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Activation of Phospholipase A_2 by the Human Endothelin Receptor in Chinese Hamster Ovary Cells Involves G_i Protein-Mediated Calcium Influx

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The signalling pathways used by the human endothelin A receptor to activate phospholipase A_2 in Chinese hamster ovary cells were investigated. Pertussis toxin caused a partial but significant reduction in endothelin-1-induced arachidonic acid release although cAMP-dependent kinase inhibitors did not mimic its action. Extracellular calcium and its entry into the cell was essential for activation of phospholipase A_2 as its removal from media or incubation with an intracellular calcium chelator-reduced activation. Nifedipine had no effect on endothelin-1-induced arachidonic acid release while divalent cations caused a significant reduction indicating the possible role of CRAC. Thapsigargin caused an increase in arachidonic acid release which was completely inhibited by pertussis toxin treatment. This further supports the involvement of CRAC in calcium influx and activation of phospholipase A_2 by the human endothelin A receptor.

The endothelins are a family of polypeptides with a wide range of effects in many tissues and cell types and are of considerable physiological and pharmacological importance (1,2,3). A number of signal transduction pathways are used by the endothelin receptors to mediate their actions (4). The $ET_A{}^r$ has been shown to increase intracellular calcium via PKC and PLC in a number of cell types (5,6). Similarly, PKC and PLC have been implicated in the activation of plA_2 (which is known to involve calcium) by other seven-transmembrane helix receptors, in both cases involving PTX sensitive G proteins (e.g. 7). It appears that a common mechanism of receptor mediated calcium entry into cells is the opening of the so-called Ca^{2+}- release-activated Ca^{2+} channels (CRAC) in response to depletion of intracellular stores (8,9). Recent reports indicate that the opening of the channel is dependent on a PTX sensitive G protein in hepatocytes (10). In

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Abbreviations: CHO, Chinese hamster ovary cells; CRAC, Ca^{2+}-release-activated channel; ET-1, endothelin 1; $hET_A{}^r$, human endothelin A receptor; IP_3, inositol 1,4,5-triphosphate; PBS, phosphate buffered saline; PKC, protein kinase C; plA_2, phospholipase A_2; PLC, phospholipase C; PTX, pertussis toxin.

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this investigation we have examined the role of calcium and PTX in activation of plA_2 by the hET_A^r in CHO cells and propose that the receptor opens CRAC in response to lowered intracellular calcium stores through a member of the G_i family of proteins.

MATERIALS AND METHODS

Materials

All cell culture materials, thapsigargin and nifedipine were obtained from Life Technologies (Paisley, Renfrewshire, Scotland). [5,6,8,9,11,12,14,15-^{3}H] Arachidonic acid was obtained from Amersham (Little Chalfont, Bucks, England). ET-1 was supplied by CRB (Northwhich, Cheshire, England). PTX, H89 and K5720 were purchased from Calbiochem (Nottingham, Nottinghamshire, England). All other chemical reagents were obtained from Sigma (Poole, Dorset, England).

Cell Culture and Generation of Cell Lines Expressing hET_A^r

CHODG44 expressing the hET_A^r were routinely grown in Dulbecco's Modified Essential Medium (DMEM) plus 10% foetal calf serum (FCS), 1X non-essential amino acids (NEAA), 1x hypoxanthine/thymidine, penicillin and streptomycin as previously described (11).

Arachidonic Acid Release Assay

Arachidonic acid assays were carried out as follows. Clones were seeded into 24 well plates so that the following day the cultures were confluent or approaching confluence. Cells were then incubated with [^{3}H] arachidonic acid (1μCi in a volume of 1ml of growth medium) overnight. Monolayers were then washed twice with 1ml of pre-warmed (37°C) incubation medium (DMEM plus HEPES (20mM) plus 0.2% bovine serum albumin ((BSA), pH7.4) and then incubated at 37°C in this medium for 30 minutes. Test agents were then added to the cells in incubation medium (final volume 1ml) and incubated with the cells at 37°C for 30 minutes (unless otherwise stated). Supernatants were removed and spun down at 13,000 rpm in a microcentrifuge for 5 minutes at room temperature to remove cells and cellular debris which contains [^{3}H] arachidonic acid and the released radioactivity quantified.

RESULTS

Effect of Calcium on plA_2 Activation

Cells assayed in calcium free media (PBS) were used to determine the effect of extracellular calcium on the ET-1 stimulated release of arachidonic acid. As shown in Table 1 the addition of ET-1 to the cells in a calcium free environment did not stimulate the release of arachidonic acid above background as did calcium alone. However, when calcium was added at 1.8mM (the concentration present in DMEM) levels of release were similar to those observed through ET-1 stimulation in normal media. This suggests that extracellular calcium plays an essential role in the ET-1 stimulated release of arachidonic acid. MAPTAM, a chelator of intracellular calcium, sequesters any free calcium within the cell. Table 1 shows that the addition of MAPTAM to the assay decreases the release of arachidonic acid to background level suggesting that calcium must be free within the cell to activate plA_2. Calcium is able to enter the cell through several different types of channels. Nifedipine, which selectively blocks voltage operated channels, was used to determine if these channels are involved in the influx of the ion. Pre-treatment of the

Table 1. The Effect of Extracellular and Intracellular Calcium Availability on ET-1 Stimulated Arachidonic Acid Release

CONDITIONS	AVERAGE +/- SD
PBS	949.67+/- 57.83
PBS + Ca^{2+} (1.8mM)	847.00+/- 169.00
PBS + ET-1 (100nM)	1018.67+/- 119.44
PBS + ET-1 (100nM) + Ca^{2+}(1.8mM)	3111.67+/- 610.83
PBS + ET-1 (100nM) + Ca^{2+}(1.8mM) + MAPTAM (250μM)	1194.00+/- 516.46
Media + ET-1 (100nM)	2808.67+/- 738.41
Media Only	563.33+/- 96.13

cells with 300μM nifedipine had no significant effect on the ET-1 stimulated release of arachidonic acid. The pretreated and untreated cells released 5111.3 (+/- 597.1) cpm and 4682.77 (+/- 519.23) cpm respectively (not significant at P>0.05). Selectively blocking the voltage operated calcium channels appeared to have no effect on the ET-1 stimulated release of arachidonic acid. Certain calcium channels are not selective for calcium but are permeable to other divalent cations and saturation of the media with divalent cations prevents these channels from taking up calcium, the level of inhibition differing for various cations. As shown in Figure 1 divalent cations have a significant inhibitory effect on the ET-1 stimulated release of arachidonic acid. Co^{2+}, Mn^{2+} and Ni^{2+}, at a concentration of 10mM, reduced arachidonic acid

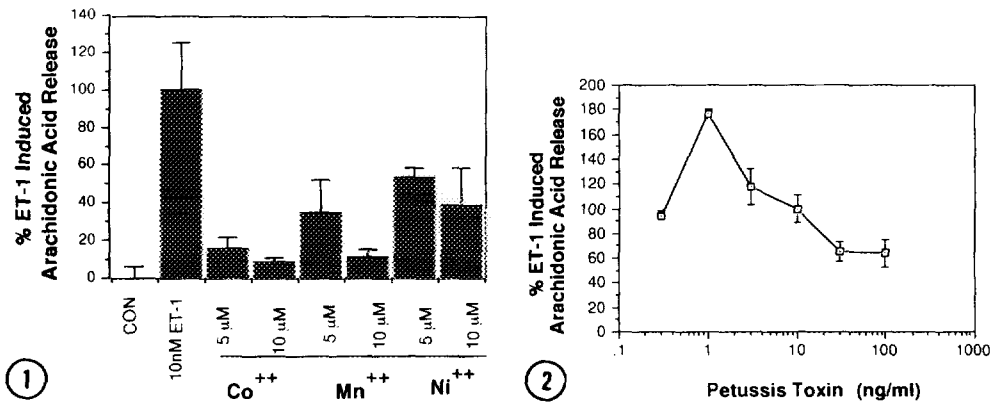

Figure 1. Effect of divalent cations (5μM and 10μM) on the ET-1-stimulated release of [^{3}H] arachidonic acid by DG44-h$ET_A{}^r$ cells. Results are the mean +/- S.D. (n=3, control n=2).

Figure 2. Effect of PTX on ET-1-stimulated [^{3}H] arachidonic acid release by DG44-h$ET_A{}^r$ cells. The cells were pretreated with PTX for six hours before being assayed. Results are mean +/- S.D. (n=3).

release by 92%, 89% and 61% respectively. The lack of activity of nifedipine and the ability of divalent cations to reduce plA_2 activation by ET-1 suggests the possible involvement of CRAC (8,9).

Pertussis Toxin and Thapsigargin

Figure 2 shows the effect that PTX has on ET-1 stimulated arachidonic acid release. At higher concentrations PTX inhibited the release of arachidonic acid by 40%. At lower concentrations however there was an almost two fold significant increase in the counts compared to ET-1 stimulation of untreated cells. This biphasic response could be explained by the action of two (or more) PTX sensitive G proteins which are inhibited at different pertussis toxin concentrations and that they mediate activation and inhibition of ET-1 induced arachidonic acid release.

As stated earlier it has been reported that opening of CRAC is caused by a reduction in intracellular calcium (8,9) and that the activation of the channels involves G_i proteins (10). Thapsigargin mimicked the response of ET-1 stimulation by preventing re-uptake of calcium into the sarcoplasmic reticulum and this reduction in intracellular calcium stores activates CRAC. If a similar pathway is operating for the $hET_A{}^r$ in CHO cells then thapsigargin should be able to mimic ET-1 and its activation of plA_2 should be abolished by PTX. Table 2 shows that the addition of thapsigargin to the cells stimulates an arachidonic acid release similar to ET-1 stimulation. Pertussis toxin had the same inhibitory effect on thapsigargin stimulated arachidonic acid release as on ET-1 release reducing the counts to background level. From this it seems that the PTX sensitive G protein is acting downstream from any IP_3-dependent mobilisation of intracellular calcium stores.

cAMP Dependent Protein Kinase

Two highly selective cAMP-dependent protein kinase inhibitors, H-89 (13) and KT5720 (12), were used to determine the involvement of the cAMP signalling pathway in the ET-1 stimulated arachidonic acid release. Figure 3 shows the effect of inhibiting cAMP-dependent protein kinase in the assay. Blocking cAMP-dependent protein kinase did not result in a decrease in

Table 2. Effect of PTX on ET-1 and Thapsigargin Stimulated Arachidonic Acid Release

ADDITIONS	Arachidonic Acid Release (cpm) AVERAGE +/- SD
Media Only	170.87+/- 52.02
ET-1 (100nM)	1870.77 +/- 288.37
PTX (100ng/ml) + ET-1 (100nM)	521.40+/- 20.82
Thapsigargin (100nM)	6472.73+/- 817.07
PTX(100ng/ml) + Thapsigargin (100nM)	309.63+/- 59.17

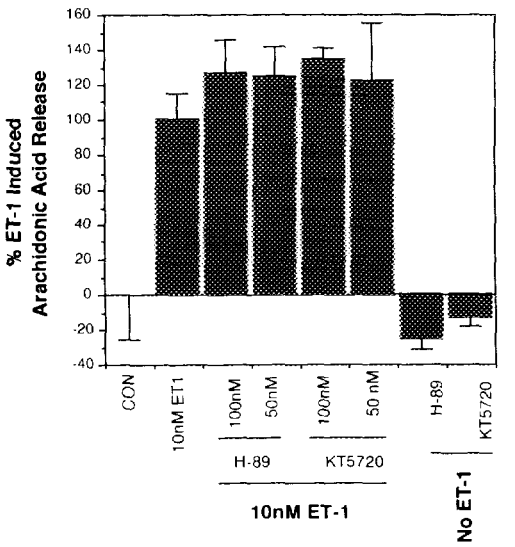

Figure 3. Effect of cAMP-dependent protein kinase Inhibitors on the release of [^{3}H] arachidonic acid stimulated by ET-1. Concentration of H-89 and KT5720 without ET-1 is 100nM. Results are mean +/- S.D. (n=3).

arachidonic acid release. In fact an unpaired students t test showed a significant, but small, increase in arachidonic acid release for both the inhibitors (P> 0.05).

DISCUSSION

Extracellular calcium has been shown to play an essential role in cell signalling by ET-1 (4,6) and in the agonist stimulated arachidonic acid release by plA2 in number of cell types (e.g.14, 15) including endothelial cells (17). We have found that the entry of extracellular calcium into the cell is essential for activation of plA2 in CHO cells expressing the $hETA^r$. Removal of extracellular calcium from the medium or incubation of cells with intracellular calcium chelator MAPTAM caused levels of arachidonic acid release to drop to background levels. While the need for extracellular and free intracellular calcium in ET-1 stimulated response has been well documented less is known about the mechanism by which calcium enters the cell.

A possible candidate for the calcium influx involved in ET-1 stimulated arachidonic acid release is CRAC linked to the capacitative model for calcium influx (8,9). Activation of these channels is triggered by a depletion of intracellular calcium stores and they respond to any drug which does so without requiring receptor occupancy. Thapsigargin which prevents the re-uptake of calcium into the sarcoplasmic reticulum caused release of arachidonic acid presumably by activation of plA2 due to a calcium influx. The inability of nifedipine to block arachidonic acid release suggests that voltage-operated calcium channels are not responsible for this calcium influx. This result is also in agreement with the suggestion that CRAC is involved, as an insensitivity to classical voltage-gated calcium channel inhibitors is a characteristic of CRAC (8,9). Further support for the involvement of CRAC is given by the effect of the divalent cations on the ET-1 stimulated release of arachidonic acid. CRAC has been shown to be inhibited by divalent cations

to varying degrees. Hoth and Penner (18) found that the order of potency for the blocking of CRAC of the cations we assayed (among others) was $Ni^{2+} < Mn^{2+} = Co^{2+}$. In this paper saturation of the media with divalent cations inhibited the release of arachidonic acid and the order of potency was similar to that found for inhibition of CRAC ($Ni^{2+} < Mn^{2+} < Co^{2+}$).

PTX sensitive G proteins also plays a role in activation of plA_2 by receptors (5,7,15) and in the activity of ET-1 (4). Pre-treatment of the cells with PTX had a biphasic response on arachidonic acid release in response to ET-1. At lower concentrations of PTX pre-treatment the release of arachidonic acid increased to 200% before decreasing to 40% when higher concentrations ($100\mu gml^{-1}$) were present. This could be explained by the simultaneous action of two PTX sensitive G-proteins which differ in their sensitivity to the toxin. One G-protein, inhibited at the lower concentrations, may be involved in opening the calcium influx channels as the level of calcium in the intracellular stores drops. The second G-protein, unaffected by low concentrations of PTX, may act to inhibit this inflow of extracellular calcium. The involvement of a PTX sensitive G-protein in store-operated calcium influx has been shown in several agonist stimulated pathways (5,7). Thapsigargin stimulated arachidonic acid release was also completely blocked by pre incubation with PTX suggesting that a sensitive G-protein is involved in the signalling which opens the calcium influx channels in response to a decrease of intracellular calcium stores. The inability of cAMP dependent protein kinase inhibitors to alter the release of arachidonic acid indicates that the PTX sensitive proteins do not activate plA_2 via cAMP but by another pathway.

In conclusion, we have shown that the ET-1 stimulated release of arachidonic acid is dependent on the entry of extracellular calcium into the cell and that entry is probably via the Ca^{2+}-release-activated channels. This influx of calcium seems to be dependent on a decrease in the intracellular stores of calcium since the actions of ET-1 can be mimicked by the addition of thapsigargin. Further we believe a PTX sensitive G-protein also seems to be involved in this release by acting as a messenger between the intracellular calcium stores and the calcium influx channels. Our results also suggest that there may be more than one PTX sensitive G-protein involved. These results are further evidence of the importance of CRAC channels in cells signalling.

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