was selected for more detailed study of its interaction with the gastrin-binding protein. The IC_{50} value of 450 μM determined by computer fitting of full titration curves was similar to the IC_{50} value of 200 μM reported previously for benzotript (Baldwin, 1994).

3.2. Affinity of CCK receptor antagonists for the gastrin-binding protein

In order to explore the possibility that other CCK receptor antagonists might be more potent inhibitors of the binding of gastrin to the gastrin-binding protein than benzotript, several CCK_1- and CCK_2-selective agonists and antagonists (Fig. 1) were tested for their ability to inhibit the cross-linking of [^{125}I]gastrin to the gastrin-binding protein (Fig. 2). Most of the agonists and antagonists bound to the gastrin-binding protein, with affinities which ranged from 20 μM for PD 149164 (benzenebutanoic acid, 4-fluoro-!b/-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo-[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-, [*R*-(*R**,*S**)]-), to 4.0 mM for CR 1409 (D,L-4-(3,4-dichlorobenzoylamino)-5-(di-*N*-pentyl amino)-5-oxo-pentanoic acid) (Table 3). No compound was observed to bind to the gastrin-binding protein with an affinity equal to or higher than its affinity for either the CCK_1 or CCK_2 receptor.

3.3. CCK_1 and CCK_2 receptor selective antagonists do not inhibit fatty acid oxidation

Oxidation of long chain fatty acids by human fibroblasts was assayed in the presence and absence of each receptor antagonist at a concentration of 50 μM, by measuring the release of tritiated water from [^{3}H]palmitic acid. The antagonists PD 134308 (Butanoic acid, 4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo-[3,3,1,1,^{3,7}]dec-2-yloxy)carbonyl]amino]propyl]amino]-1-phenylethyl]amino]-4-oxo-, [*R*-(*R**,*R**)]-, *N*-methyl-D-glucamine salt) and PD 142898 (!b/-Alanine, *N*-[!a/-methyl-*N*-[[(2-methylcy-

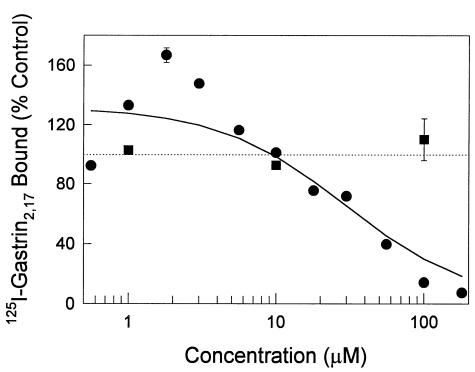

Fig. 2. Binding of CCK receptor antagonists to the gastrin-binding protein. [^{125}I]–[Nle^{15}]gastrin-(2–17) was cross-linked to the porcine gastrin-binding protein with disuccinimidylsuberate in the absence (control) or presence of increasing concentrations of the CCK receptor antagonist PD 149164 (circles) and its ethyl ester PD 149650 (squares) as previously described (Baldwin, 1994; Baldwin et al., 1986). Reaction products were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the radioactivity associated with the gastrin-binding protein was detected and quantified with a phosphorimager. No inhibition was observed with PD 149650. The IC_{50} value for PD 149164 (29 μM), and the level of [^{125}I]–[Nle^{15}]gastrin-(2–17) bound in the absence of competitor (132%), were estimated with the program SIGMASTAT (Jandel Scientific, San Rafael, CA) by nonlinear regression to the equation $y = a/(1 + x/b)$, where y is the amount of iodinated gastrin bound expressed as a percentage of the value a observed in the absence of binding inhibitor, x is the concentration of binding inhibitor, and b is the IC_{50} value. This IC_{50} value was combined with the values obtained in two other experiments to obtain the mean values presented in Table 3.

Table 3
Comparison of affinities of CCK receptor antagonists for the CCK_1 and CCK_2 receptors and for the gastrin-binding protein
The structures of the antagonists PD 149164, SR 27897B, LY 288513 and RP 73870A are shown in Fig. 1 and the names of all compounds tested in this study are given in Table 1. IC_{50} values for the porcine protein were determined by competition for covalent cross-linking of [^{125}I]–[Nle^{15}]gastrin-(2–17) to the protein with disuccinimidylsuberate as described in the Fig. 2 legend. IC_{50} values for the CCK_1 and CCK_2 receptors were determined by competition for [^{125}I]CCK-8 or [^{125}I]CCK-33 binding to membranes from pancreas (CCK_1 receptor) or cerebral cortex (CCK_2 receptor), or to intact COS or CHO cells transfected with cDNAs encoding the human gallbladder CCK_1 receptor (Ulrich et al, 1993) or the human brain CCK_2 receptor (Pisegna et al., 1992). PD 134308 and PD 149164 act as agonists at rat CCK_1 receptors (Hughes et al., 1996). Values are for the human receptors unless indicated as follows: [a]guinea pig; [b]rat; [c]mouse. Manufacturer's names are abbreviated as follows: MSD, Merck, Sharp and Dohme; RPR, Rhone-Poulenc Rorer. References are as follows: 1. Baldwin et al., 1986; 2. Ulrich et al., 1993; 3. Pisegna et al., 1992; 4. Lin et al., 1989; 5. Lin et al., 1990; 6. Baldwin, 1994; 7. Hahne et al., 1981; 8. Chang et al., 1985; 9. Boden et al., 1993; 10. Lotti and Chang, 1989; 11. Gully et al., 1993; 12. Hughes et al., 1990; 13. Hughes et al., 1996; 14. Rasmussen et al., 1993; 15. Pendley et al., 1995.

	IC_{50} for binding (μM)			Ref.
	Gastrin-binding protein	CCK_1 receptor	CCK_2 receptor	
Agonists				
$Gastrin_{17}$	0.2	0.08	0.0064	1, 2, 3
A57,696	20 ± 10	14[a]	0.0027[a]	4
A71,378	80 ± 90	4.2×10^{-4}[a]	1.2[a]	5
Non-selective CCK receptor antagonists				
Proglumide	5,100	600[b]	875[a]	6, 7, 8
Benzotript	200	102[b]	84[a]	6, 7, 8
PD 142898	140 ± 80	0.0039	0.0042	9
CCK_1 receptor-selective antagonists				
CR 1409	4,000 ± 1,500	0.0022[a]	2.2[a]	10
L364,718	> 200	0.0001	0.5	2, 3, 6
SR 27897B	250 ± 110	0.0006[b]	0.49[a]	11
CCK_2 receptor-selective antagonists				
PD 132461	> 10,000	0.780	0.0063	9
PD 134308	30 ± 10	2.7[b]	0.0017[c]	12
PD 149164	20 ± 4	0.071	8.4×10^{-5}	13
PD 149650	> 10,000			
L365,260	> 200	9	0.01	2, 3, 6
LY 288513	610 ± 50	> 30	0.016	14
RP 73870A	280 ± 35	1.6[a]	0.00048[a]	15

clohexyl)oxy]carbonyl]-D-tryptophyl]-L-3-(phenylmethyl)-, (1S-trans)-, compd. 1-deoxy-1-(methylamino)-D-glucitol (1:1)) had no effect on long chain fatty acid oxidation compared with the control where no antagonist was added (Fig. 3). Because a slight inhibition was observed in the presence of PD 149164, its ethyl ester (PD 149650, Benzenebutanoic acid, 4-fluoro-!b/-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2-[[(tricyclo-[3.3.1.1^{3,7}]dec-2-yloxy)carbonyl]amino]propyl] amino]-, ethyl ester, [*R*-(*R**,*S**)]-)) was also assayed to determine whether or not passage across the cell membrane was limiting the extent of inhibition observed. Inhibition by PD 149650 was indeed greater than inhibition by PD 149164, even though PD 149650 did not appear to bind to the isolated gastrin-binding protein (Table 3). Attempts to assay PD 149650 at higher concentrations were prevented by precipitation of the antagonist in the aqueous medium. The other CCK receptor antagonists tested slightly increased long chain fatty acid oxidation compared to the control.

Neither PD 149164 nor PD 149650 in the concentration range 6–60 μM had any significant effect on proliferation of the human colorectal carcinoma cell line LIM 1215 (data not shown). Higher concentrations could not be tested because of precipitation of the compounds in the tissue culture medium over the 24 h assay period.

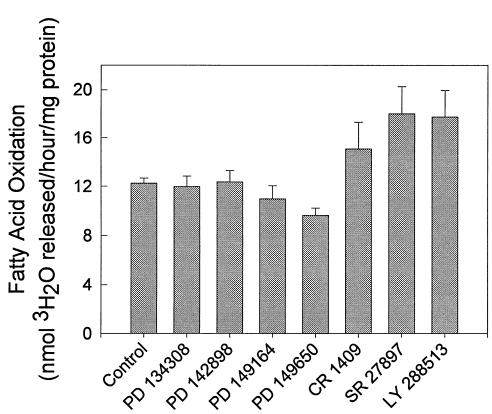

Fig. 3. Effect of CCK receptor antagonists on long chain fatty acid oxidation. Oxidation of the long chain fatty acid palmitate by normal human fibroblasts in the absence and presence of the indicated CCK receptor antagonists (50 μM) was measured as described in the Methods section. Values are the mean ± standard error of quadruplicates.

4. Discussion

The results reported here indicate that the CCK_2 receptor antagonist PD 149164 also inhibits binding of gastrin to the gastrin-binding protein. The IC_{50} value of 20 μM is 10-fold higher than the IC_{50} value of the most potent inhibitor of the binding of gastrin to the gastrin-binding protein known previously. Most other antagonists selective for either the CCK_1 or CCK_2 receptors also bind to the gastrin-binding protein (Table 3). This observation is not unexpected since all three proteins do bind gastrin, albeit with different affinities. Thus the IC_{50} values for the binding of gastrin to the CCK_1 and CCK_2 receptors are 80 nM and 6.4 nM, respectively, while the gastrin-binding protein binds gastrin with a lower affinity of 200 nM (Shulkes and Baldwin, 1997). In no case was the affinity of the antagonist for the gastrin-binding protein equal to or higher than the affinity of the antagonist for either the CCK_1 or CCK_2 receptor. Presumably the lack of correlation between the affinities of the antagonists for the CCK_1 or CCK_2 receptor, and for the gastrin-binding protein, reflects the fact that the receptors and the gastrin-binding protein belong to structurally very diverse families.

Further information about the binding of inhibitors to the gastrin-binding protein can be obtained from comparison of the binding of benzotript analogues (Table 2). The observation that the L- and D-isomers of benzotript bound with similar affinity indicates that interchanging two of the four groups surrounding the chiral carbon has no effect on binding, and hence implies that only two of the groups are bound by the enzyme. Since alteration of the position and nature of the substituent on the benzoyl group did not necessarily result in loss of binding (Table 2), the chlorine atom in the 4-position on the benzoyl group is unlikely to contribute significantly to binding to the enzyme. Further work will be required to determine the relative contributions of the benzoyl, indole and carboxyl substituents to inhibitor binding.

The effects of the antagonists on fatty acid oxidation in intact fibroblasts were also tested. Although PD 134308 and PD 142898 had no effect on fatty acid oxidation compared with the control, some inhibition was observed in the presence of PD 149164. The fact that three antagonists which bound to the isolated gastrin-binding protein with relatively high affinity had little effect on fatty acid oxidation in whole cells at concentrations higher than the IC_{50} values observed in vitro may be due to the compounds' failure to penetrate the fibroblast cell membrane. This explanation is supported by the observation that greater inhibition of fibroblast fatty acid oxidation is observed in the presence of PD 149650, which is the ethyl ester of PD 149164, than in the presence of PD 149164 itself. Presumably the greater lipid solubility of PD 149650 allows more of the drug to reach the cell interior. Once inside the cell an esterase may remove the ethyl group to generate PD 149164, which might then bind to and inhibit the α-subunit of the mitochondrial trifunctional protein. The reason for the slight stimulation of long chain fatty acid oxidation observed in fibroblasts treated with the other CCK receptor antagonists shown in (Fig. 3) is not clear at present.

In conclusion, the present study has demonstrated that the CCK_2 receptor antagonist PD 149164 also binds to the gastrin-binding protein. The observed IC_{50} value of 20 μM is 10-fold higher than the value for the most potent binding inhibitor available previously. Most of the other CCK receptor antagonists tested also bound to the gastrin-binding protein, but in no case was the affinity equal to or greater than the affinity of the compound for either the preferred CCK_1 or CCK_2 receptor. The findings of the fatty acid oxidation assays were not conclusive, and did not provide evidence for a role of the antagonists in inhibiting the putative fatty acid oxidation function of the gastrin-binding protein. More specific binding inhibitors need to be designed so that a clearer picture is obtained of the physiological role of the gastrin binding protein.

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