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Transfected adenosine A_1 receptor-mediated modulation of thrombin-stimulated phospholipase C and phospholipase A_2 activity in CHO cells

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Abstract

Thrombin receptor activation in Chinese hamster ovary (CHO) cells stimulates the hydrolysis of inositol phospholipids and the release of arachidonic acid. Our previous studies have shown that activation of the human transfected adenosine A_1 receptor in CHO cells (CHO-A1) potentiates the accumulation of inositol phosphates elicited by endogenous P_{2U} purinoceptors and CCK_A receptors. In this study we have investigated whether adenosine A_1 receptor activation can modulate thrombin-stimulated arachidonic acid release and/or inositol phospholipid hydrolysis in CHO-A1 cells. Thrombin stimulated [^{3}H]arachidonic acid release and total [^{3}H]inositol phosphate accumulation in CHO-A1 cells. Both these responses to thrombin were insensitive to pertussis toxin. The protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), potentiated thrombin-stimulated [^{3}H]arachidonic acid. In marked contrast, PMA inhibited thrombin-stimulated [^{3}H]inositol phosphate accumulation. The selective protein kinase C inhibitor Ro 31-8220 (3-{1-[3-(2-isothioureido)propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione) had no effect on thrombin-stimulated [^{3}H]arachidonic acid release but reversed the potentiation of thrombin-stimulated [^{3}H]arachidonic acid release elicited by PMA. The selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA) augmented the release of [^{3}H]arachidonic acid produced by thrombin. Co-activation of the adenosine A_1 receptor also potentiated thrombin-stimulated [^{3}H]inositol phosphate accumulation. The synergistic interactions between the adenosine A_1 receptor and thrombin were abolished in pertussis-toxin-treated cells. The potentiation of [^{3}H]arachidonic acid release by CPA was blocked by the protein kinase C inhibitors Ro 31-8220 and GF 109203X (3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione). In conclusion, thrombin receptor activation in CHO-A1 cells stimulates the accumulation of [^{3}H]inositol phosphates and the release of [^{3}H]arachidonic acid through pertussis-toxin-insensitive G-proteins. Experiments using PMA suggest that protein kinase C differentially regulates thrombin receptor activation of phospholipase C and phospholipase A_2. Co-activation of the transfected human adenosine A_1 receptor augments thrombin-stimulated phospholipase C and phospholipase A_2 activity. Finally, the augmentation of phospholipase A_2 activity by the adenosine A_1 receptor is inhibited by selective protein kinase C inhibitors, suggesting the involvement of protein kinase C.

Keywords: Thrombin receptor; Adenosine A_1 receptor; P_{2U} purinoceptor; Inositol phosphate; Arachidonic acid; CHO (Chinese hamster ovary) cell; Protein kinase C

1. Introduction

The thrombin receptor is a member of the seven transmembrane spanning G-protein-coupled receptor superfamily which is activated by a unique proteolytic cleavage mechanism (Coughlin, 1994; Grand et al., 1996). The cloned human thrombin receptor stimulates inositol phospholipid hydrolysis and inhibition of adenylyl cyclase in Rat 1 cells (Hung et al., 1992). The inhibition of adenylyl cyclase in these cells is sensitive to pertussis toxin, suggesting coupling to G_i/G_o proteins, whereas the stimulation of inositol phospholipid hydrolysis is largely insensitive to pertussis toxin, indicating the involvement of G_q/G_{11} proteins (Hung et al., 1992). However, in platelets and vascular smooth muscle cells, thrombin-stimulated inositol phospholipid hydrolysis has been reported to be sensitive to pertussis toxin, implicating the involvement of G_i/G_o proteins (Crouch and Lapetina, 1988; Huang and Ives, 1989). The thrombin receptor has been shown to couple to G_{12} in platelets (Offermanns et al., 1994) and recently Aragay et al. (1995) demonstrated a specific role

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for $G\alpha_{12}$ in the mitogenic response to thrombin in 1321N1 astrocytoma cells.

Thrombin can also stimulate a wide variety of other signalling pathways (for review, see Grand et al., 1996). For example, Lin et al. (1992) demonstrated that the thrombin receptor can stimulate cytoplasmic phospholipase A_2 activity in Chinese hamster ovary (CHO) cells. Cytoplasmic phospholipase A_2 catalyzes the release of arachidonic acid from the *sn*-2 position of phospholipids, predominantly phosphatidylcholine (Dennis, 1994). In CHO cells, thrombin-stimulated release of arachidonic acid has been reported to be sensitive to pertussis toxin, suggesting the involvement of G_i/G_o proteins (Winitz et al., 1994).

Thrombin can also stimulate inositol phospholipid hydrolysis and Ca^{2+} mobilisation in CHO cells (Jacksowski et al., 1988; Mitsuhashi et al., 1989; Banno et al., 1994; Mochizuki-oda et al., 1994). In contrast, to the phospholipase A_2 response, pertussis toxin pre-treatment produced only a small attenuation of thrombin-stimulated inositol phospholipid hydrolysis, suggesting that coupling to phospholipase C occurs predominantly through a G_q/G_{11} proteins (Banno et al., 1994).

Our recent studies have focused on CHO-K1 cells transfected with the human brain adenosine A_1 receptor (Townsend-Nicholson and Shine, 1992). In these cells activation of the human transfected adenosine A_1 receptor leads to the inhibition of forskolin-stimulated cAMP production and the stimulation of inositol phospholipid hydrolysis and Ca^{2+} mobilisation (Townsend-Nicholson and Shine, 1992; Iredale et al., 1994; Megson et al., 1995). The inhibition of adenylyl cyclase and the stimulation of inositol phospholipid hydrolysis are inhibited by pertussis toxin, suggesting the involvement of G_i/G_o protein(s) in both signalling pathways. Furthermore, we have shown that in these cells, activation of the adenosine A_1 receptor potentiates the accumulation of inositol phosphates elicited by endogenous P_{2U} puronoceptors and CCK_A receptors (Megson et al., 1995; Dickenson and Hill, 1996).

The aim of the present study was therefore to compare the effect of adenosine A_1 receptor stimulation on the two different intracellular responses to thrombin (arachidonic acid release and inositol phospholipid hydrolysis) in CHO-K1 cells.

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 Prof. John Shine (Garvan Institute, Sydney, Australia). CHO-A1 cells were cultured in 75 cm^2 flasks (Costar) in Dulbeco's modified Eagle's Medium (DMEM)/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 confluence and were subcultured (1:5 split ratio) using trypsin (0.05%, w/v)/EDTA (0.02%, w/v). Cells for [^{3}H]inositol phosphate and [^{3}H]arachidonic acid determinations were grown in 24-well cluster dishes (Costar).

2.2. Measurement of [^{3}H]arachidonic acid release

Confluent monolayers of CHO-A1 cells were loaded for 3 h with [^{3}H]arachidonic acid (15 kBq/well) in 24-well cluster dishes in DMEM/F-12 containing 1% foetal calf serum. Labelled cells were then washed once with 1 ml/well Hanks/HEPES buffer, pH 7.4, supplemented with 0.2% bovine serum albumin. Washed cells were incubated at 37°C for 20 min in 250 μl/well of the same medium. Agonists were added in 250 μl of medium and the incubation continued for 15 min at 37°C. After agonist stimulation, 400 μl aliquots were removed and centrifuged to remove non-adherent cells. Radioactivity was quantified by scintillation counting in the liquid phase (scintillator plus, Packard).

2.3. Inositol phospholipid hydrolysis

At confluence, cell monolayers were loaded for 24 h with [^{3}H]*myo*-inositol (37 kBq/well) in 24-well cluster dishes in inositol-free DMEM containing 1% foetal calf serum. 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). Agonists were then added in 10 μl of medium and the incubation continued for 40 min 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).

2.4. Data analysis

Agonist EC_{50} values (concentration of agonist producing 50% of the maximal stimulation) were obtained by computer-assisted curve fitting by use of the computer programme InPlot (GraphPAD, San Diego, CA, USA). Statistical significance was determined by Student's un-

paired 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 thrombin-receptor-stimulated [^{3}H]inositol phosphate accumulation was performed as follows. Concentration–response curves to thrombin (first agonist) were generated in the absence and presence of a fixed concentration of CPA (1 μM; second agonist; see Fig. 5). A curve was then generated which showed the predicted additive response for the first agonist in the presence of the second agonist. This was obtained by adding the response to the fixed concentration of the second agonist (in this case CPA) to that obtained with each concentration of first agonist alone (in this case thrombin; see Fig. 5). Fold-over-basal increases in [^{3}H]inositol phosphate accumulation were determined for the maximally effective concentrations of thrombin (0.1 unit/ml) and CPA (1 μM). Statistical analysis (Student's unpaired t-test) was performed by comparing the predicted additive fold-over-basal increases in [^{3}H]inositol phosphates (achieved by adding together the responses obtained for 0.1 unit/ml thrombin and 1 μM CPA alone) with the fold-over-basal increases in [^{3}H]inositol phosphates obtained from the simultaneous addition of thrombin (0.1 unit/ml) and CPA (1 μM). Finally, statistical analysis (Student's unpaired t-test) of phospholipase A_2 data was performed by comparing fold-over-basal increases in [^{3}H]arachidonic acid release.

2.5. Materials

[2-^{3}H]*Myo*-inositol and [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid were from New England Nuclear and Amersham International (Amersham, UK), respectively. Bovine thrombin was purchased from Organon Teknika. Uridine 5′-triphosphate (UTP) was supplied by Boehringer. N^6-Cyclopentyladenosine (CPA) and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma (Poole, UK), respectively. Pertussis toxin was obtained from Porton Products Ltd. Ro 31-8220 (3-{1-[3-(2-isothioureido) propyl]indol-3-yl}-4-(1-methylindol-3-yl)-3-pyrrolin-2,5-dione) was from Calbiochem (Nottingham, UK). GF 109203X (3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione) was from Research Biochemicals International (Natick, MA, USA). Dulbecco's modified Eagle's Medium/Nutrient Mix F-12 (1:1) and foetal calf serum were from Sigma. All other chemicals were of analytical grade.

3. Results

3.1. Thrombin-stimulated [^{3}H]arachidonic acid release and [^{3}H]inositol phosphate accumulation in CHO-A1 cells

Thrombin stimulated a significant and concentration-dependent (EC_{50} = 0.018 ± 0.005 unit/ml (n = 4)) increase in [^{3}H]arachidonic acid release in CHO-A1 cells (Fig. 1a). Fifteen minutes stimulation with the maximally effective concentration of thrombin (1 unit/ml) produced a 1.7 ± 0.1 (n = 8)-fold increase over basal levels in [^{3}H]arachidonic

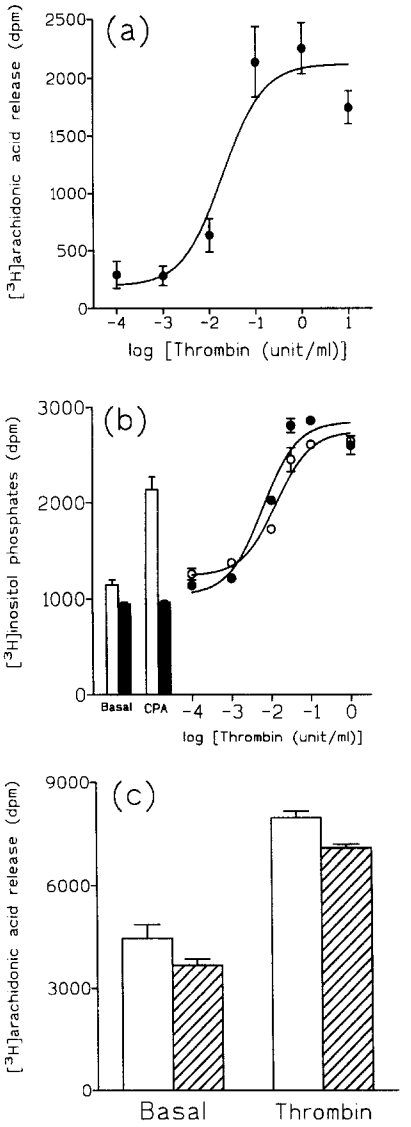

Fig. 1. Effect of thrombin on arachidonic acid release and inositol phospholipid hydrolysis in CHO-A1 cells. (a) Concentration–response curve for thrombin-stimulated [^{3}H]arachidonic acid release in CHO-A1 cells. Values represent the mean ± S.E.M. obtained from four independent experiments measured in triplicate. Results are expressed as the increase in [^{3}H]arachidonic acid obtained after subtracting the basal release of [^{3}H]arachidonic acid (basal = 7906 ± 572 dpm; n = 4). (b) Effect of thrombin on total [^{3}H]inositol phosphate accumulation in CHO-A1 cells. Concentration–response curves to thrombin in control cells (○) and cells pre-treated for 24 h with 100 ng/ml pertussis toxin (●). Basal and CPA (1 μM)-stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. Values represent the mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three other experiments. (c) Effect of pertussis toxin on thrombin-stimulated [^{3}H]arachidonic acid release. Control cells (open columns) and cells pre-treated for 24 h with 100 ng/ml pertussis toxin (shaded columns) were stimulated with thrombin (1 unit/ml) for 15 min. Values represent the mean ± S.E.M. of triplicate determinations in a single experiment. Similar data were obtained in three other experiments.

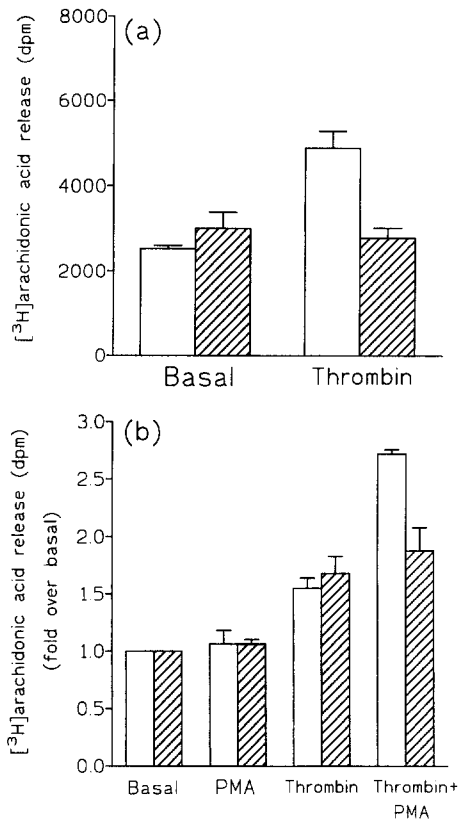

Fig. 2. The regulation of thrombin-stimulated arachidonic acid release by extracellular calcium and protein kinase C in CHO-A1 cells. (a) The effect of removing extracellular Ca^{2+} on thrombin-stimulated [^{3}H]arachidonic acid release. Cells were stimulated with thrombin (1 unit/ml) in the presence (open columns) or absence (shaded columns) of extracellular Ca^{2+}. Values represent the mean ± S.E.M. of triplicate determinations in a single experiment. Similar results were obtained in two further experiments. (b) Effect of protein kinase C activation and inhibition on thrombin-stimulated [^{3}H]arachidonic acid release. In these experiments cells were initially pre-incubated for 20 min with 10 μM Ro 31-8220 (shaded columns) or 0.1% dimethyl sulphoxide (open columns). Cells were then stimulated with 1 μM PMA for 10 min in the continued absence or presence of 10 μM Ro 31-8220 before stimulating with thrombin (1 unit/ml) for 15 min. Results are expressed as fold-over-basal increase in [^{3}H]arachidonic acid release. Basal [^{3}H]arachidnic acid release was 1549 ± 161 dpm ($n = 3$) and 1703 ± 154 dpm ($n = 3$) in control and Ro 31-8220-treated cells, respectively. Each column represents the mean ± S.E.M. of three independent experiments each measured in triplicate.

acid release ($P < 0.05$). Pre-treatment of cells with pertussis toxin (100 ng/ml for 24 h) had no significant effect on thrombin-stimulated [^{3}H]arachidonic acid release (1 unit/ml of thrombin elicited a 1.8 ± 0.10 ($n = 3$)-fold increase in [^{3}H]arachidonic acid release in cells treated with pertussis toxin; Fig. 1c). The maximally effective concentration of thrombin (1 unit/ml) obtained in this study is comparable to values reported previously for thrombin-stimulated arachidonic acid release in CHO cells (Banno et al., 1994; Gupta et al., 1990). As we have previously reported, this concentration of pertussis toxin and length of pre-incubation completely inhibits the accumulation of [^{3}H]inositol phosphates elicited by the selective adenosine A_1 receptor agonist N^6-cyclopentyladenosine (CPA; 1 μM; see Fig. 1b; Megson et al., 1995; Dickenson and Hill, 1996).

Thrombin (1 unit/ml) also stimulated a significant increase in the accumulation of total [^{3}H]inositol phosphates in CHO-A1 cells (2.20 ± 0.35-fold increase over basal levels; $n = 8$; $P < 0.05$). The [^{3}H]inositol phosphate response to thrombin was concentration-dependent with an EC_{50} (0.014 ± 0.002 unit/ml; Fig. 1b) similar to that obtained for [^{3}H]arachidonic acid release. Pre-treatment of cells with pertussis toxin (100 ng/ml for 24 h) had no significant effect on thrombin-stimulated [^{3}H]inositol

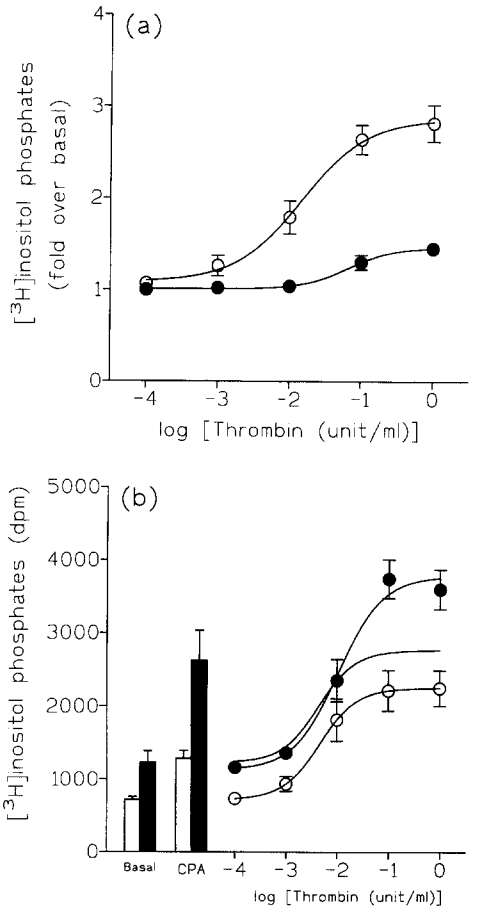

Fig. 3. The effect of protein kinase C activation and inhibition on thrombin stimulated inositol phospholipid hydrolysis in CHO-A1 cells. (a) Concentration–response curves for thrombin-stimulated [^{3}H]inositol phosphate accumulation in control cells (○) and cells pre-treated for 10 min with 1 μM phorbol 12-myristate 13-acetate (●). Values represent the mean ± S.E.M. obtained from three independent experiments each measured in duplicate. (b) Concentration–response curves for thrombin-stimulated [^{3}H]inositol phosphate accumulation in control cells (○) and cells pre-treated for 20 min with 10 μM Ro 31-8220 (●). Values represent the mean ± S.E.M. obtained from four independent experiments each measured in triplicate. The solid line represents the predicted additive response to thrombin (in the absence of Ro 31-8220) and the increase in basal [^{3}H]inositol phosphate accumulation observed in Ro 31-8220-treated cells. CPA (1 μM)-stimulated [^{3}H]inositol phosphate accumulation was also measured in the absence and presence of Ro 31-8220 during these experiments.

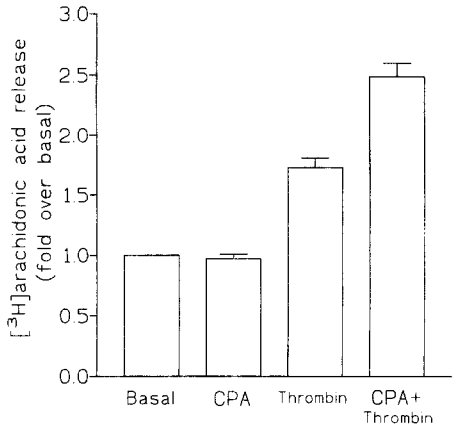

Fig. 4. The effect of adenosine A_1 receptor activation on thrombin-stimulated arachidonic acid release in CHO-A1 cells. CHO-A1 cells were loaded with [^{3}H]arachidonic acid for 3 h before being stimulated with the CPA (1 μM), thrombin (1 unit/ml) or a combination of CPA (1 μM) and thrombin (1 unit/ml) for 15 min. Results are expressed as fold-over-basal increase in [^{3}H]arachidonic acid release (basal = 4302 ± 427 dpm; $n = 8$). Each column represents the mean ± S.E.M. of eight independent experiments, each measured in triplicate.

phosphate accumulation (Fig. 1b). In cells pre-treated with pertussis toxin, thrombin (1 unit/ml) stimulated a 2.1 ± 0.2 ($n = 4$)-fold increase in [^{3}H]inositol phosphates with an EC_{50} of 0.011 ± 0.002 unit/ml ($n = 4$). These results suggest that thrombin-stimulated increases in [^{3}H]arachidonic acid release and [^{3}H]inositol phosphate accumulation are mediated through pertussis-toxin-insensitive G_q/G_{11} protein(s).

Cytoplasmic phospholipase A_2 activity is known to be regulated by increases in intracellular Ca^{2+} and by protein kinase C-mediated phosphorylation (Nemenoff et al., 1993). Thrombin-stimulated [^{3}H]arachidonic acid release

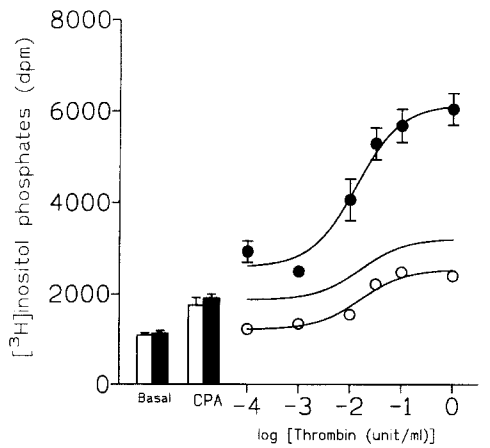

Fig. 5. Synergistic interactions between adenosine A_1 and thrombin-receptor-stimulated inositol phospholipid hydrolysis in CHO-A1 cells. Concentration–response curves to thrombin in the absence (○) or presence (●) of CPA (1 μM). The solid line represents the predicted additive response to thrombin and CPA calculated as described in Section 2. Basal and 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.

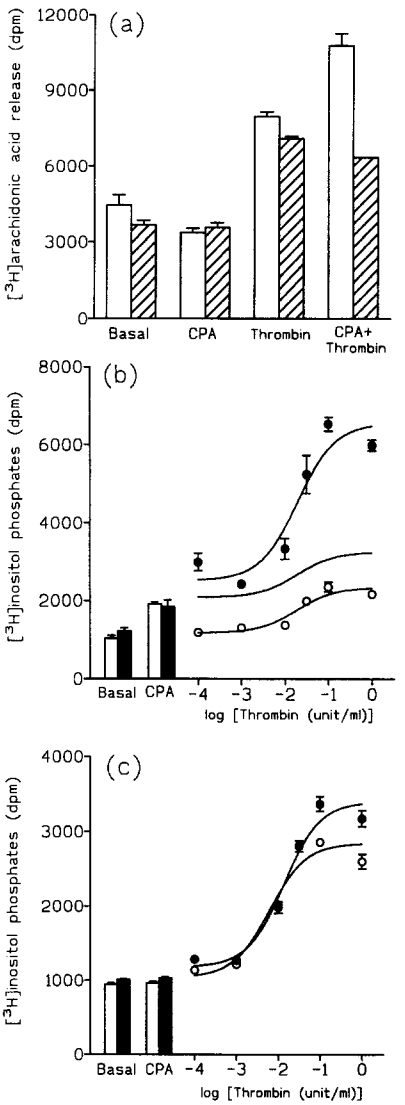

Fig. 6. Effect of pertussis toxin pre-treatment on the interactions between CPA and thrombin in CHO-A1 cells. (a) Effect of pertussis toxin on [^{3}H]arachidonic acid release. Control cells (open columns) and cells pre-treated with 100 ng/ml pertussis toxin for 24 h (shaded columns) were stimulated with CPA (1 μM), thrombin (1 unit/ml) or a combination of CPA (1 μM) and thrombin (1 unit/ml) for 15 min. Values represent the mean ± S.E.M. of triplicate determinations in a single experiment obtained on the same experimental day. Similar data were obtained in two further experiments. (b) and (c) Effect of pertussis toxin on [^{3}H]inositol phosphate accumulation in response to CPA and thrombin. (b) Control experiment showing concentration–response curves to thrombin in the absence (○) or presence (●) of CPA (1 μM). The solid line represents the predicted additive response to thrombin and CPA calculated as described in Section 2. Basal and CPA (1 μM)-stimulated [^{3}H]inositol phosphate accumulation measured in each experiment are represented by histograms. (c) As in (b), but cells pre-treated for 24 h with 100 ng/ml pertussis toxin. The direct effect of CPA on [^{3}H]inositol phosphate accumulation and the potentiation of thrombin-stimulated [^{3}H]inositol phosphate production are abolished by pertussis toxin. The values represent the mean ± S.E.M. of triplicate determinations in a single experiment obtained on the same experimental day. Similar data were obtained in two other experiments.

was abolished in experiments performed in the absence of extracellular calcium (Fig. 2a). These data suggest that thrombin-mediated [^{3}H]arachidonic acid release is dependent upon Ca^{2+} influx. It should be noted that in the absence of extracellular calcium thrombin stimulates a transient increase in $[Ca^{2+}]_i$ in CHO-A1 cells (data not shown). These data imply that the transient increase in $[Ca^{2+}]_i$ due to Ca^{2+} release from intracellular stores is not sufficient to activate cytoplasmic phospholipase A_2 in CHO-A1 cells. The possible involvement of protein kinase C in thrombin-stimulated [^{3}H]arachidonic acid release was investigated using the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), and the selective protein kinase C inhibitor, Ro 31-8220 (Davis et al., 1989). Pre-treatment with PMA (1 μM; 10 min) had no significant effect on basal [^{3}H]arachidonic acid release (Fig. 2b). However, PMA (1 μM) significantly potentiated thrombin-stimulated [^{3}H]arachidonic acid release (Fig. 2b). In these experiments, thrombin alone (1 unit/ml) stimulated a 1.6 ± 0.1 ($n = 3$)-fold increase in [^{3}H]arachidonic acid release, whereas in the presence of PMA thrombin stimulated a 2.70 ± 0.05 ($n = 3$)-fold increase in [^{3}H]arachidonic acid release (Fig. 2b; $P < 0.05$). Pre-treatment with the protein kinase C inhibitor, Ro 31-8220 (10 μM; 20 min), had no significant effect on thrombin-stimulated [^{3}H]arachidonic acid release but completely reversed PMA-induced augmentation of thrombin-stimulated [^{3}H]arachidonic acid release (Fig. 2b). Finally, we determined the effect of protein kinase C activation (using PMA) and inhibition (using Ro 31-8220) on thrombin-stimulated [^{3}H]inositol phosphate responses. Pre-treatment with 1 μM PMA (10 min) inhibited thrombin-stimulated [^{3}H]inositol phosphate accumulation by $75 \pm 2\%$ ($n = 3$; Fig. 3a). In contrast, Ro 31-8220 (10 μM; 20 min) produced a small augmentation of thrombin-stimulated [^{3}H]inositol phosphate accumulation (Fig. 3b).

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

In CHO-A1 cells, activation of the human transfected adenosine A_1 receptor leads to the inhibition of adenylyl cyclase (Townsend-Nicholson and Shine, 1992; Megson et al., 1995) and the stimulation of inositol phospholipid hydrolysis and Ca^{2+} mobilisation (Iredale et al., 1994; Megson et al., 1995). In the current study the selective adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA; 1 μM), did not stimulate a measurable release of [^{3}H]arachidonic acid in CHO-A1 cells (Fig. 4). We have previously reported that the transfected adenosine A_1 re-

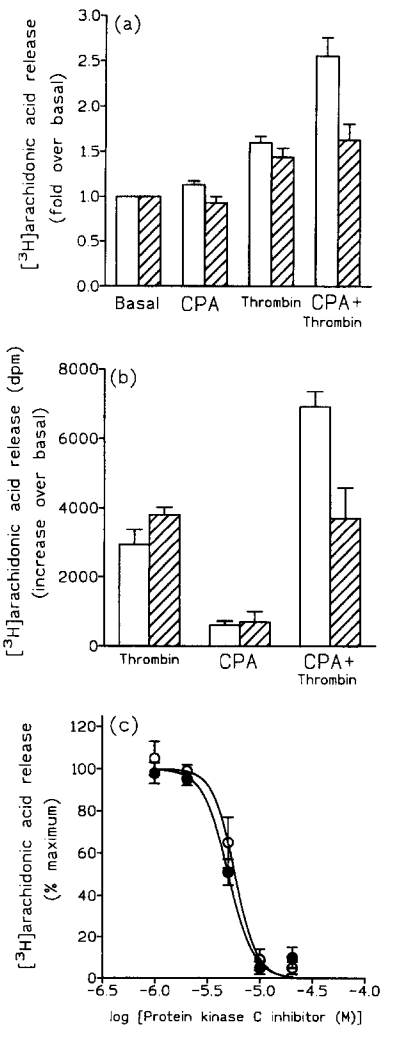

Fig. 7. Effect of the selective protein kinase C inhibitors Ro 31-8220 and GF 109203X, on the potentiation of thrombin-stimulated arachidonic acid release by co-activation of the adenosine A_1 receptor in CHO-A1 cells. (a) Control cells (open columns) and cells pre-incubated for 20 min with 10 μM Ro 31-8220 (shaded columns) were stimulated with with CPA (1 μM), thrombin (1 unit/ml) or a combination of CPA (1 μM) and thrombin (1 unit/ml) for 15 min. Results are expressed as fold-over-basal increases in [^{3}H]arachidonic acid release. Basal [^{3}H]arachidonic acid release was 4069 ± 476 dpm ($n = 6$) and 3933 ± 295 dpm ($n = 6$) in control and Ro 31-8220-treated cells, respectively. Each column represents the mean ± S.E.M.of six independent experiments each measured in triplicate. (b) Control cells (open columns) and cells pre-incubated for 20 min with 10 μM GF 109203X (shaded columns) were stimulated with with CPA (1 μM), thrombin (1 unit/ml) or a combination of CPA (1 μM) and thrombin (1 unit/ml) for 15 min. Results are expressed as the increase in [^{3}H]arachidonic acid release after subtracting basal release. The small increase in [^{3}H]arachidonic acid release observed with CPA was not statistically significant from basal release. Basal [^{3}H]arachidonic acid release was 11818 ± 213 dpm ($n = 4$) and 12055 ± 849 dpm ($n = 4$) in control and GF-109203X-treated cells, respectively. Each column represents the mean ± S.E.M. of four independent experiments each measured in triplicate. (c) Concentration–response curves for the effect of Ro 31-8220 (○) and GF 109203X (●) on the potentiation of thrombin-stimulated arachidonic acid release by the adenosine A_1 receptor. The basal response was subtracted from all data and then the residual net stimulated response expressed as a percentage of that obtained in with 1 unit/ml thrombin in the presence of 1 μM CPA (% maximum). Data are the mean ± S.E.M. of three independent experiments each measured in triplicate.

ceptor directly stimulates [^{3}H]inositol phosphate accumulation and synergistically enhances the [^{3}H]inositol phosphate responses elicited by endogenous P_{2U} purinoceptors and CCK_A receptors in CHO-A1 cells (Megson et al., 1995; Dickenson and Hill, 1996). Fig. 4 shows the effect of CPA (1 μM) on thrombin-stimulated [^{3}H]arachidonic acid release in CHO-A1 cells. CPA augmented the release of [^{3}H]arachidonic acid produced by thrombin. In these experiments, the response to thrombin (1 unit/ml) increased from 1.7 ± 0.1 (n = 8)-fold to 2.5 ± 0.1 (n = 8)-fold over basal in the presence of 1 μM CPA ($P < 0.05$). The potentiation of thrombin-stimulated [^{3}H]arachidonic acid release by CPA was abolished in cells pre-treated with pertussis toxin (100 ng/ml for 24 h; Fig. 6a).

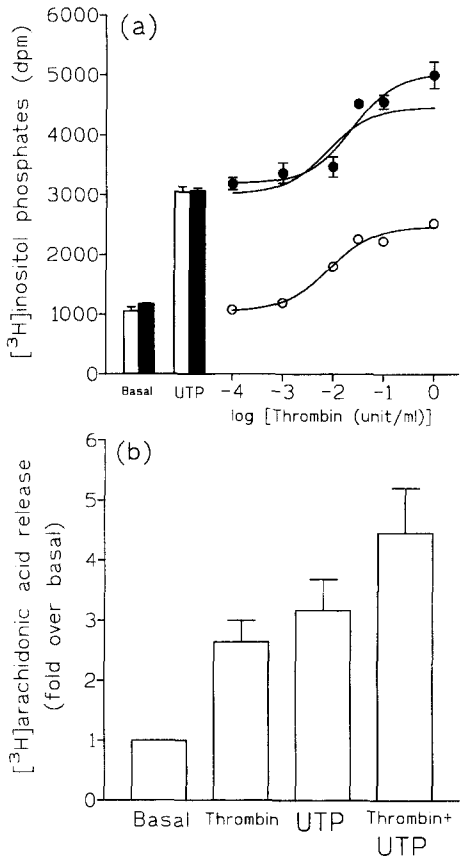

Fig. 8. Effect of co-activation of P_{2U} purinoceptors and thrombin receptors on inositol phospholipid hydrolysis and arachidonic acid release in CHO-A1 cells. (a) Concentration–response curves for thrombin-stimulated [^{3}H]inositol phosphate accumulation in the absence (○) or presence (●) of UTP (100 μM). The solid line represents the predicted additive response to thrombin and UTP calculated as described in Section 2. Basal and UTP (100 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. (b) The effect of co-activation of thrombin receptors and P_{2U} purinoceptors on [^{3}H]arachidonic acid release. Cells were stimulated with thrombin (1 unit/ml), UTP (100 μM) or a combination of thrombin (1 unit/ml) and UTP (100 μM) for 15 min. Results are expressed as fold-over-basal increase in [^{3}H]arachidonic acid release. Each column represents the mean ± S.E.M. of three independent experiments each measured in triplicate.

The potentiation of thrombin-stimulated [^{3}H]arachidonic acid release by co-activation of the adenosine A_1 receptor may be secondary to increased protein kinase C activity brought about by the ability of CPA to augment receptor-mediated (G_q/G_{11} coupled) inositol phospholipid hydrolysis in CHO-A1 cells (Megson et al., 1995; Dickenson and Hill, 1996). Therefore, we investigated whether CPA could potentiate thrombin-stimulated [^{3}H]inositol phosphate accumulation. Co-activation of CHO-A1 cells with CPA and thrombin produced a synergistic increase in total [^{3}H]inositol phosphate accumulation (Fig. 5). In these experiments CPA (1 μM) and thrombin (0.1 unit/ml) alone produced increases in [^{3}H]inositol phosphates of 1.6 ± 0.1 (n = 3) and 2.1 ± 0.2 (n = 3)-fold, respectively. A combination of CPA (1 μM) and thrombin (0.1 unit/ml) produced a 4.6 ± 0.3 (n = 3)-fold increase in [^{3}H]inositol phosphates, which is significantly greater than the predicted additive response of 2.7 ± 0.2-fold (n = 3). The synergistic interaction between CPA and thrombin-stimulated [^{3}H]inositol phosphate accumulation was abolished in cells pre-treated with for 24 h with 100 ng/ml pertussis toxin (Fig. 6c). These results demonstrate that co-activation of the human transfected adenosine A_1 receptor can potentiate thrombin-receptor-stimulated [^{3}H]inositol phosphate accumulation. The ability of CPA to potentiate thrombin-stimulated inositol phospholipid hydrolysis should additionally increase diacylglycerol production leading to increased protein kinase C activation. Consequently, enhanced protein kinase C activity may account for the augmentation of thrombin-stimulated [^{3}H]arachidonic acid release by CPA. The selective protein kinase C inhibitors Ro 31-8220 (10 μM) and GF 109203X (Toullec et al., 1991; 10 μM) completely abolished the ability of CPA to augment thrombin-stimulated [^{3}H]arachidonic acid release, implicating the involvement of protein kinase C (Fig. 7). Concentration–response curves for Ro 31-8220 (EC_{50} = 5.6 ± 0.8 μM; n = 3) and GF 109203X (EC_{50} = 4.3 ± 0.9 μM; n = 3) are shown in Fig. 7. Both protein kinase C inhibitors had no significant effects at concentrations below 2 μM. However, the inhibitory concentrations of Ro 31-8220 (10 μM) and GF 109203X (10 μM) employed in this work are similar to those used in other studies (Bunn and Saunders, 1995; Hoiting et al., 1995).

3.3. Interactions between thrombin and P_{2U} purinoceptors

We have previously reported that synergy is not observed when co-activating two G_q-coupled receptors in CHO-A1 cells (i.e., P_{2U} purinoceptors and CCK_A receptors; Dickenson and Hill, 1996). In this study, co-activation of thrombin and P_{2U} purinoceptors produced additive increases in [^{3}H]inositol phosphate accumulation and [^{3}H]arachidonic acid release (Fig. 8).

4. Discussion

The thrombin receptor has previously been shown to couple to both pertussis-toxin-sensitive (G_i/G_o) and pertussis-toxin-insensitive (G_q/G_{11} and G_{12}) G-proteins in a wide range of tissues and cell types (for review, see Grand et al., 1996). In the present study we have examined thrombin-receptor-stimulated cell signalling in CHO-K1 cells, which have been transfected with the human adenosine A_1 receptor. In these cells, thrombin stimulated inositol phospholipid hydrolysis in a manner which was completely insensitive to inhibition by pertussis toxin, suggesting the involvement of $G_{q/11}$ proteins in the coupling to phospholipase C. These data are consistent with previous work in CHO-K1 cells (Jacksowski et al., 1988; Mitsuhashi et al., 1989; Banno et al., 1994), although in some studies a small pertussis-toxin-sensitive component of the response has been detected (Banno et al., 1994).

Thrombin also stimulated the release of [^{3}H]arachidonic acid from CHO-A1 cells. However, in marked contrast to previous studies in CHO-K1 cells (Lin et al., 1992; Winitz et al., 1994), thrombin-stimulated [^{3}H]arachidonic acid release from CHO-A1 cells was insensitive to inhibition by pertussis toxin. The reason for this difference is unclear, but it may reflect the fact that the expression of thrombin receptors in the CHO-A1 cell line is not sufficient to couple to the $G_{i\alpha 2}$ protein, which appears responsible for the pertussis toxin sensitivity observed in previous studies (Winitz et al., 1994). It is notable, in the study of Winitz et al. (1994), that the [^{3}H]arachidonic acid release induced by thrombin was not completely attenuated by pertussis toxin, suggesting that the residual component of the response was mediated by $G_{q/11}$ proteins. Furthermore, the pertussis-toxin-sensitive component of the response to thrombin appeared to involve the activation of protein kinase C (Winitz et al., 1994), In the present study, we have therefore investigated whether G_i-protein activation by adenosine A_1-receptors can augment thrombin-induced [^{3}H]arachidonic acid release via a mechanism involving protein kinase C.

In CHO-A1 cells, thrombin-induced release of [^{3}H]arachidonic acid release was not attenuated by the selective protein kinase C inhibitors Ro 31-8220 or GF 109203X (Davis et al., 1989; Toullec et al., 1991). These data would seem to suggest that protein kinase C is not involved in the phospholipase A_2 response to thrombin alone. However, activation of protein kinase C, using the phorbol ester PMA (phorbol 12-myristate 13-acetate), potentiated thrombin-stimulated [^{3}H]arachidonic acid release in CHO-A1 cells. A similar augmentation of agonist-stimulated [^{3}H]arachidonic acid release has been observed in CHO cells transfected with the substance P receptor (Garcia et al., 1994). However, the precise mechanism underlying this effect of protein kinase C on cytosolic phospholipase A_2 activity remains to be established. It is known that cytoplasmic phospholipase A_2 can be activated by a wide variety of agents, with full activation of the enzyme requiring both phospholipase A_2 phosphorylation and increases in intracellular calcium (for translocation of phospholipase A_2 from the cytoplasm to the membrane). Recent studies have indicated that cytoplasmic phospholipase A_2 can be phosphorylated, and thus activated, by protein kinase C and mitogen-activated protein kinase (Lin et al., 1993; Nemenoff et al., 1993). Indeed, human cytoplasmic phospholipase A_2 can be phosphorylated by protein kinase C at sites distinct from those altered by mitogen-activated protein kinase (Nemenoff et al., 1993). It is possible, therefore, that protein kinase C may enhance phospholipase A_2 activity which has been initially stimulated by mitogen-activated protein kinase. The initial activation of mitogen-activated protein kinase could be secondary to the rise in intracellular calcium and may involve the newly discovered proline-rich tyrosine kinase 2 (PYK2) which stimulates the mitogen-activated protein kinase pathway in response to increases in intracellular calcium (Lev et al., 1995). It may involve the pertussis-toxin-insensitive $G_{\alpha 12}$ protein which has been reported to couple thrombin receptors to the mitogen-activated protein kinase pathway in 1321N1 cells (Aragay et al., 1995). Interestingly, a GTPase-deficient mutant of α_{12} stimulates arachidonic acid release from NIH 3T3 cells (Xu et al., 1993). Alternatively, protein kinase C may indirectly activate the mitogen-activated protein kinase pathway. Protein kinase C is known to activate the mitogen-activated protein kinase pathway via direct phosphorylation and activation of the serine/threonine protein kinase, Raf-1 (Kolch et al., 1993).

Of particular interest in the present study is the differential effect of acute protein kinase C activation (10 min pre-incubation with 1 μM PMA) on thrombin-stimulated [^{3}H]arachidonic acid release and [^{3}H]inositol phosphate accumulation in CHO-A1 cells. Thus, while protein kinase C activation significantly augmented the phospholipase A_2 activity, PMA pre-treatment virtually abolished the inositol phosphate response to thrombin in these cells. The amino acid sequence of the human thrombin receptor reveals that there is one potential protein kinase C phosphorylation site within the third intracellular loop (Vouret-Craviari et al., 1995). Furthermore, the thrombin receptor can be rapidly phosphorylated in response to β-phorbol-12,13 dibutyrate (Vouret-Craviari et al., 1995), although protein kinase C activation does not appear to promote thrombin receptor desensitization (Paris et al., 1988). The inhibitory effects of phorbol esters on thrombin-stimulated inositol phospholipid hydrolysis are therefore most likely due to downstream inhibition at the level of the G-protein or phospholipase C. The fact that thrombin-stimulated [^{3}H]arachidonic acid release is not similarly affected by phorbol esters would suggest that phospholipase C is the more likely target. Indeed, phospholipase C-β1 has been reported to be a target for protein kinase C-mediated attenuation in several cell types (Ryu et al., 1990). The data obtained with

Ro 31-8220 suggest that the diacylglycerol produced by phospholipase C activation (in response to thrombin in CHO-A1 cells) is sufficient to produce negative feedback (via protein kinase C) on inositol phospholipid hydrolysis.

One of the objectives of the current study was to investigate the modulation of the thrombin receptor signalling by co-activation of the human transfected adenosine A_1 receptor in CHO-A1 cells. We have shown in the present study that adenosine A_1 receptor activation can potentiate thrombin-stimulated [^{3}H]arachidonic acid release from CHO-A1 cells. Similarly, Akbar et al. (1994) demonstrated that adenosine A_1 receptor activation enhanced carbachol-stimulated [^{3}H]arachidonic acid release from CHO cells, co-transfected with adenosine A_1 and muscarinic M_3 receptor cDNA. Our previous studies have shown that the human transfected adenosine A_1 receptor can also potentiate the accumulation of inositol phosphates elicited by endogenous G_q/G_{11}-coupled P_{2U} purinoceptors and CCK_A receptors in CHO-A1 cells (Megson et al., 1995; Dickenson and Hill, 1996). In the current study, co-activation of the human transfected adenosine A_1 receptor synergistically enhances thrombin-receptor-stimulated [^{3}H]inositol phosphate production. The intracellular mechanism(s) underlying the potentiation of G_q/G_{11}-mediated phospholipase C responses by receptors coupled to pertussis toxin-sensitive G_i/G_o proteins are not known, although we and others have speculated on the involvement of G_i protein $\beta\gamma$ subunits (see Gerwins and Fredholm, 1992a,b; Dickenson and Hill, 1994, 1996).

Similarly, the precise mechanism(s) responsible for the potentiation of thrombin-stimulated [^{3}H]arachidonic acid release by the adenosine A_1 receptor are not entirely clear. However, it is possible that the augmentation of thrombin-stimulated inositol phospholipid hydrolysis may yield sufficient diacylglycerol for a protein kinase C-mediated increase in phospholipase A_2 activity. Support for this contention is provided by the finding that the protein kinase C inhibitors Ro 31-8220 and GF 109203X negated the potentiation of thrombin-stimulated [^{3}H]arachidonic acid release by co-activation of the adenosine A_1 receptor.

In conclusion, the present study has shown that thrombin stimulates the release of [^{3}H]arachidonic acid from CHO-A1 cells through a mechanism that is protein kinase C-independent and pertussis-toxin-insensitive. Thrombin-stimulated release of [^{3}H]arachidonic acid release is potentiated by acute activation of protein kinase C and by co-activation of human transfected adenosine A_1 receptor. The augmentation of [^{3}H]arachidonic acid release by the adenosine A_1 receptor is prevented by selective protein kinase C inhibitors, suggesting a role for protein kinase C. In contrast, thrombin-stimulated inositol phospholipid hydrolysis is virtually abolished by acute activation of protein kinase C. The data presented clearly demonstrate that protein kinase C activation has a differential effect on thrombin receptor signalling in CHO cells.

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