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European Journal of Pharmacology 302 (1996) 141–151

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Synergistic interactions between human transfected adenosine A_1 receptors and endogenous cholecystokinin receptors in CHO cells

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Received 17 August 1995; revised 7 December 1995; accepted 12 January 1996

Abstract

The effect of G_i coupled receptor activation (adenosine A_1 and 5-HT_{1B} receptors) on cholecystokinin receptor-stimulated inositol phosphate accumulation has been investigated in Chinese hamster ovary cells transfected with the human adenosine A_1 receptor cDNA (CHO-A1). CHO cells constitutively express the 5-HT_{1B} receptor [Berg, Clarke, Sailstad, Saltzman and Maayani (1994) Mol. Pharmacol. 46, 477–484]. Our previous studies using CHO-A1 cells have revealed that both the adenosine A_1 and 5-HT_{1B} receptor are negatively coupled to adenylyl cyclase activity and stimulate increases in $[Ca^{2+}]_i$ through a pertussis toxin-sensitive pathway. In the present study the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine stimulated a pertussis toxin-sensitive increase in total [^{3}H]inositol phosphate accumulation. The sulphated C-terminal octapeptide of cholecystokinin (CCK-8) stimulated a robust and pertussis toxin-insensitive increase in [^{3}H]inositol phosphate accumulation through the activation of CCK_A receptors. Co-stimulation of CHO-A1 cells with N^6-cyclopentyladenosine and CCK-8 produced a synergistic increase in [^{3}H]inositol phosphate accumulation. The synergistic interaction between N^6-cyclopentyladenosine and CCK-8 was abolished in pertussis toxin-treated cells. Synergy between N^6-cyclopentyladenosine and CCK-8 still occurred in the absence of extracellular calcium. The 5-HT_{1B} receptor agonist 5-carboxyamidotryptamine did not stimulate a measurable increase in [^{3}H]inositol phosphate accumulation. Furthermore, 5-carboxyamidotryptamine had no significant effect on CCK-8 mediated [^{3}H]inositol phosphate production. Activation of endogenous P_{2U} receptors (G_q/G_{11} coupled) with ATPγS produced a significant increase in [^{3}H]inositol phosphate accumulation. Co-stimulation of CHO-A1 cells with ATPγS and CCK-8 produced additive increases in [^{3}H]inositol phosphate accumulation. These data indicate that CHO-A1 cells may prove a useful model system in which to investigate further the mechanisms underlying the intracellular 'cross-talk' between phospholipase C coupled receptors (G_q/G_{11} linked) and G_i/G_o coupled receptors.

Keywords: CCK_A receptor; Adenosine A_1 receptor; 5-HT_{1B} receptor; P_{2U} purinoceptor; Inositol phosphate; CHO (Chinese hamster ovary) cell

1. Introduction

In the smooth muscle cell line, DDT_1MF-2, activation of the endogenous adenosine A_1 receptor leads to the inhibition of adenylyl cyclase activity (Ramkumar et al., 1990) and the stimulation of inositol phospholipid hydrolysis and Ca^{2+} mobilisation (Gerwins and Fredholm, 1992a, b; White et al., 1992; Dickenson and Hill, 1993a). The inhibition of adenylyl cyclase and the apparent direct coupling to phospholipase C are inhibited by pertussis toxin suggesting the involvement of G_i/G_o protein(s) in both signalling pathways. Furthermore, in DDT_1MF-2 cells the adenosine A_1 receptor synergistically interacts with several receptors which are coupled to phospholipase C through pertussis toxin-insensitive G protein(s), e.g. histamine H_1 receptor (Dickenson and Hill, 1993b), bradykinin and ATP (Gerwins and Fredholm, 1992a; Gerwins and Fredholm, 1992b) and α_{1B}-adrenoceptors (Dickenson, 1994).

Adenosine A_1 receptor agonists in FRTL-5 thyroid cells and RINm5F cells also potentiate inositol phospholipid hydrolysis and/or Ca^{2+} mobilisation elicited by a range of phospholipase C coupled receptors (Okajima et al., 1989a, b; Nazarea et al., 1991; Sho et al., 1991; Biden and Browne, 1993). Furthermore, in NG108-15 cells δ-opioid receptor activation (which again is a G_i/G_o coupled receptor) enhanced P_2 receptor and bradykinin induced $Ins(1,4,5)P_3$ formation (Okajima et al., 1993). In the above examples, adenosine A_1 receptor activation on its own had no effect on inositol phospholipid hydrolysis and/or Ca^{2+} mobilisation.

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PII S0014-2999(96)00039-8

Interactions between receptors coupled negatively to adenylyl cyclase (linked to pertussis toxin-sensitive G_i proteins) and receptors coupled to phospholipase C (linked to pertussis toxin-insensitive G_q/G_{11} proteins) are clearly prevalent in a variety of cell types. However, the precise mechanism underlying G_i/G_o coupled receptor augmentation of phospholipase C activity remains unclear.

Studies using Chinese hamster ovary cells transfected with the human brain adenosine A_1 receptor (CHO-A1 cells) have revealed that the A_1 receptor can inhibit forskolin stimulated cAMP production and stimulate the mobilisation of intracellular Ca^{2+} through pertussis toxin-sensitive mechanisms (Townsend-Nicholson and Shine, 1992; Iredale et al., 1994). In addition, CHO cells endogenously express a 5-hydroxytryptamine $(5\text{-HT})_{1B}$-like receptor that is negatively coupled to adenylyl cyclase activity through a pertussis toxin-sensitive pathway (Berg et al., 1994; Giles et al., 1994). Furthermore, we have recently shown that 5-HT_{1B} receptor activation in CHO-A1 cells also stimulates a pertussis toxin-sensitive increase in $[Ca^{2+}]_i$, suggesting a possible coupling to phospholipase C (Dickenson and Hill, 1995). It is important to note that the human transfected adenosine A_1 receptor is expressed at moderate levels (200 fmol/ mg of protein (Iredale et al., 1994)), whereas the endogenous 5-HT_{1B} receptor is expressed at levels that are not detectable using [^{3}H]5-HT or [^{125}I]cyanopindolol binding (Giles et al., 1994).

CHO cells also contain an endogenous cholecystokinin receptor linked to inositol phospholipid hydrolysis (Freund et al., 1994). Cholecystokinin receptors are classified into two major subtypes, CCK_A and CCK_B, both of which couple to phospholipase C through pertussis toxin-insensitive G_q/G_{11} proteins (De Weerth et al., 1993). Therefore, the aim of this investigation was to study the possible regulation of cholecystokinin receptor stimulated [^{3}H]inositol phosphate accumulation by co-activation of G_i coupled receptors in CHO-A1 cells, i.e. the human transfected adenosine A_1 receptor and the endogenous 5-HT_{1B} receptor.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary cells (CHO-K1) transfected with the human brain adenosine A_1 receptor sequence (CHO-A1) were a generous gift from Dr. Andrea Townsend-Nicholson and Professor John Shine, Garvan Institute, Sydney, Australia. Untransfected CHO-K1 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, UK). CHO cells were cultured in 75 cm^2 flasks (Costar) in Dulbecco's modified Eagles medium/nutrient F12 (1 : 1) supplemented with 2 mM L-glutamine and 10% (v/v) foetal calf serum. Cells were maintained at 37°C in a humidified 10% CO_2 atmosphere until confluency and were subcultured (1 : 5 split ratio) using trypsin (0.05% w/v)/EDTA (0.02% w/v). Cells for [^{3}H]inositol phosphate determinations were grown in 24 well cluster dishes (Costar).

2.2. Inositol phospholipid hydrolysis

At confluence, cell monolayers were loaded for 24 h *myo*-[^{3}H]inositol (37 kBq/well) in 24 well cluster dishes in inositol-free DMEM containing 0.1% foetal calf serum. Pre-labelled cells were then washed once with 1 ml/well Hanks/Hepes buffer pH 7.4 and incubated at 37°C for 30 min in the presence of 20 mM LiCl (290 μl/well). Where appropriate antagonists were added at the beginning of this incubation period. Agonists were then added in 10 μl of medium and the incubation continued for 40 min (unless otherwise stated) at 37°C. Incubations were terminated by aspiration of the incubation medium and the addition of 900 μl cold (−20°C) methanol/0.12 M HCl (1 : 1, v/v). Cells were left a minimum of 2 h at −20°C before isolation of total [^{3}H]inositol phosphates in the supernatant of the disrupted cell monolayers by anion exchange chromatography. 800 μl aliquots of the supernatant were neutralised by the addition of 135 μl 0.5 M NaOH, 1 ml 25 mM Tris. HCl (pH 7.0) and 3.1 ml distilled water and added to columns of Dowex 1 anion exchange resin (X8, 100–200 mesh, chloride form). [^{3}H]inositol and [^{3}H]glycerophosphoinositol were removed with 20 ml of distilled water and 10 ml 25 mM ammonium formate respectively. Total [^{3}H]inositol phosphates were then eluted with 3 ml of 1 M HCl and the columns regenerated with 10 ml 1 M HCl followed by 20 ml distilled water. Radioactivity was quantified by scintillation counting in the gel phase (Scintillator plus, Packard).

2.3. Measurement of inositol 1,4,5-triphosphate formation

CHO-A1 cells were grown in 24 well cluster dishes and when confluent washed once with 200 μl/well Hanks/Hepes buffer, pH 7.4 and allowed to stabilise for 5 min at 37°C. The buffer was then replaced with 100 μl of Hanks/Hepes buffer with or without the appropriate agonists for the indicated time periods. The reaction was terminated by the addition of 33 μl 7.5% (w/v) perchloric acid and placed on ice for 5 min. Samples were then neutralised with 66 μl 1.2 M $KHCO_3$ and frozen at −20°C until required. $Ins(1,4,5)P_3$ concentration was determined using the radioreceptor assay described by Challis et al. (1988). Assays were performed for 30 min at 4°C in a total volume of 120 μl which consisted of 30 μl of 100 mM Tris-HCl, 4 mM EGTA, pH 8.0; 30 μl of [2-^{3}H]inositol 1,4,5-triphosphate (diluted in distilled water to give 5000–6000 dpm/assay tube); 30 μl of standard $Ins(1,4,5)P_3$ concentrations (0.036–36 pmol $Ins(1,4,5)P_3$/assay) or 30 μl of prepared sample and 30 μl of bovine adrenal cortical $Ins(1,4,5)P_3$ binding protein. The assay

Table 1
Synergistic interactions between adenosine A_1 and CCK_A receptor stimulated [^{3}H]inositol phosphate production in CHO-A1 cells

Agonist 1 Concentration response curve	Agonist 2 Fixed concentration	Incubation time (min)	CCK-8 (1 μM)	CPA (1 μM)	Predicted additive response	Observed response CCK-8 and CPA	Agonist 1 EC_{50} (nM)	Agonist 1 EC_{50} (nM) in the presence of agonist 2	*n*
CCK-8	CPA (1 μM)	40	5.5±0.7	1.60±0.10	6.1±0.6	9.5±0.9 [a]	18± 5.0	11± 1.0	5
CPA	CCK-8 (1 μM)	40	5.8±0.8	1.70±0.10	6.5±0.6	9.3±0.5 [a]	67±18	37±11	4
[b] CCK-8	CPA (1 μM)	40	3.3±0.2	1.80±0.08	4.1±0.1	8.1±0.7 [a]	29±15	10± 0.1	3
[b] CPA	CCK-8 (1 μM)	40	2.2±0.3	1.50±0.02	2.7±0.3	4.9±0.5 [a]	20± 9	21± 3	3
CCK-8	CPA (1 μM)	5	2.2±0.1	1.30±0.01	2.5±0.1	4.7±0.2 [a]	13± 1.0	30±12	3

Concentration response curves to agonist 1 were obtained in the absence or presence of a fixed concentration of agonist 2. Fold increases over basal levels in [^{3}H]inositol phosphate production were determined for CCK-8 (1 μM) and N^6-cyclopentyladenosine (CPA; 1 μM) alone. Statistical analysis (Student's unpaired *t* test) was performed by comparing the fold increase in [^{3}H]inositol phosphates obtained from the simultaneous addition of CCK-8 (1 μM) and N^6-cyclopentyladenosine (CPA; 1 μM) with the predicted fold increase in [^{3}H]inositol phosphates determined by simply adding together the responses of CCK-8 and N^6-cyclopentyladenosine (CPA) alone. There was no significant difference between the EC_{50} values obtained for agonist 1 in the absence or presence of agonist 2. Values represent means ± S.E.M. of *n* experiments.

[a] Statistically significant ($P < 0.05$) from the predicted additive fold increase in [^{3}H]inositol phosphates.

[b] Experiments performed in calcium-free media containing 0.1 mM EDTA.

was stopped by the addition of 3 ml of ice-cold 25 mM Tris-HCl, 1 mM EGTA, pH 8.0 to the assay tube, rapid filtration through Whatman GF/B filters, followed by two 3 ml washes of the assay tube with ice-cold 25 mM Tris-HCl, 1 mM EGTA, pH 8.0. Filters were transferred to scintillation vial inserts and 4 ml of Emulsifier-Safe scintillator (Packard) added. The filters were left at room temperature over night before liquid-scintillation counting.

2.4. Data analysis

EC_{50} and IC_{50} (concentrations of drug producing 50% of the maximal stimulation or inhibition) values were obtained by computer assisted curve fitting by use of the computer programme InPlot (GraphPAD, California, USA). Statistical significance was determined by Student's unpaired t test ($P < 0.05$ was considered statistically significant). All data are presented as mean ± S.E.M. The n in the text refers to the number of separate experiments.

Statistical analysis of the synergistic interactions between adenosine A_1 and cholecystokinin receptor co-activation were performed as follows. Concentration response curves to CCK-8 (first agonist) were generated in the absence and presence of a fixed concentration of N^6-cyclopentyladenosine (1 μM; second agonist; see Fig. 2A and Table 1). A curve representing the predicted additive response was subsequently generated by adding the response to the fixed concentration of the second agonist (in this case N^6-cyclopentyladenosine) to that obtained with each concentration of first agonist alone (in this case CCK-8; see Fig. 2A). Fold over basal increases in [^{3}H]inositol phosphate accumulation were determined for the maximally effective concentrations of CCK-8 (1 μM) and N^6-cyclopentyladenosine (1 μM). Statistical analysis (Student's unpaired t test) was performed by comparing the predicted additive fold increases in [^{3}H]inositol phosphates (achieved by adding together the responses obtained for 1 μM CCK-8 and 1 μM N^6-cyclopentyladenosine alone; see Table 1) with the fold increases in [^{3}H]inositol phosphate obtained from the simultaneous addition of CCK-8 (1 μM) and N^6-cyclopentyladenosine (1 μM).

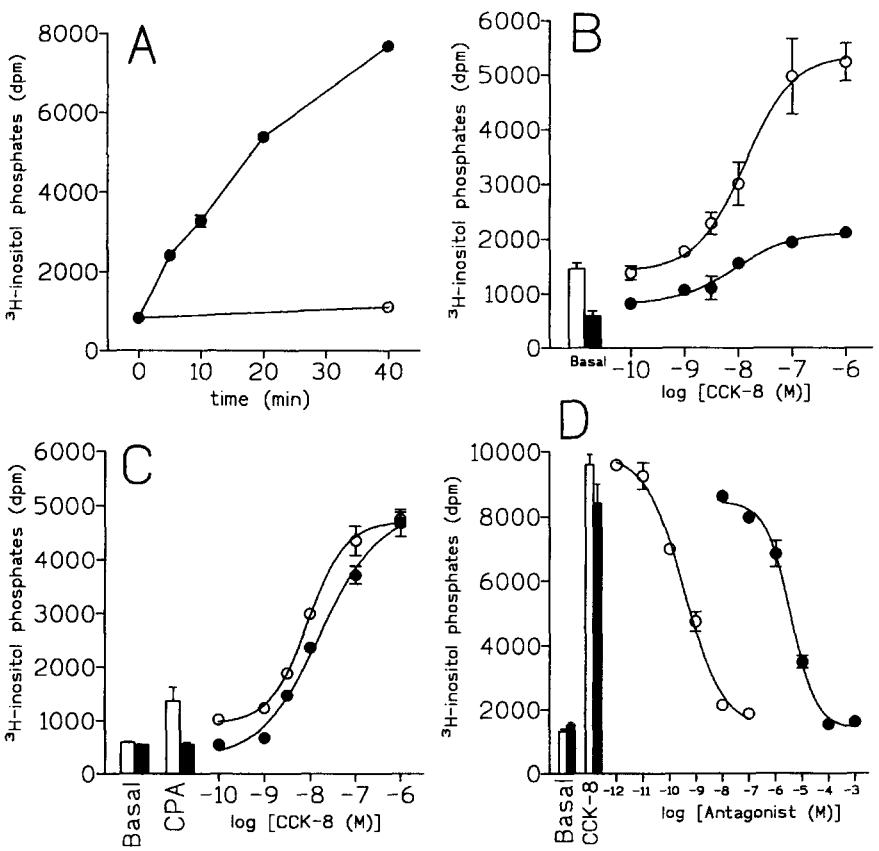

Fig. 1. Effect of the sulphated C-terminal octapeptide of cholecystokinin (CCK-8) on total [^{3}H]inositol phosphate accumulation in CHO-A1 cells. A: time course for the accumulation of total [^{3}H]inositol phosphate in response to 1 μM CCK-8 (●). The basal accumulation of [^{3}H]inositol phosphates after 40 min is also shown (○). B: concentration response curves to CCK-8 in the presence (○) and absence (●) of extracellular calcium. C: concentration response curves to CCK-8 in control cells (○) and cells pre-treated for 24 h with 100 ng/ml pertussis toxin (●). In these experiments the response to N^6-cyclopentyladenosine (CPA; 1 μM) was completely abolished by pertussis toxin. D: inhibition of CCK-8 (100 nM) stimulated [^{3}H]inositol phosphate production by devazepide (○) and PD-135,158 (●). Values represent of mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in at least two further experiments.

2.5. Chemicals

Myo-[2-^{3}H]inositol and [2-^{3}H]inositol 1,4,5-triphosphate were from New England Nuclear. The sulphated C-terminal octapeptide of cholecystokinin (CCK-8), fura-2 acetoxy methyl ester and ionomycin were from Calbiochem/Novobiochem (Nottingham, UK). Adenosine 5′-*O*-(3-thiotriphosphate) and N^6-cyclopentyladenosine were obtained from Boehringer and Sigma Chemical Co. (Poole, Dorset, UK) respectively. Inositol 1,4,5-triphosphate, 5-carboxyamidotryptamine, lorglumide ((±)-4-[3,4-dichlorobenzoylamino]-5-(dipentylamino)-5-oxopentanoate) and PD 135,158 ([1*S*-[1α,2β[*S**(*S**)]]-4-[[2-[[3-(1*H*-indol-3-yl)-2-methyl-1-oxo-2[[[1,7,7-trimethylbicyclo[2.2.1]hept-2-yl)oxy]carbonyl]-amino]-propyl]amino]-1-phenylethyl]-amino]-4-oxo-butanoic acid *N*-methyl-D-glucamine salt) were purchased from Research Biochemicals International. Pertussis toxin was obtained from Porton Products. The kind gift of devazepide (L-364,718; (S)-*N*-(2,3-dihydro-1-methyl-2-oxo-5-phenyl-1*H*–1,4-benzodiazepin-3-yl)-1*H*-indole-2-carboxamide) from Merck Sharp and Dohme is gratefully acknowledged. Dulbecco's modified Eagles medium/nutrient mix F-12 (1 : 1) and foetal calf serum were from Sigma Chemical Co. (Poole, Dorset, UK). All other chemicals were of analytical grade.

3. Results

3.1. Cholecystokinin stimulated [^{3}H]inositol phosphate accumulation in CHO-A1 cells

The sulphated C-terminal octapeptide of cholecystokinin (CCK-8; 1 μM) elicited a significant increase in the accumulation of total [^{3}H]inositol phosphates in CHO-A1 cells in the presence of 20 mM LiCl (6.3 ± 0.6 fold increase over basal levels; $n = 9$; $P < 0.05$).The accumulation of [^{3}H]inositol phosphates in response to CCK-8 stimulation was essentially linear during the 40 min incubation period (Fig. 1A). The response to CCK-8 (40 min incubation) was concentration-dependent with an EC_{50} value of 13 ± 3 nM ($n = 9$; Fig. 1B). In the absence of extracellular Ca^{2+} (experiments performed in nominally calcium-free Hanks/Hepes buffer containing 0.1 mM EGTA) the maximal [^{3}H]inositol phosphate accumulation in response to 1 μM CCK-8 was significantly reduced (51 ± 5% of that obtained in the presence of 2 mM calcium; $n = 6$; Fig. 1B). There was no significant change in the EC_{50} value for CCK-8 in the absence of extracellular calcium (25 ± 10 nM; $n = 6$). These data suggest that Ca^{2+}-dependent activation of phospholipase C contributes significantly to the total [^{3}H]inositol phosphate response produced by CCK-8.

Pre-treatment of cells with pertussis toxin (100 ng/ml for 24 h) had no significant effect on CCK-8 stimulated [^{3}H]inositol phosphate accumulation (Fig. 1C). This concentration of pertussis toxin and length of pre-treatment is sufficient to uncouple transfected muscarinic M_2 acetylcholine receptors from endogenous $G\alpha_{i2}$ and $G\alpha_{i3}$ proteins in CHO cells (Hunt et al., 1994). In pertussis toxin treated cells 1 μM CCK-8 elicited a 6.0 ± 0.9 ($n = 4$) fold increase in [^{3}H]inositol phosphates with an EC_{50} of 14 ± 2 nM ($n = 4$). These data suggest that CCK-8 stimulated increases in [^{3}H]inositol phosphates are mediated through a pertussis toxin-insensitive G_q/G_{11} protein. The pharmacological characterisation of the cholecystokinin receptor subtype mediating increases in [^{3}H]inositol phosphates in CHO-A1 cells was investigated using the selective CCK_A receptor antagonists devazepide and lorglumide and the CCK_B receptor antagonist PD 135,158. Antagonist inhibition concentration response curves were obtained in the presence of a fixed concentration of CCK-8 (100 nM). The inhibition curves for devazepide ($IC_{50} = 0.26 \pm 0.06$ nM; $n = 3$) and PD 135,158 ($IC_{50} = 4.8 \pm 0.5$ μM; $n = 4$) are shown in Fig. 1D. Lorglumide also potently inhibited CCK-8 stimulated [^{3}H]inositol phosphate accumulation with an IC_{50} of 58 ± 6 nM ($n = 4$). These data suggest the CCK_A receptor subtype mediates CCK-8 stimulated increases in [^{3}H]inositol phosphates in CHO-A1 cells.

3.2. Interactions between CCK_A and adenosine A_1 receptors in CHO-A1 cells

Previous studies using CHO cells transfected with human brain adenosine A_1 receptor cDNA (CHO-A1 cells) have shown that the transfected A_1 receptor can inhibit adenylyl cyclase activity and stimulate the mobilisation of intracellular calcium (Townsend-Nicholson and Shine, 1992; Iredale et al., 1994). The inhibition of adenylyl cyclase and the apparent direct coupling to phospholipase C are inhibited by pertussis toxin suggesting the involvement of G_i/G_o protein(s) in both signalling pathways. In the current study the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (1 μM) produced a small but significant 1.6 ± 0.1 fold increase in [^{3}H]inositol phosphates in CHO-A1 cells ($n = 9$; $P < 0.05$) which was completely inhibited by pertussis toxin ($n = 7$; see Fig. 1C and Fig. 4B). Fig. 2A shows the effect of N^6-cyclopentyladenosine (1 μM) on the concentration response curve for CCK-8 stimulated [^{3}H]inositol phosphate formation in CHO-A1 cells. Clearly, co-activation of CHO-A1 cells with N^6-cyclopentyladenosine and CCK-8 produced a synergistic increase in total [^{3}H]inositol phosphate accumulation. For example, in these experiments CCK-8 (1 μM) and N^6-cyclopentyladenosine (1 μM) alone produced increases in [^{3}H]inositol phosphates of 5.5 ± 0.7 ($n = 5$) and 1.6 ± 0.1 ($n = 5$) fold, respectively. A combination of N^6-cyclopentyladenosine (1 μM) and CCK-8 (1 μM) produced a 9.5 ± 0.9 ($n = 5$) fold increase in [^{3}H]inositol phosphates which is significantly greater than the predicted additive response of 6.1 ± 0.6 fold ($n = 5$). N^6-cyclopentyladenosine had no significant effect on the EC_{50} value for CCK-8 stimulated [^{3}H]inositol phosphate produc-

tion (see Table 1). Fig. 2B shows a concentration response curve for N^6-cyclopentyladenosine stimulated [^{3}H]inositol phosphate accumulation in the absence of CCK-8, and in the presence of a fixed concentration of CCK-8 (1 μM). The data from these experiments are summarised in Table 1. The potentiation of CCK-8 stimulated [^{3}H]inositol phosphate production by N^6-cyclopentyladenosine (1 μM) was also observed when the agonist incubation time was reduced from 40 min to 5 min (Fig. 3; Table 1). The synergistic interaction between N^6-cyclopentyladenosine and CCK-8 was abolished in cells pre-treated with for 24 h with 100 ng/ml pertussis toxin (Fig. 4). Finally, the synergy between N^6-cyclopentyladenosine and CCK-8 was also evident in experiments performed in the absence of extracellular calcium (Fig. 5; Table 1). These data clearly demonstrate a synergistic interaction between the human transfected adenosine A_1 receptor and the endogenous CCK_A receptor in CHO-A1 cells.

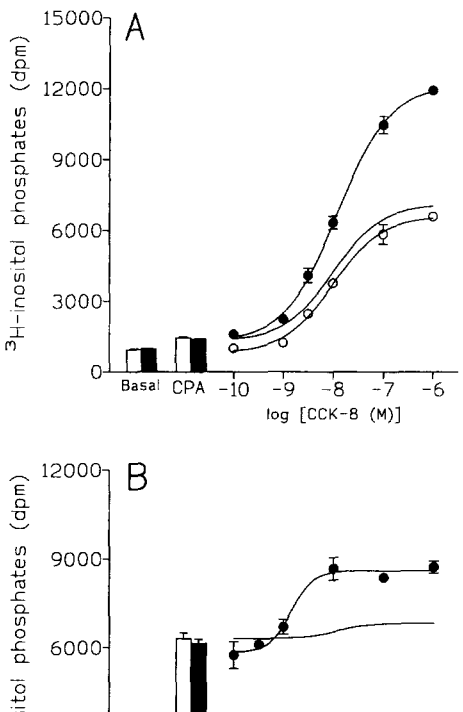

Fig. 2. Synergistic interactions between adenosine A_1 and CCK_A receptors in CHO-A1 cells. A: concentration response curves to CCK-8 in the absence (○) or presence (●) of N^6-cyclopentyladenosine (CPA; 1 μM). The solid line represents the predicted additive response to CCK-8 and N^6-cyclopentyladenosine calculated as described under Materials and methods. Basal and N^6-cyclopentyladenosine (1 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. B: concentration response curves to N^6-cyclopentyladenosine in the absence (○) or presence (●) of CCK-8 (1 μM). The solid line represents the predicted additive response to both agonists as calculated as described under Materials and methods. Basal and CCK-8 (1 μM) stimulated [^{3}H]inositol phosphate measured in each experiment are represented by histograms. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in four (A) and three (B) other experiments.

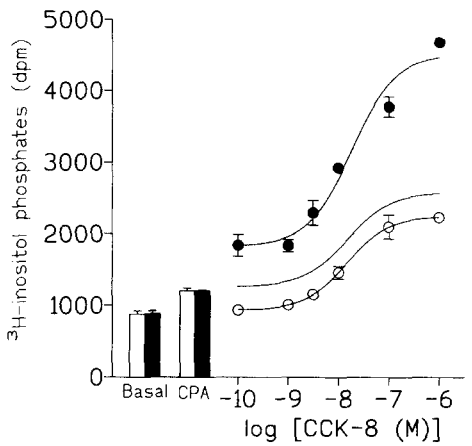

Fig. 3. Synergistic interactiions between adenosine A_1 and CCK_A receptors following 5 min agonist stimulation. Concentration response curves to CCK-8 were obtained in the absence (○) or presence (●) of N^6-cyclopentyladenosine (1 μM). The solid line represents the predicted additive response to CCK-8 and N^6-cyclopentyladenosine calculated as described under Materials and methods. Basal and N^6-cyclopentyladenosine (CPA; 1 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

3.3. Interactions between CCK_A and P_{2U}-purinoceptors

CHO cells endogenously express a P_{2U} purinoceptor linked to pertussis toxin-insensitive increases in $[Ca^{2+}]_i$ (Iredale et al., 1994; Iredale and Hill, 1994). Hence, we investigated whether CCK_A and P_{2U} purinoceptors interact synergistically to increase [^{3}H]inositol phosphate accumulation. Activation of the P_{2U} receptor with ATPγS (100 μM; $EC_{50} = 3.5 \pm 1.0$ μM; $n = 3$) elicited a 3.0 ± 0.2 ($n = 6$) fold increase in [^{3}H]inositol phosphate production in CHO-A1 cells. Fig. 6A shows the effect of ATPγS (100 μM) on the concentration response curve for CCK-8 stimulated [^{3}H]inositol phosphate formation in CHO-A1 cells. Clearly, co-activation of P_{2U} and CCK_A receptors in CHO-A1 cells produced increases in total [^{3}H]inositol phosphate accumulation which were purely additive. In these experiments ATPγS (100 μM) and CCK-8 (100 nM) alone produced increases in [^{3}H]inositol phosphates of 3.1 ± 0.2 ($n = 3$) and 5.2 ± 0.1 ($n = 3$) fold respectively. The fold increase in [^{3}H]inositol phosphate accumulation in response to simultaneous addition of 100 μM ATPγS and 100 nM CCK-8 (7.2 ± 0.6; $n = 3$) was similar to the predicted additive value (7.4 ± 0.1; $n = 3$). ATPγS had no significant effect on the EC_{50} value for CCK-8 stimulated [^{3}H]inositol phosphate production (14.5 ± 2.1 nM ($n = 3$) in the absence of ATPγS and 15 ± 1.2 nM ($n = 3$) in the presence of ATPγS). Similar, additive increases in [^{3}H]in-

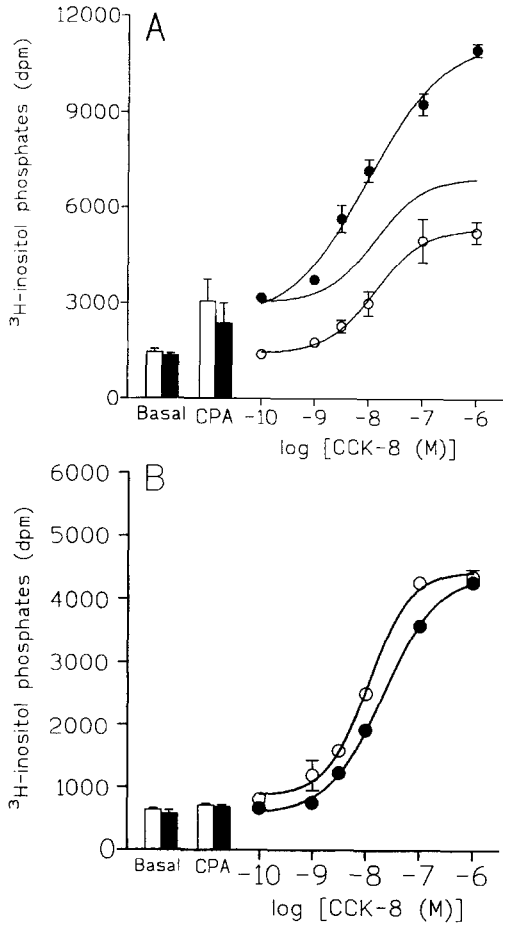

Fig. 4. Effect of pertussis toxin pre-treatment on the synergistic interaction between N^6-cyclopentyladenosine and CCK-8 in CHO-A1 cells. A: control experiment showing concentration response curves to CCK-8 in the absence (○) or presence (●) of N^6-cyclopentyladenosine (1 μM). The solid line represents the predicted additive response to CCK-8 and N^6-cyclopentyladenosine calculated as described under Materials and methods. Basal and N^6-cyclopentyladenosine (CPA; 1 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. B: as in A but cells pre-treated for 24 h with 100 ng/ml pertussis toxin. Both the direct effect of N^6-cyclopentyladenosine on [^{3}H]inositol phosphate accumulation and the potentiation of CCK_A receptor stimulated [^{3}H]inositol phosphate production are abolished by pertussis toxin. The values represent mean ± S.E.M. of triplicate determinations in a single experiment obtained on the same experimental day. Similar data were obtained in four (A) and two (B) other experiments.

ositol phosphate accumulation were observed when examining the effect of CCK (1 μM) on the concentration response curve for ATPγS (Fig. 6B).

3.4. Interactions between CCK_A and 5-HT_{1B} receptors

CHO cells endogenously express a 5-HT_{1B}-like receptor that inhibits adenylyl cyclase activity through a pertussis toxin-sensitive mechanism (Berg et al., 1994). Our recent studies have shown that 5-HT_{1B} receptor activation also inhibited forskolin stimulated [^{3}H]cAMP production in CHO-A1 cells (Dickenson and Hill, 1995). In addition, we have shown that activation of the 5-HT_{1B}-like receptor in CHO-A1 cells stimulates a pertussis toxin-sensitive increase in $[Ca^{2+}]_i$ (Dickenson and Hill, 1995). The pertussis toxin sensitivity of these 5-HT_{1B}-like receptor mediated responses suggests the involvement of G_i/G_o protein(s) in both signalling pathways. Based on these findings it would be predicted that 5-HT_{1B} receptor activation in CHO-A1 cells would also stimulate increases in [^{3}H]inositol phosphates and potentiate the [^{3}H]inositol phosphate response produced by CCK-8. The 5-HT_1 receptor selective agonist 5-carboxyamidotryptamine (10 μM) did not stimulate a

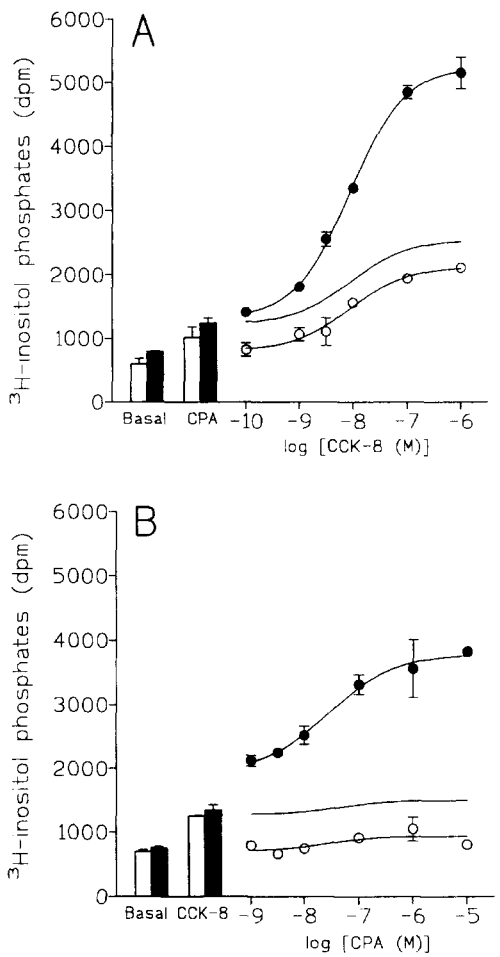

Fig. 5. Synergistic interactions between adenosine A_1 and CCK_A receptors in the absence of extracellular calcium. A: concentration-response curves to CCK-8 in the absence (○) or presence (●) of N^6-cyclopentyladenosine (1 μM) were obtained in calcium-free media containing 0.1 mM EDTA. The solid line represents the predicted additive response to CCK-8 and N^6-cyclopentyladenosine calculated as described under Materials and methods. Basal and N^6-cyclopentyladenosine (CPA; 1 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. B: concentration-response curves to N^6-cyclopentyladenosine in the absence (○) or presence (●) of CCK-8 (1 μM). The solid line represents the predicted additive response to both agonists as described under Materials and methods. Basal and CCK-8 (1 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

measurable increase in [^{3}H]inositol phosphates in CHO-A1 cells (Fig. 7A). It should be noted that 5-carboxyamidotryptamine stimulates a concentration-dependent increase in $[Ca^{2+}]_i$ in CHO-A1 cells with an EC_{50} value of 12 nM (Dickenson and Hill, 1995). Furthermore, 5-carboxyamidotryptamine (10 μM) had no significant effect on CCK-8 mediated [^{3}H]inositol phosphate production (see Fig. 7A). Finally, 5-carboxyamidotryptamine had no significant effect on adenosine A_1 receptor mediated [^{3}H]inositol phosphate production (Fig. 7B).

3.5. Inositol 1,4,5 triphosphate formation in CHO-A1 cells

The effect of adenosine A_1, CCK_A and 5-HT_{1B} receptor activation on Ins(1,4,5)P_3 levels in CHO-A1 cells is shown

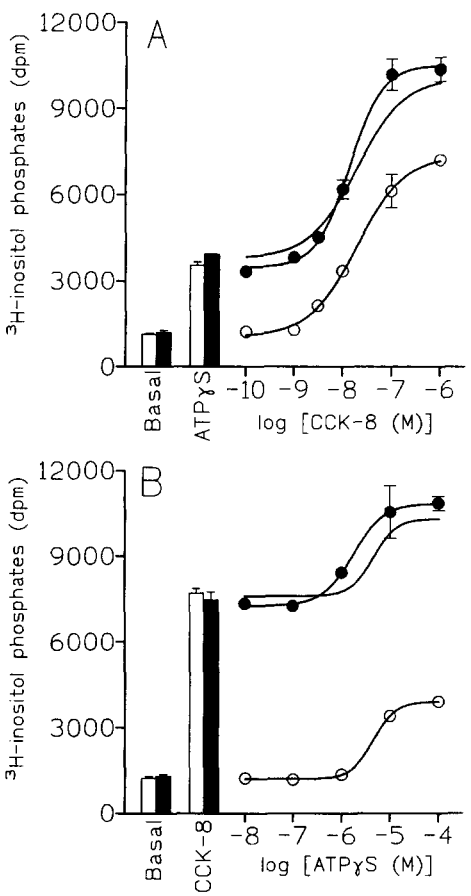

Fig. 6. Effect of co-activation of P_{2U}-purinoceptor and CCK_A receptors on [^{3}H]inositol phosphate accumulation in CHO-A1 cells. A: concentration response curves to CCK-8 in the absence (○) or presence (●) of ATPγS (100 μM). The solid line represents the predicted additive response to CCK-8 and ATPγS calculated as described under Materials and methods. Basal and ATPγS (100 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. B: concentration response curves to ATPγS in the absence (○) or presence (●) of CCK-8 (1 μM). The solid line represents the predicted additive response to both agonists as described under Materials and methods. Basal and CCK-8 (1 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

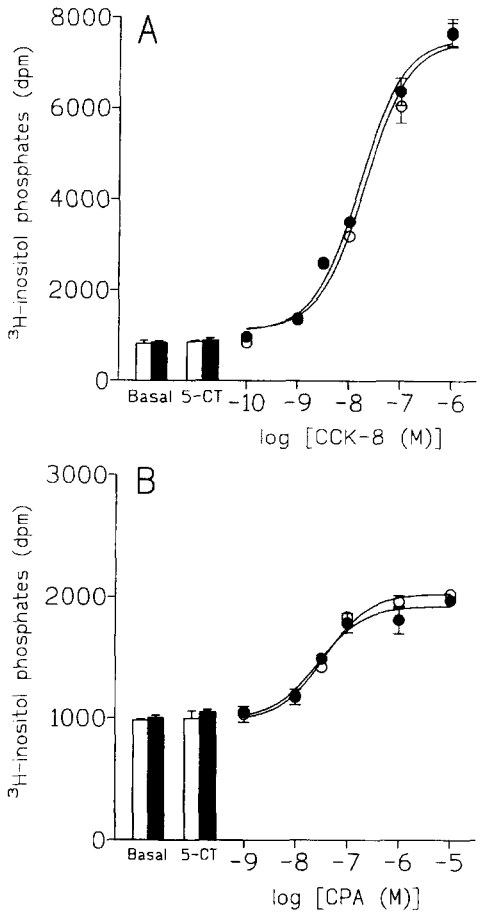

Fig. 7. Effect of 5-HT_{1B} receptor activation on CCK_A and adenosine A_1 receptor stimulated [^{3}H]inositol phosphate accumulation in CHO-A1 cells. A: concentration response curves to CCK-8 in the absence (○) or presence (●) of 5-carboxyamidotryptamine (10 μM). Basal and 5-carboxyamidotryptamine (5-CT; 10 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. B: concentration response curves to N^6-cyclopentyladenosine in the absence (○) or presence (●) of 5-carboxyamidotryptamine (10 μM). Basal and 5-CT (10 μM) stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. Values represent mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in two other experiments.

in Fig. 8. Neither, 1 μM CCK-8, 1 μM N^6-cyclopentyladenosine, 10 μM 5-carboxyamidotryptamine or a combination of CCK-8 and 5-carboxyamidotryptamine caused any significant increase in basal levels of Ins(1,4,5)P_3. In contrast, co-stimulation with CCK-8 (1 μM) and N^6-cyclopentyladenosine (1 μM) produced a significant increase in Ins(1,4,5)P_3 levels (Fig. 8A). In these experiments Ins(1,4,5)P_3 levels increased significantly from basal levels of 3.9 ± 1.0 pmol/well to 15.9 ± 1.4 pmol/well within 10 sec ($P < 0.05$; Fig. 8B).

4. Discussion

As mentioned in the Introduction, CHO cells endogenously express cholecystokinin receptors coupled to inosi-

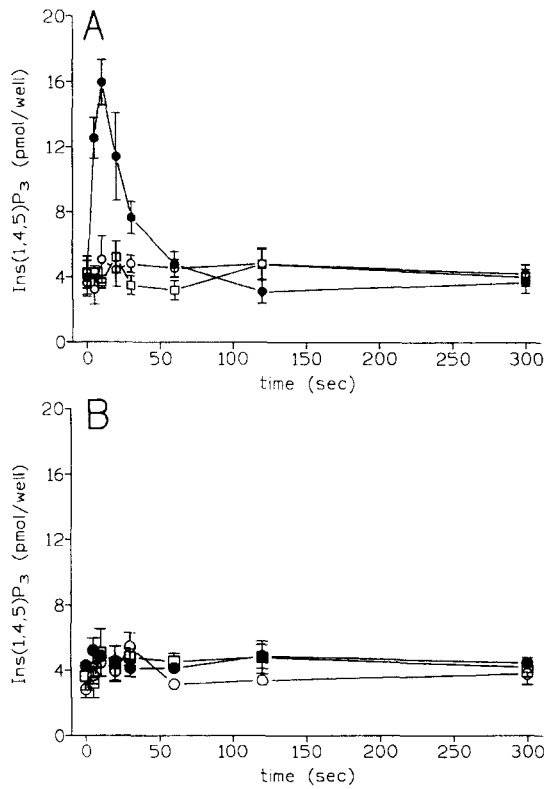

Fig. 8. The effect of adenosine A_1, CCK_A and 5-HT_{1B} receptor activation on Ins(1,4,5)P_3 formation in CHO-A1 cells. A: cells were stimulated with 1 μM CCK-8 (○), 1 μM N^6-cyclopentyladenosine (□) or a combination of 1 μM CCK-8 and 1 μM N^6-cyclopentyladenosine (●) for the indicated times. B: cells were stimulated with 1 μM CCK-8 (○), 10 μM 5-carboxyamidotryptamine (□; 5-CT) or a combination of 1 μM CCK-8 and 10 μM 5-CT (●) for the indicated times. Results are expressed as the mean ± S.E.M. from three independent experiments each performed in triplicate. Statistical analysis (Student's unpaired *t* test) showed that co-stimulation with CCK-8 and N^6-cyclopentyladenosine significantly increased Ins(1,4,5)P_3 levels compared to basal Ins(1,4,5)P_3 levels.

tol phospholipid hydrolysis. The aims of the present study were: (i) to pharmacologically characterise the cholecystokinin receptor expressed by CHO cells and (ii) to investigate the effect of adenosine A_1 and 5-HT_{1B} receptor activation on cholecystokinin stimulated [^{3}H]inositol phosphate accumulation. The data presented show that the sulphated C-terminal octapeptide of cholecystokinin (CCK-8) stimulated a pertussis toxin-insensitive increase in [^{3}H]inositol phosphate accumulation in CHO-A1 cells. The lack of pertussis toxin sensitivity of the CCK-8 response suggests coupling to phospholipase C via a G_q/G_{11} protein. CCK-8 stimulated [^{3}H]inositol phosphate accumulation was potently inhibited by the CCK_A receptor selective antagonists devazepide (IC_{50} = 0.26 nM) and lorglumide (IC_{50} = 58 nM). The potency of devazepide is similar to the IC_{50} value (0.93 nM) reported for the inhibition of CCK_A receptor stimulated [^{3}H]inositol phosphate production in CHP212 neuroblastoma cells (Barrett et al., 1989). The low potency of the CCK_B receptor selective antagonist PD 135,158 (IC_{50} = 4.8 μM) confirmed that CCK_A receptors are involved in CCK-8 stimulation of [^{3}H]inositol phosphate production in CHO-A1 cells. PD 135,158 inhibits [^{125}I]CCK binding to the mouse cortex CCK_B receptor with an IC_{50} value of 2.8 nM (Hughes et al., 1990).

Our previous studies have shown that the human transfected adenosine A_1 receptor and the endogenous 5-HT_{1B} receptor inhibit forskolin stimulated cAMP production and stimulate increases in $[Ca^{2+}]_i$ in CHO-A1 cells (Iredale et al., 1994; Dickenson and Hill, 1995). In this study we have demonstrated that A_1 receptor activation also stimulates a pertussis toxin-sensitive increase in [^{3}H]inositol phosphate accumulation. In marked contrast to the transfected A_1 receptor, the endogenous 5-HT_{1B} receptor did not stimulate a measurable increase in total [^{3}H]inositol phosphate accumulation. These data are in agreement with Berg et al. (1994) who were also unable to demonstrate inositol phosphate accumulation in CHO cells in response to 5-HT or 5-carboxyamidotryptamine. These data were rather surprising given the ability of various 5-HT_{1B} receptor agonists to stimulate increases in $[Ca^{2+}]_i$ in CHO-A1 cells (Dickenson and Hill, 1995). This apparent discrepancy may be due to 5-HT_{1B} receptors stimulating small increases Ins(1,4,5)P_3 which although sufficient to increase $[Ca^{2+}]_i$ are too small to detect in either total [^{3}H]inositol phosphates experiments (measured in the presence of LiCl) or mass Ins(1,4,5)P_3 determinations.

The data presented in this study demonstrate that co-activation of the human transfected adenosine A_1 receptor synergistically enhances the CCK_A receptor stimulated [^{3}H]inositol phosphate production. In contrast, the endogenous 5-HT_{1B} receptor had no significant effect on CCK-8 stimulated [^{3}H]inositol phosphate accumulation. One notable difference is that the human transfected adenosine A_1 receptor is expressed at moderate levels (200 fmol/mg of protein (Iredale et al., 1994)), whereas the endogenous 5-HT_{1B} receptor is expressed at levels that are not detectable using specific [^{3}H]5-HT or [^{125}I]cyanopindolol binding (Giles et al., 1994). Therefore, the inability of 5-HT_{1B} receptor activation to potentiate CCK_A receptor stimulated [^{3}H]inositol phosphate accumulation may be a consequence of low 5-HT_{1B} receptor number. Alternatively, the synergy between adenosine A_1 and CCK_A receptors may reflect the coupling of the adenosine A_1 receptor to a particular pertussis toxin-sensitive G protein. At the present time we do not know whether adenosine A_1 and 5-HT_{1B} receptors couple to the same or different PTX-sensitive G proteins in CHO cells. Western blot analysis has revealed that CHO cells express $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_{o2}$ subunits (Prather et al., 1994).

The activation of phospholipase C through receptors coupled to pertussis toxin-sensitive G_i proteins is thought to be mediated by G_i protein $\beta\gamma$ subunits (Camps et al., 1992; Clapham and Neer, 1993). The β isoforms of phospholipase C (PLC-β_{1-3}) are regulated by both $G\alpha$

subunits belonging to the G_q class of G proteins ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{15}$ and $G\alpha_{16}$) and by $G\beta\gamma$ subunits (Clapham and Neer, 1993; Offermanns and Schultz, 1994). Furthermore, the $G\alpha$ subunits activate the phospholipase C β isoforms in the order PLC $\beta_1 \geq$ PLC $\beta_3 \gg$ PLC β_2, whereas $\beta\gamma$ subunits activate in the order of PLC $\beta_3 >$ PLC $\beta_2 >$ PLC β_1 (Park et al., 1993). Using Western blot analysis we have screened CHO-A1 cell membrane and cytosolic fractions for the presence phospholipase C β_{1-3} isoforms. Unfortunately, no immunoreactive proteins in the expected molecular weight range for phospholipase C β isoforms, i.e. circa 150 kDa were detected (Dickenson and Hill, unpublished observations). The identity of the phospholipase β isoform(s) present in CHO-A1 cells and the role $\beta\gamma$ subunits play in adenosine A_1 receptor coupling to phospholipase C remain to be established.

The mechanism underlying the synergy between receptors coupled to pertussis toxin-sensitive and insensitive G proteins is not known although several authors have speculated on the involvement of G_i protein $\beta\gamma$ subunits (Gerwins and Fredholm, 1992a, b; Biden and Browne, 1993; Dickenson and Hill, 1993b; Dickenson, 1994; Okajima et al., 1993). For example, exogenous $\beta\gamma$ subunits potentiated a P_{2Y}-purinoceptor activation of phospholipase C in turkey erythrocyte membrane preparations (Boyer et al., 1989). We recently investigated the effects of G protein $\beta\gamma$ subunits on phospholipase C activity in DDT_1MF-2 cells (Dickenson et al., 1995). These studies revealed that cytosolic and membrane fractions prepared from DDT_1MF-2 cells contain phospholipase C activity which can stimulated by Ca^{2+} ions and G protein $\beta\gamma$ subunits (Dickenson et al., 1995). Furthermore, the $\beta\gamma$-dependent activation of phospholipase C, which was virtually absent in the presence of low Ca^{2+}, increased markedly with increasing free Ca^{2+} ion concentration. These findings suggest that Ca^{2+} ions and $\beta\gamma$ subunits may interact to enhance phospholipase C activity in DDT_1MF-2 cells. Such an interaction may account for the synergy observed between receptors coupled to pertussis toxin-sensitive and insensitive G proteins in DDT_1MF-2 cells (Dickenson and Hill, 1993b). Similar experiments using membranes prepared from CHO-A1 cells have revealed the presence of phospholipase C activity which can be stimulated by $\beta\gamma$ subunits in a Ca^{2+}-dependent manner (Dickenson and Hill, unpublished observations). It is therefore possible that $\beta\gamma$ subunits released from G proteins coupled to the adenosine A_1 receptor may enhance Ca^{2+}-dependent phospholipase C activity in CHO-A1 cells. However, our data clearly show synergy between adenosine A_1 and CCK_A receptors in the absence of extracellular calcium ions. These observations would seem to argue against the possibility that $\beta\gamma$ subunits enhance Ca^{2+}-dependent phospholipase C activity.

Alternatively, the potentiation mechanism may involve the synergistic activation of phospholipase C by $G\alpha_q$ and $G\beta\gamma$ subunits. For example, co-expression of $G\alpha_{16}$ and $G\beta_1\gamma_1$ subunits synergistically activated recombinant phospholipase C β_2 in COS-7 cells (Wu et al., 1993). Similarly, $G\alpha_q/_{11}$ and $\beta\gamma$ subunits synergistically activated purified rat brain phospholipase C β_3 (Smrcka and Sternweis, 1993). These observations are comparable to the potentiation of α_s stimulated adenylyl cyclase II activity by G_i protein derived $\beta\gamma$ subunits (Federman et al., 1992). Therefore, in a model system, $\beta\gamma$ subunits derived from pertussis toxin-sensitive G proteins (released following adenosine A_1 receptor activation) would interact synergistically with $G\alpha_q$ subunits (released following CCK_A receptor activation) to stimulate phospholipase C.

In summary, the present study has illustrated marked synergism between the human transfected adenosine A_1 receptor and the endogenous CCK_A receptor in CHO-A1 cells. The CHO-A1 cell line may provide an ideal model system that can be used to decipher the molecular mechanisms underlying the interactions between receptors coupled to pertussis toxin-sensitive and insensitive G proteins.

Acknowledgements

We thank the Wellcome Trust for financial support (grant reference 038757/z/93/z/1/1.5)

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